DIVERSITY OF OOMYCETES ASSOCIATED WITH SOYBEAN SEEDLING DISEASES

By

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ABSTRACT

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In the United States, soybeans are produced on 76 million acres of highly productive land, but can be severely impacted by diseases caused by oomycetes. Oomycetes are part of the microbial community that is associated with plant roots and the rhizosphere, which is a dynamic and complex environment subject to the interaction of different microbes and abiotic factors that could affect the outcome of the phytobiome interaction. Depending on environmental and edaphic conditions, some oomycete species will thrive causing root and seedling rot. The identity and distribution of these pathogen species is limited. Therefore, the main questions driving my research are what oomycetes are associated with soybean seedlings and what are the roles of these species causing disease? What factors increase or reduce the impact of these species on the soybean production system? With these questions in mind, the goals of my research are: (1) to characterize the oomycete diversity associated with seedling and root rot diseases of soybean; (2) determine the role of environmental and edaphic factors on the distribution of oomycete species; (3) develop molecular diagnostic tools for *Phytophthora sojae* and *P. sansomeana* and (4) evaluate the community structure of the oomycete species associated with soybean root diseases under different conditions.

We initially utilized a two-year culture-based survey to study oomycetes associated with soybean seedlings from 11 states in the Midwest, characterizing the communities and profiling phenotypic traits such as pathogenicity and aggressiveness. With this approach, a total of 84 oomycete species were identified and characterized. Of those 84 oomycete species, 43 species

had detrimental effects on soybean seedlings being pathogenic and 17 of those pathogenic species also caused disease on soybean seeds. In addition, the ecology of oomycetes was studied by correlating abundance and diversity of oomycetes with different environmental and edaphic parameters. Our main findings were that the community structure of oomycetes (presence/absence) associated with soybean seedlings was similar geographically, but their abundances differed. By using the environmental and edaphic data, it was observed that latitude was correlated with oomycete diversity with increasing diversity observed in samples from higher latitudes. Other parameters such as temperature and precipitation affected community composition within and across years. Soil parameters like pH, clay content and cation exchange capacity also influenced the oomycete community structure.

The survey served as a basis to develop markers for diagnostics, providing a collection of *Phytophthora sojae* isolates, a major soybean pathogen, and at the same time identifying and providing cultures for *Phytophthora sansomeana* as a threat for soybean. A hierarchical detection system for quantitative PCR and isothermal amplification was developed to identify these pathogens at the genus and species-specific level. Both assays were validated under laboratory and field conditions. Finally, an amplicon-based community analysis was adapted and developed to further investigate and characterize oomycete species and communities associated with agricultural systems. The loci targeted were the ITS of the rDNA, the D1-D3 regions of the 28S and the *cox*I gene. The data generated from the amplicon-based approach, in conjunction with the phenotype (pathogenicity/virulence) and species distribution, is being used to evaluate the role of climatic, edaphic and biotic factors on the oomycete community structure. Improved understanding of the oomycete community, especially in the root system, and the factors that influence it will enable improved disease management and enhance plant health.

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Problem statement

Soybean has been designated as a key crop for global food security and oilseed production (Singh et al., 2007). Production worldwide is around 223 million tons, which ranks soybean as the fourth most important crop in the world and second in the U.S. in terms of land area planted (FAOSTAT 2010, http://faostat.fao.org/default.aspx). In US, the production of soybean has grown in the last years with the harvested area around 24 million hectares with a crop value of $35 billion dollars (http://www.soystats.org). This commodity significantly influences Michigan's agriculture providing indirect and direct economic impacts. Soybean is only surpassed by corn, which is the most valuable crop in Michigan; the planted area for this crop is close to 2.6 million acres with a value of $2.2 billion dollars (USDA, NASS Statistics, 2012). The production costs for corn and soy are around $332 and $143 dollars per acre, respectively, where seed alone represents 30 to 40% of operating costs. If seed treatments are included these operating costs increase to 35% and 54%, which emphasizes the importance of management and understanding of diseases at the seedling stage.

Crop germination and stand are key factors for a successful cropping season for growers. During seed establishment, seedlings are subject to attack by a number of soilborne pathogens, resulting in lack of germination, damping off or plant death. Poor plant stands due to disease result in replanting and increased costs to growers. The impact of these soilborne diseases is not only limited to the beginning of the season, as root infections can occur at later stages, often reducing yield without significant above ground symptoms. In 2005, loss to soybean seedling diseases in the US was estimated at 829 tons (Wrather and Koenning, 2006). From 2006 to 2009, soybean yield losses due to seedling diseases have increased considerably ranking second

only to soybean cyst nematode (Koenning and Wrather, 2010). These soilborne diseases have been attributed to several pathogens most of them fungi and fungi-like organisms (oomycetes).

The crop establishment is key factor for soybean growers at the beginning of the season where most of the time, the seedlings are subject of the attack of soilborne pathogens, resulting in damping off or poor plant establishment. The impact of these diseases is not only limited at the beginning of the season, as a matter of fact, root infections can occur at later stages also reducing the crop performance. In 2005, the loss to seedling diseases in the US was estimated on 829 tons (Wrather and Koenning 2006). Different factors may influence this output, such as seed quality, the environment and their interaction with the soil inhabitants, which many could play a role on these catastrophic outputs (Broders et al. 2009). These soilborne diseases have been attributed to several pathogens most of them fungi and oomycetes, but the key species playing a role in disease are not known completely (Broders et al. 2007). In fact, the oomycetes *Pythium* and *Phytophthora* are two of the main causal agents of soilborne diseases, where just on soybean have increased losses by four-fold in the last ten years (Koenning and Wrather 2010). This increased incidence is related to some of cultural practices now being used by growers, like no till and early planting. These practices and the environment interact strongly with the microbial communities present in the soil, causing shifts in the different species that exist in this habitat (Arcate et al. 2006). The diversity of species causing soybean seedling diseases in the soybean belt in the US is limited.

The understanding of these soil communities and the pathogens there present has traditionally been done using culture based methods. These provide are representation of the community and have been helpful on describing some of diversity present in soybean and their role as pathogens (Broders et al. 2007, Zitnick-Anderson and Nelson 2015). The caveat for the

media-based isolation is the introduction significant biases due to differential growth of organisms such as influenced by temperature, medium preference, and antibiotic/fungicide sensitivity. Even semi-selective medium designed specifically to increase the recovery rate of a target organism or group of organisms introduce biases such as selection of isolates within the target species or group reducing representation of isolates within the target group that are slower growing and more sensitive to antibiotics. Nowadays, culture independent microbial community analysis provides a significant advantage over culture based methods as no selection pressure is placed on the group of organisms and a far more complete snap shot of organisms present can be achieved, at least at the level for which primers are designed. Therefore, we hypothesized that there are multiple oomycete species associated with soybean seedling diseases and the abundance of these could be affected by different parameters, including environmental, soil (edaphic) and microbiome present on this system.

The goals of my doctoral research are: (i) determine the diversity of oomycete species associated with soybean seedling diseases and establish the pathogenicity of those species found on symptomatic and asymptomatic soybean seedlings, (ii) evaluate the co-relation of different environmental and edaphic parameters with the abundance and distribution of oomycetes associated with soybean seedling diseases, (iii) develop genus and species specific diagnostic tools for major pathogens of soybean, using different approaches that facilitate the detection and accurate diagnostic of pathogens, and (iv) develop and use culture-independent approaches to understand ecology of oomycete communities associated to soybean plants and the role of different factors: host, environment and management practices in the ecology of these organisms.

Oomycete community ecology: tools and challenges from a plant pathology perspective

This chapter was written as review on the state of the art of oomycete ecology and the use of next-generation sequencing to study oomycete ecology applied to plant pathology, it will be submitted for publication to Fungal Ecology.

Introduction

Oomycetes are eukaryotic organisms classified within the kingdom Stramenopila, which also contains the brown algae. However, oomycetes were originally classified within the kingdom Fungi due to their morphological resemblance of the members of this group (Beakes et al. 2012). Despite the taxonomical distinction of oomycetes and fungi, there are comparable traits such as those involving nutrition mechanisms, through the secretion of enzymes and nutrient absorption. These characteristics also result in analogous ecological functions that influence the ecosystem (Richards et al. 2006). Oomycetes are known to contain mainly plant and animal pathogenic species, and a few saprotrophs surviving in agricultural, aquatic and forest/terrestrial systems (Agrios 2005, Strittmatter et al. 2008). Among the different oomycete species, plant pathogenic species are the most studied and characterized, containing genera such as *Phytophthora* and *Pythium* (Jiang and Tyler 2012). In the last 20 years, the description of new oomycete species has increased exponentially resulting in over one hundred species belonging within each of these two genera (Figure 1.1) and new genera such as *Phytopythium* and *Pythiogeton*. Since 2000, a total of 290 new oomycete species have been described (http://www.mycobank.org/, visited May 2016), composed of 93 correspond to *Phytophthora* spp., 60 *Pythium* spp., 36 *Hyaloperonospora* spp., 18 *Phytopythium* spp., 13 *Albugo* spp. and 7 *Pythiogeton* spp. among others.

Recent reviews have pointed out the general impact of oomycetes as a posed threat for agricultural and natural ecosystems (Fisher et al. 2013). The increasing diversity being currently recognized has shown that some species are moving to new habitats and have the potential to be emerging and re-emerging pathogens. There are multiple examples of oomycete plant pathogen introductions in nature (Jules et al. 2002, Cline et al. 2008, Grünwald et al. 2012) and agricultural systems (Thines et al. 2009, Cohen et al. 2015), for example sudden oak death, caused by *Phytophthora ramorum* and emerging diseases caused by downy mildews on different crops. Outside of agriculture, oomycetes have been associated with ecosystem services in natural systems, where they serve to maintain a natural equilibrium. In forests, the role of *Pythium* spp. and other soilborne oomycetes is to cause seedling diseases to help maintain the plant diversity in the ecosystem (Gilbert 2002). This process is known as the Janzell-Connell hypothesis, where it is hypothesized that seedling mortality is caused by *Pythium* spp., maintaining and supporting the ecosystem diversity by controlling the emerging seedlings. In addition, the role of these organisms has a distance-dependent effect, where oomycete species specialize on local plant species resulting in a negative density- and distance-dependent regulation (Benítez et al. 2013). However, the true role of these species in an ecosystem is limited and not well studied, and for the basal oomycetes or those species that are not plant pathogens it is even less studied.

Studies of oomycete plant pathogens generally focus on the interaction of single species with a single plant host. To date, the majority of concentrated effort has been on studying organisms belonging to the genus *Phytophthora*, where most of the aggressive plant pathogenic species belong. Investigations have mainly addressed the population genetics of single species (Fry et al.

2009, Schoebel et al. 2014, Kamvar et al. 2015), surveying diversity (Knaus et al. 2015, Nagel et al. 2015, Sims et al. 2015) and understanding their basic biology (Goss et al. 2011, Martin et al. 2014, Na et al. 2014). Although as recent as 2012, only six *Phytophthora* genomes were sequenced, a recent effort initiative approach was undertaken in 2015 to sequence and catalogue the genomes of multiple species across the 10 designated clades (Kronmiller et al. 2015). This effort has resulted in 29 more *Phytophthora* genomes currently being sequenced to understand the functional variability of members of this genus. Currently, there are only 37 oomycete genomes published in NCBI (http://www.ncbi.nlm.nih.gov/genome), that correspond to 20 *Phytophthora* spp., 7 *Pythium* spp., 2 *Saprolegnia* spp., which reflects the lack of characterization of oomycetes.

Extensive sampling and isolation on semi-selective media have been used to describe new oomycete species across different ecosystems, especially in agricultural systems, where these species have a significant impact. Community composition at a local scale has been studied for *Pythium* spp. associated with crops such as soybean, corn, wheat, and carrot (Higginbotham et al. 2004, Broders et al. 2007, Suffert and Guibert 2007, Zitnick-Anderson and Nelson 2015). These studies have resulted in the association of multiple species with these hosts and the characterization of aggressive species. Typically, the same species are found across different agricultural hosts, including *Py. ultimum* sensu lato, *Py. sylvaticum* and *Py. irregulare*. *Phytophthora* community analyses are broader and greater in number with regard to the ecosystems studied. Forest systems have been of special interest due to the recent epidemics caused by *Phytophthora* spp., altering the balance of tree species in these ecosystems. For instance, a survey of these species in oaks across the Midwestern U.S. was carried out sampling

healthy and declining trees, finding multiple *Phytophthora* spp. associated (Balci et al. 2007). Surveys have also focused on riparian systems, as a surveillance method to search for potential pathogenic or invasive oomycete species that could represent a threat to native systems (Nagel et al. 2015, Sims et al. 2015). With respect to agriculture, Parke et al. (2014) used a survey-approach to characterize critical points within a nursery production system that could bring oomycete pathogenic species into a system, identifying soil as the main source for *Phytophthora* spp. There are also studies focusing on the diversity of *Phytophthora* species causing disease on floral crops (Hwang and Benson 2005), fruit trees (Latorre et al. 2001) and grapevines (Spies et al. 2011a).

Despite the importance of oomycetes for agriculture and natural ecosystems, the ecological study of these organisms is limited and has been restricted to specific systems. The availability of novel tools like next-generation sequencing (NGS) have opened a new window to understand not only the functional genomics of oomycetes, but also their diversity, evolution and ecology within different ecosystems. The description of oomycete species was limited by morphological features before the genomic era, sequencing has enabled a systematics revolution, resulting in an exponential increase in the description of species in the last 20 years (Figure 1.1). The use of rDNA has allowed the study of communities in relation with their environment. The goal of this review is to examine all the different methods and approaches that were taken to study the role of oomycetes in the environment, especially in agriculture. Also, evaluate the state of the art on oomycete ecology, identifying challenges, and where this research can be taken to improve our understanding of plant-oomycete complex systems. This is an exciting time for oomycete biology and ecology. The availability of genomic tools enables studies that can focus on

questions related to the interaction of species at the community level, the effect of different anthropogenic and natural processes on the diversity of these organisms.

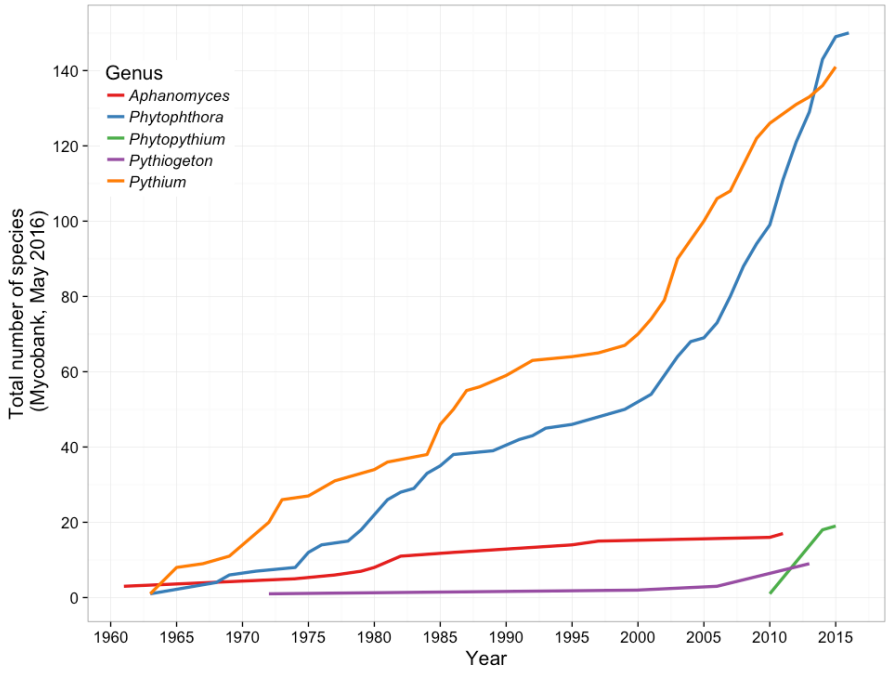

Figure 1.1. Number of species described since 1960 in the genera *Aphanomyces*, *Phytophthora, Phytopythium, Pythiogeton* and *Pythium*. Information obtained from Mycobank (mycobank.org) accessed May 2016.

Traditional molecular tools to study oomycete diversity and ecology

During the study of oomycetes, different approaches have been taken to understand the diversity of these organisms in different ecosystems. Ranging from the traditional and valuable culture-based approach to newer approaches involving next generation sequencing. Most of the diversity of oomycetes has been described through culture approaches by baiting, direct isolation from soil, plant tissue, and animal tissue (Anderson 1987, Arcate et al. 2006, Weiland 2011, Van Buyten and Höfte 2013, Duffy et al. 2015, Nagel et al. 2015). Those efforts and others have resulted in much of the descriptions of oomycete species done in the last 50 years. However, the advance of molecular biology has brought to the field different techniques that when applied will greatly aided the understanding of the diversity of oomycetes in different environments,

examples off the different approaches are listed in Table 1.1. Direct sequencing from cultures was the starting point, but when analyzing a soil or water sample or even plant tissue, some studies have used a cloning library after amplifying environmental DNA with oomycete specific primers (Arcate et al. 2006, Wielgoss et al. 2009). Thus facilitating the analysis of communities to detect relevant OTUs that later could be isolated and evaluated for specific phenotypes. For instance, Nelson and Karp (2013) evaluated the oomycete community associated with *Phragmites* invasive and non-invasive species to determine the impact of the invasive species and how oomycetes responded in the presence of these hosts. In the following study done by Crocker et al. (2015), isolates were collected for those species found in the first study and evaluated for pathogenicity/virulence on the different plant species of this ecosystem, including *Phragmites* spp. Another example of clone libraries was reported by Scibetta et al. (2012), which focused on *Phytophthora* spp. and amplifying the whole ITS region or just the ITS1, which resulted in the later being the most reliable and sensitive method to detect *Phytophthora* spp.

Table 1.1. Summary of approaches to study diversity and ecology of oomycete species in agricultural and natural ecosystems.

Method	Target	Focus of the study	Reference
Clone library	ITS library generated from total DNA from rhizosphere	Identify oomycetes associated with soil and rhizosphere of different plants	(Arcate et al. 2006)
Clone library	ITS library generated from DNA extracted from litter	Identify oomycete species associated with *Phragmites* in a litoral zone	(Wielgoss et al. 2009)

Table 1.1 (cont'd)

Clone library	ITS library generated from plant tissue	Identification of oomycete species associated with native and non-native plant species	(Nelson and Karp 2013)
PCR-RFLP	DNA Fingerprint of *cox*I and *cox*II	Identification of *Phytophthora* spp.	(Martin and Tooley 2004)
PCR-RFLP	DNA fingerprint of the ITS	Identification of *Phytophthora* spp. in tree nurseries	(Knaus et al. 2015)
T-RFLP	Fragment analysis of ITS	Identification of oomycete pathogens in organic crops	(Benitez 2008, Summers et al. 2014)
PCR-SSCPs	Single strand conformation of the ITS1	Identification of pure cultures of *Pythium* and *Phytophthora* spp.	(Kong et al. 2003, Kong et al. 2004, Kong et al. 2005)
PCR-SSCPs	Single strand conformation of the ITS1	Identification of oomycetes associated with soybean fields	(Broders et al. 2009)
PCR-DGGE	DNA fingerprints from environmental DNA	Evaluate eukaryote community structure associated with rice roots	(Ikenaga et al. 2004)
PCR-DGGE	DNA fingerprints from environmental DNA	Evaluation of the effect of soil fumigation to control apple replant	(Yao et al. 2006)
Macroarrays	Array with probes that hybridize to ITS sequences	Detection of *Pythium* and *Phytophthora* species in soil samples	(Summerbell et al. 2005, Tambong et al. 2006, Chen et al. 2013)

Table 1.1 (cont'd)

qPCR	Probes based detection for ITS, SSU, mtDNA	Detection of specific species of *Pythium* on different plant or soil tissues	(Schroeder et al. 2006, Schroeder et al. 2013)
qPCR	Probe based detection for ITS, mtDNA	Detection of specific species of *Phytophthora* on different plant or soil tissues	(Bilodeau et al. 2007, Cooke et al. 2007, Schena et al. 2008, Bilodeau et al. 2014)
454 sequencing	Pyrosequencing of the fungal ITS1 amplicons from root tissue	Identify fungal and oomycete pathogens associated with pea roots	(Xu et al. 2012)
454 sequencing	Pyrosequencing of oomycete ITS1 amplicons	Validation of ITS1 as marker to identify *Phytophthora* spp.	(Vettraino et al. 2012)
454 sequencing	Pyrosequencing of oomycete ITS1 amplicons from soil samples	Identification of *Phytophthora* spp. in forests	(Coince et al. 2013, Vannini et al. 2013)
454 sequencing	Pyrosequencing of ITS1 amplicons form soil and plant tissue	Identification of oomycetes from plant and soil tissues	(Sapkota and Nicolaisen 2015)
454 sequencing	Pyrosequencing of *cox*I amplicons from soil samples	Identification of oomycete species in soybean fields	(Coffua et al. 2016)

Apart from cloning and clone sequencing, other approaches have used the variability of the rDNA and the ITS region to apply different techniques in order to study diversity or simplify the identification of isolates without sequencing all of them. An example is PCR followed by restriction fragment length polymorphism (PCR-RFLP), where ITS amplified from genomic DNA obtained from a culture is digested with a restriction enzyme and the product is separated

on a gel. Molecular types are designated based on the fingerprint and only one or a few are confirmed by sequencing, simplifying the process of identification of different oomycetes or within oomycete genera (Cooke et al. 2000b, Nechwatal et al. 2008, Knaus et al. 2015). The RFLP approach was also used to identify *Phytophthora* spp. using *cox*I and *cox*II mitochondrial markers (Martin and Tooley 2004). A variant of the PCR-RFLP was developed using labeled fragments, designated terminal restriction fragment length polymorphism (T-RFLP) was also adapted to oomycetes and used to characterized oomycete communities in agricultural systems (Benitez 2008, Summers et al. 2014). The method is based on the amplification of the ITS region followed by digestion, the labeled amplified fragments are compared with an *in silico* database to determine the identity of the major species.

Another approach that was proposed also based on PCR was the single strand conformation polymorphism (PCR-SSCP) that was developed by Kong et al. (2003) to characterize *Phytophthora* spp. and later the method was also adapted to *Pythium spp.* (Kong et al. 2004). The method was proposed as new approach to determine species level in plant and environmental samples, however, it still required some form of isolation since the PCR product results from a colony. The colony PCR was carried out on pure cultures, or colonies around 2 mm of diameter after soil dilution method or water filtration (Kong et al. 2005). This technique is limited since it requires extra steps to study communities. Nonetheless, the techniques have been applied to community studies in agricultural systems. Broders et al. (2009) used SSCPs to study the diversity of *Pythium* species in soybean fields in Ohio and their association with soil and chemical properties. However, SSCPs does require the isolation of organisms, while other methods like PCR-denaturing gradient gel electrophoresis (PCR-DGGE) was used on

environmental samples, extracting total DNA from plant tissue and then carrying out PCR with specific primers (Ikenaga et al. 2004, Yao et al. 2006). In this case, bands must be compared to a database to determine the identity or bands of specific interest need to be cut and sequenced for further investigation, but certainly the approach revealed changes in the community composition.

A diagnostic oriented method used to evaluate the presence and absence of certain oomycete species was based on the use of macroarrays, creating a library of probes of species of interest that hybridize either to genomic DNA or environmental DNA. Tambong et al. (2006) developed an oligonucleotide array to detect *Pythium* spp. This method was tested with a collection of isolates and validated with soil DNA and root baits to determine the presence of different *Pythium* spp. (Summerbell et al. 2005). A similar approach was used by other groups, where a macroarray with a library of pathogens commonly found in tomato and sugar beets was also used to establish the presence of pathogens, including different species of oomycetes (Summers et al. 2014, Liebe et al. 2015). Despite the power of the technique and its multiple applications to plant pathology studies, the method is only qualitative and depending on the probe design, it may result in cross-reactivity issues. Moving forward in diagnostics, the quantitative PCR (qPCR) could be an effective way to detect and quantify specific pathogens from environmental samples. Multiple assays have been developed for specific species (Schroeder et al. 2006, Cooke et al. 2007, Bienapfl et al. 2011, Martin et al. 2012, Schena et al. 2013, Schroeder et al. 2013, Gangneux et al. 2014), which can be easily applied to detect different oomycete species. Some of these developments allowed the multiplex detection of pathogens. There is the potential to use different assays in systems like the TaqMan array cards or the WaferGen, allowing the detection of multiple species, manly plant pathogenic oomycetes, within environmental samples.

However, it will be necessary to standardize assays to similar conditions of amplification to be used at the same time in this systems (Saunders 2013).

Next-generation sequencing (NGS) to study oomycete diversity and ecology

The advances of next-generation sequencing (NGS) has made a great advance in ecology studies by providing tools to researchers interested in this area and other fields, increasing the scale of the number of species or OTUs that can be obtained from samples. The transition from chain termination sequencing (e.g. Sanger) that produced 0.08 MB of data with read lengths of 500-700 bp per run to the high-throughput sequencing methods, 454 pyrosequencing and illumina improved the amount of data obtained and the costs. For instance, identifying 500 isolates sequencing ITS in both directions with Sanger will cost around $2500 and it is only 0.35 MB of data. While, 454 pyrosequencing run will cost $1,100 and produces 400-600 MB of data with read lengths of ~500 bp and the illumina MiSeq run costs $900 produces 7 GB of data with ~ 400 bp (2X250 bp). These two NGS methods have been the most commonly used to study fungal and oomycete communities to date. There are different strategies to carry out the sequencing on either platform that were originally developed for bacterial communities and have been adapted to fungal communities (Kozich et al. 2013, Lundberg et al. 2013, D'Amore et al. 2016). The selection of method depends on the barcode and output necessary to effectively answer the question presented in the system under study. The trade-offs are especially on read length and the amount of data generated, where 454 sequencing provides longer reads and relatively small output, whereas illumine can provide assembled reads of 400 bp and larger outputs in comparison to the 454. There are multiple studies that have used these technologies to study oomycete communities.

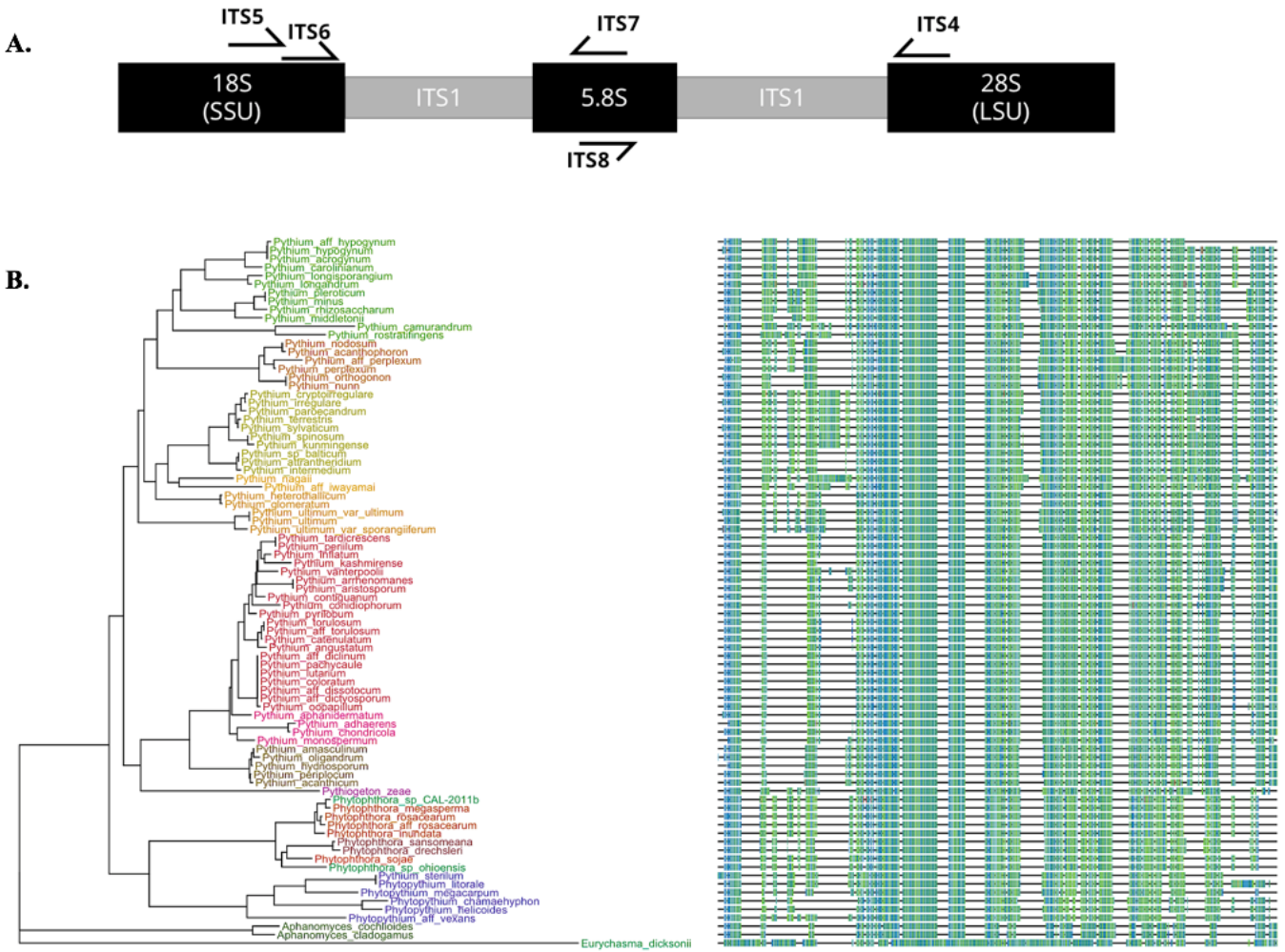

Figure 1.2. (a) Schematic representation of oomycete rDNA including the internal transcribed spacers (ITS1 and ITS2), indicating location of primers commonly used for amplification of this region. (b) Neighbor Joining tree of *Pythium* and *Phytophthora* ITS sequences.

There is different application of NGS to study oomycete diversity, and these are listed in Table 1.1. One of the first studies that obtained amplicon sequences of oomycetes used the fungal ITS1 primers, which are not ideal for the task, but they can still amplify certain oomycetes. The research was focused on fungal community structure across pea fields with different levels of disease, found the oomycete pathogens *Pythium* (1.2%) and *Aphanomyces* (0.02%) were present in low frequencies in comparison with other fungal root rot pathogens (Xu et al. 2012). These could be due to primer biases, but also the database used. Vettraino et al. (2012) used an artificial community composed of 8 *Phytophthora* spp. and a *Phytopythium* (*Pythium vexans*) to examine the potential of ITS1 amplified by primers ITS6 and ITS7 and sequenced by 454 (Figure 1.2a). An important finding of this study was that due to error rates and threshold, the number of unmatched reads changes quickly, for instance at 97% threshold the number of false OTUs is around 4 and the percent of unmatched reads is <5%, while a threshold of 99% results in one false OTU and 20% unmatched reads. After the proof of concept, Vannini et al. (2013) followed up with the application of 454 pyrosequencing of the ITS1 to study the diversity of *Phytophthora* spp. in chestnut forests by collecting bulk soil samples from two sites with disease symptoms. The amplicon data was matched to a database of 15 *Phytophthora* spp., which resulted in *Ph. plurivora* being the most abundant followed by *Ph. capsici* and *Ph. gonapodyides*, matching some the isolations. A total of 9 species were isolated and identified by the amplicon sequencing. Other studies listed in Table 1.1 were focused on the genus *Phytophthora* using 454 to examine diversity of this genera in forests and plantations in Northern Spain (Català et al. 2016). A two step PCR approach was used amplifying the full ITS, followed by *Phytophthora* ITS1 specific primers (Scibetta et al. 2012). The approach was used on soil and water samples, which soil samples revealed 13 *Phytophthora* spp. in comparison to 35 species in

water samples. The two most common species found were *Ph. gonapodyides* and *Ph. cryptogea*, however non-target species were also detected, such as *Hyaloperonospora, Peronospora* and *Pythium*.

Coince et al. (2013) also used a 454 approach to study fungal and oomycete communities in beech forests. Oomycete amplicons were also produced by a two-step PCR was used, which resulted in only 10 oomycete OTUs represented by 463 sequences out of 55,936 quality filtered reads. The same study used primers fungal primers that proved to be specific for this group, resulting in no oomycete reads obtained. Recently, Sapkota and Nicolaisen (2015) also used a nested PCR approach using primers ITS6, ITS4 and ITS7 to study oomycete communities (Figure 1.2). The authors addressed the issue observed by Coince et al. (2013), where very limited oomycete sequences were found using oomycete ITS designed primers. The evaluation of primer sequences revealed mismatches in the 3' region of the primer when compared with fungi and plant, suggesting some degree of specificity. To address this issues, primer annealing temperature for the amplification was increased to 59ºC. Soil samples and carrot tissue with cavity spot disease symptoms caused by different *Pythium* spp. were used as validation samples for this approach, resulting in 94-95% reads matching oomycetes and 5-6% reads matching plant and fungi. A total of 67 OTUs were determined and the OTU identification revealed 70-80% Pythiales (*Pythium* and *Phytophthora*), 5% Saprolegniales and 1% Peronosporales. The latest study published on oomycete community analysis also used 454 pyrosequencing of the *cox*I mitochondrial marker to evaluate the diversity of oomycete species in soybean fields in Pennsylvania (Coffua et al. 2016). The *cox*I marker could be aligned allowing phylogenetic

inference of the species observed across the communities, 49 OTUs were observed and matched to known oomycete species.

To date, there are not studies published using illumina approaches focused only on oomycete communities. Nonetheless, different groups have reported the use of illumina sequencing to study *Phytophthora* spp. communities (Cooke et al. 2014, Morales-Rodríguez et al. 2014). A recent paper proposed the used of peptide nucleic acid (PNA) probes to block amplification of non-target sequences and also developed or modified existing primers to study microbial diversity associated with plant hosts (Agler et al. 2016). Primers for oomycete ITS 1 and ITS2 were developed, but amplification of non-targets was not an issue in ITS1, however, the ITS2 had less than 5% non-target amplification that was eliminated with the PNA probes. The study proposed a holistic approach looking at different markers for bacteria (16S), fungi (ITS1 and ITS2), oomycetes (ITS1 and ITS2), and other lower eukaryotes (18S) at the same time capturing a mixed-kingdom community.

Barcodes for identification of oomycetes

The study of oomycete species through molecular methods has progressed rapidly in the last 20 years, due to the limitations of the morphology-based characterization, which fell short in classification of the different isolates found in nature within specific species (Erwin and Ribeiro 1996). Following the path of fungal ecology and biology, the rDNA was targeted to identify different species among the oomycetes in the late 1980s and early 1990s. Regions targeted were 18S, 5.8S or even 28S, using restriction analysis and hybridization (Figure 1.2) (Lévesque 2011). It was not until the mid-1990s that partial sequences of the large subunit (LSU) and small subunit (SSU) of the rDNA were used for the identification of *Pythium* and *Phytophthora* and other

oomycete species (Briard et al. 1995, Dick et al. 1999). The ITS region of rDNA was not considered until 1992, when Lee and Taylor (1992) used the internal transcribed spacer (ITS) sequences to identify five *Phytophthora* spp. The use of ITS as marker to identify and group the oomycetes was extended by Cooke et al. (2000a) and Lévesque and De Cock (2004) who developed comprehensive phylogenies of *Phytophthora* and *Pythium* spp., respectively. These studies provided the bases and propagated the use of ITS to study oomycetes, increasing the number of oomycete species reported. By using the rDNA as region, Beakes et al. (2012) and others revealed a greater diversity, demonstrating the existence of basal clades outside of saprolegnian and peronosporolean groups proposed earlier (Lévesque 2011). More recent studies, have focused on plant pathogenic species revealing a greater diversity even within well-studied groups in the oomycetes by using multi-locus approaches, resulting in new genera such as *Phytopythium* (Huang et al. 2013, Thines 2014, De Cock et al. 2015).

The full ITS region, comprising ITS1 and ITS2 (Figure 1.2a), has enough variability to resolve most oomycete species (Robideau et al. 2011), providing enough interspecific and intraspecific distances separating major genera, features desired in a barcode marker. However, the ITS as barcode has limitations in some of oomycete species, downy mildews that have multiple repeats in ITS2 making this region longer 1000 bp, which causes issues with amplification and molecular analyses of this marker due to the length variability of the ITS (Thines et al. 2005). The differences in length across different species results in an alignment with multiple gaps, which is undesirable for phylogenetics and other approaches (Figure 1.2b). A second limitation is the similarity of the ITS sequences in closely related species, having a similarity higher than 98%, which will cause issues in the identification of specific species

(Robideau et al. 2011). An additional barcode marker proposed was the Cytochrome c oxidase subunit I (*cox*I), which is a mitochondrial marker which could be used for multiple organisms other than oomycetes, including plant and lower animals (Hebert et al. 2003, Chase et al. 2007). In comparison to the rDNA, this marker does not have length variability across different oomycete species, but there is enough variability to resolve genera and species, allowing the alignment of multiple sequences of this protein-coding gene. Robideau et al. (2011) evaluated the performance *cox*I as a barcode marker across oomycete genera, mainly *Pythium* and *Phytophthora* species. The evaluation of *cox*I resulted in a more robust separation of certain species, but as the ITS, *cox*I was not able to separate species of oomycetes, like *Ph. capsici* from *Ph. mexicana* or *Py. acrgynum* from *Py. hypogynum*.

The number of sequences available for the ITS are the most comprehensive, currently over 15 thousand sequences have been deposited in genbank representing over 150 species, however this resource should be used with caution, since a large number of these sequences are misclassified (Kang et al. 2010). Due to the efforts of different scientists, three different resources are available for the community. The first two are the *Phytophthora* db database (Park et al. 2013) and *Phytophthora*-ID (Grünwald et al. 2010), both focus on the same genus and both provide curated sequences of different loci, including ITS and *cox*I, for the identification of *Phytophthora* species. The third option is a more comprehensive resource part of the Consortium for the Barcode of Life (http://www.barcoding.si.edu/), the database contains curated oomycete sequences for both ITS and *cox*I including chromatograms. This database contains 2114 records that represent 425 species for the ITS and 2538 records that represents 445 species for *cox*I (http://v4.boldsystems.org/, visited May 2016). The availability of such resources

facilitates the development of new projects focused on cataloging the diversity of oomycete species. In addition, these resources will aid the improvement or design of new targets that may be used for next generation sequencing to characterize communities, facilitating the ability to study the ecology of these organisms. Nonetheless, there are limitations in the extent of these databases and others, since some species only have a few sequences.

The BOLD systems database also has sequences deposited for other loci, such as *cox*II, *cox*III and atp6 that may be used for identification and phylogenetic analysis. Recently, *cox*II and the spacer between the *cox*I and *cox*II gene have also been suggested as useful barcoding markers for resolving species (Choi et al. 2015). These two regions were compared with the *cox*I to evaluate their performance as barcodes. Taking into account intra- and inter-specific distances and identification criteria, two main points were observed: (i) *cox* spacer was the most variable with higher interspecific distance, but intraspecific distances were similar for the three loci; (ii) *cox*II resulted in the lowest ambiguous and incorrect identifications, followed by *cox*I, while *cox* spacer resulted in a higher number of ambiguous identifications. However, there is still a need as a community to improve resources that could be standardized and implemented widely to provide the necessary data to answer questions on the ecology and epidemiology of the oomycetes in different ecosystems, for instance BOLD systems stands as the most useful resource. The availability of genomic resources will provide resources to further look at new potential barcodes to study this group (Lévesque et al. 2010, Adhikari et al. 2013, Kronmiller et al. 2015).

Species definition and number of species in oomycetes

An estimate of diversity within the oomycetes was proposed by Dick (1999) to contain at least 900 to a maximum of 1500 species, he also proposed to rename the oomycetes as the Peronosporomycetes. As as point of reference, fungal species were initially suggested to be 1.5 million and due to recent sequencing efforts was updated to 5 million species (Blackwell 2011). Molecular methods have increased our understanding of oomycetes just as it has in other groups, like the fungi, increasing the number of species described in the last 20 years. However, the definition of species has become an important topic for ecological studies, since it will be key to understand the diversity and abundance of these organisms. Speciation of oomycetes has been reviewed before focusing on plant pathogens (Restrepo et al. 2013), listing the reproductive isolation mechanisms: premating, postmating and postzygotic structures that will result in the delineation of new species. Especially, those arose from interspecific crosses resulting in hybrid formation.

Hybridization plays an important role in the oomycetes, and it has been widely studied in the genus *Phytophthora* with profound changes in the ecology of some species resulting in expansion of host ranges or adaptation to new environments (Stukenbrock and McDonald 2008, Goss et al. 2011, Bertier et al. 2013). This is also true for other members of the oomycetes, like some complexes in *Pythium*, where there is evidence of movement of genetic material (Spies et al. 2011b). The fact that hybrids exist in nature should be considered when working with diversity of oomycete species, usually studies present evidence based on the ITS sequences (Bertier et al. 2013, Burgess 2015), but it will not be evident from mitochondrial markers since those are inherited from one of the parental lines (Whittaker et al. 1994). Therefore, there will be

limitations and biases that should be considered when comparing across different markers for community analysis.

One of the main points that has been discussed in oomycetes and fungi is delimitation and the recognition of species, where a single gene is not enough to determine species (Giraud et al. 2008, Lévesque 2011). Rather than using single genes to determine the existence of new species, a more reliable approach should be taken, as the genealogical concordance phylogenetic species recognition proposed by Taylor et al. (2000). In ecological studies this should be acknowledge since there are limitations of individual markers, in fact, the ITS is the marker used to identify hybrids since it can delineate differences, those differences can be the result of a speciation process. Nonetheless, the use of single genes at higher taxonomic ranks allows the comparison of communities, but their performance in terms of resolution varies. The use of sequences and in some occasions DNA fingerprints allow the use of operational taxonomic units (OTU), which are defined based on sequence similarity and clustering methods (Caron et al. 2009). A threshold is required to define OTUs, however, it is important to keep in mind that the OTU concept is not a directly analogous to species, therefore caution should be taken when using the term. For instance, the fungal ITS was evaluated to determine variation intragenomically, resulting in variability higher than a 3% threshold, which can cause taxon inflation, and OTUs could represent similar species (Nilsson et al. 2008). In addition, the high variability of this region causes resolution problems at the family level (Lindahl et al. 2013). This could be a another point to take into account when working with oomycetes, since it has been observed that some members of this group also have variable ITS, this was reported in *Pythium* spp. (Schroeder et al. 2013).

Applications of NGS to study the ecology of oomycetes

The diversity of oomycetes by using NGS is a critical research topic since there is still multiple unknown species that could be present in different ecosystems, and they could represent a threat to native plant species. Most recent epidemics are result of movement of pathogens across ecosystems, therefore if there is a better knowledge of distribution and diversity of oomycete species, better control measures could be taken to avoid epidemics. It was also suggested that there could be overlooked species, since most of the species described are the result of surveys for pathogens. New species in the genera *Halophytophthora* and *Salisapilia* were discovered in estuarine and coastal environments where these species could serve as potential decomposers (Hulvey et al. 2010, Nigrelli and Thines 2013), however the ecological roles of these organisms are not well known.

The use of amplicon sequencing to characterize oomycete communities will give insight on the forces that drive diversity of these organisms. As demonstrated by the disease triangle concept, the interaction of environment, host and pathogen requires the right balance for disease development, which can result in an epidemic. The use of novel approaches can improve the knowledge of oomycete ecology, especially for agricultural and forest systems, where these organisms are major threats. In agriculture, host germplasm and its effects on the associated community remain an important question, since cultivars of the same host or cover crops could have an effect on the microbiome. For instance, Bakker et al. (2016) studied the effect of a rye cover crop before corn and found that the cover crop actually increased the density of root rot pathogens, thus increasing the potential for disease. The amplicon sequencing approach can be used to study the effect of other cover crops or rotation systems on the density of oomycete

pathogens. Other focus of the research is the effect of different disease management strategies on the oomycete community composition. The adoption of traditional practices such the use of compost or manure in traditional and organic agriculture had effects on the diversity of microbial communities (bacteria and fungi), increasing the diversity on organic crops (Hartmann et al. 2014). The increased diversity observed resulted in the enrichment of certain guilds in charge of the metabolism of certain molecules present in manure or compost. However, different crops used different practices, like in soybean and corn, where the use of tilling, seed treatments or fungicide applications can have a strong effect on the selection of the associated microbial community as it was observed in the wheat phyllosphere (Karlsson et al. 2014, Pérez-Jaramillo et al. 2015).

With respect to the role of environment on the distribution and ecology of oomycetes there is not much information, however in fungal species this question has been addressed. For example, arbuscular mycorrhizal fungi (AMF) were evaluated under a climate gradient to determine the role of environment on the colonization of plants by this group of fungi (Wilson et al. 2016). The climate gradient resulted in a decreased colonization by AMF by indirect effects of increased temperature like plant biomass, water availability and nutrient availability. Likewise, oomycete pathogens associated with different ecosystems could have different responses to the climate change, which could result in the emergence of new diseases (Anderson et al. 2004, Stukenbrock and Bataillon 2012).

The understanding of the niche concept could be an important focus of research since many oomycetes represent a threat, but it could be of great value to predict the niche of different

species, even more those that could represent a threat to food production and natural systems. It has been suggested that oomycetes and fungi niches are determined by the molecules secreted (e.g. hydrolytic enzymes, toxins, or other proteins) that are present in the genome and are part of the traits that determine the ecological niche (Soanes et al. 2007). For instance, the ability to cause disease on specific plant hosts or to degrade plant tissues, in conjunction with the actual distribution of the species will help to delineate the niche and range of oomycete species. Furthermore, using climate models, some parameters such as temperature, drought tolerance or pH (Rillig et al. 2015) could be evaluated phenotypically to provide more information to establish the niche of species (Rillig et al. 2015). These parameters could be measured under controlled conditions and provide models to determine the likelihood of survival or threats in certain ecosystems.

The microbial community around the root system and rhizosphere is a complex system that provides an environment where different interactions can occur, and could define the co-existence of species also affecting their niche. The availability of genomes of oomycete will provide an extensive array of information including effectors and molecules that can be secreted into the surrounding environment or the host (Soanes et al. 2007). These molecules could hypothetically influence the interactions with the surrounding microbiota, since these could affect bacteria, fungi or other microorganisms in the soil or colonizing the root or plant tissue (Kemen 2014). There are different studies in the interaction of oomycetes in the soil with other organisms, for instance some bacteria reduce zoospore germination and cyst formation thus reducing infection by antibiosis (Heungens and Parke 2000), other examples with bacteria also refer to enhancing or controlling oomycetes, or bacteria using hyphae to mobilize and acquire

specific substrates (Frey-Klett et al. 2011). New interactions could be identified by analyzing not only oomycetes in specific environments, but also considering fungi and bacteria to establish networks that show co-occurrence of specific taxonomies (Barberán et al. 2012).

Perspectives of oomycete ecology

Most of the development in community analysis and metagenomics have been conducted with bacterial communities, nonetheless fungal studies have also come a long way by modifying the existing methods in order to be able to actually characterize communities and understand the ecology and function (Lindahl et al. 2013). Oomycete community studies are in the early stages, where some of the methods are being translated to this group of organisms that was already paved by the fungal ecologists. Researchers interested in oomycete ecology should have an easier path towards the understanding of these organisms. Publications have referred to this field as metagenomics, however it is important to clarify that metagenomics is based on extraction of total DNA from the community, and sequencing the DNA without amplification. Therefore, amplicon sequencing should not be considered metagenomics. Metagenomics data analyses are a major task since the analyses pipelines are still in development and published studies that reported a taxonomic assignment showed that oomycetes are not usually well represented (Paula et al. 2014). The lack of representation of oomycetes could be due to different causes such as, the lack of annotation in databases, lack of sequenced genomes, and this group could just represent a small fraction of the community. Therefore, the research community should work together to improve the technical and knowledge gaps to allow oomycetes to become better represented in metagenomic studies.

Another area that is actively being discussed in bacteria as well as in fungi, is the concept of an active community, which is especially important in soil, where different dormant and/or resistant structures can be found, even naked DNA (Lennon and Jones 2011). These structures are usually resistant spores, like oospores or chlamydospores, which are often dormant, and only during the right conditions (e.g. pH, water content, host, or microbes), these spores germinate and become active. Nonetheless, it has been observed in some groups of fungi that there is just a fraction of the community that is active (Liao et al. 2014).

Different methods could be used to address the active community, but the most comprehensive method is metatranscriptomics. It refers to the sequencing of total RNA from the community to determine the gene expression of all the species present in the sample and use this data to get an idea of the taxonomic composition of the active community. This is a major challenge, since currently analyzing a transcriptomic experiment which consists of a single host inoculated with a single oomycete pathogen requires genomes of both organisms to facilitate the understanding of the interaction (Kuske et al. 2015). The transition to a complex community could become a major challenge not only because of the complexity of the analysis, but also due to the fraction of RNA that corresponds to the oomycete community. However, controlled experiments under greenhouse or growth chamber conditions using artificial communities could give us an insight into how some of those species interact together and with their host. The study of an active community will also help to provide information on traits that could be derived from the genome and transcriptome data that could define a functional guild in oomycetes, linking species with actual traits as it is currently being done with fungi (Nguyen et al. 2016). In addition, the information on traits could help to delineate better niches in oomycetes, and also

develop better experiments to understand the succession or temporal variation of the community (Aguilar-Trigueros et al. 2014).

The knowledge of trait data and niche could assist to obtain more information on new species and ways to grow them, this has been done on bacteria (Oberhardt et al. 2015) and there are some studies on fungi targeting groups actively growing on hosts (Bonito et al. 2016). The ideal situation would be using fungal and oomycete culture collections to work together to build a database containing nutrient requirements and strategies that will help to isolate specific groups of oomycetes from different ecosystems. In addition, the use of phylogenetics with existing material will help to localize new species within specific clades and depending on trait mapping, trees could provide inform on the nutritional requirements for some specific groups, aiding the recovery of new species. Finally, the understanding of the diversity and development of sound techniques could also aide in the detection and monitoring of pathogens. Studies that have focused on the use of geostatistical models and sampling of fungal communities have suggested the possibility of using some of these amplicon studies as proxy for soil health and disease prediction (Xu et al. 2012, Steere et al. 2016). Information on oomycetes could also be incorporated since these studies are mostly on plant pathogens and could be very informative for soilborne diseases where multiple species co-occur.

The study of oomycete diversity and ecology will be critical to manage diseases and control movement of pathogens into ecosystems. By characterizing the oomycete community and the role of host and environment on the community will help to device better strategies to control them. The state of the art and approaches summarized show how ecology is converging

with plant pathology, moving towards the same questions, what affects community structe? How the community is assembled? What conditions promote certain species? Since we have now the tools to study complex systems, such as the rhizosphere or phyllosphere, where multiple species co-exist and interact to produce or not disease should be a major focus of study to characterize the phytobiome.

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Chapter 2

Oomycete species associated with soybean seedlings in North America – Part I:

Identification and pathogenicity characterization

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ABSTRACT

Oomycete pathogens are commonly associated with soybean root rot, and have been estimated to reduce soybean yields in the United States by 1.5 million tons on an annual basis. Limited information exists regarding the frequency and diversity of oomycete species across the major soybean producing regions in North America. A survey was conducted across 11 major soybean producing states in the U.S. and the province of Ontario, Canada. In 2011, 2,378 oomycete cultures were isolated from soybean seedling roots on a semi-selective medium (CMA-PARPB) and identified by sequencing of the ITS region of rDNA. Sequence results distinguished a total of 51 *Pythium*, 3 *Phytophthora*, 3 *Phytopythium* and 1 *Aphanomyces* spp. in 2011, with *Py. sylvaticum* (16%) and *Py. oopapillum* (13%) being the most prevalent. In 2012, the survey was repeated, but due to drought conditions across the sampling area, fewer total isolates (n= 1,038) were collected. Additionally, in 2012, a second semi-selective medium (V8-RPBH) was included which increased the *Phytophthora* spp. isolated from 0.7% to 7% of the total isolates. In 2012, 54 *Pythium*, 7 *Phytophthora*, 6 *Phytopythium* and 1 *Pythiogeton* sp. were recovered, with *Py. sylvaticum* (14%) and *Py. heterothallicum* (12%) being recovered most frequently. Pathogenicity and virulence were evaluated with representative isolates of each of the 84 species on soybean cv. 'Sloan'. A seed rot assay identified 13 and 11 pathogenic species at 13ºC and 20ºC, respectively. A seedling root assay conducted at 20ºC identified 43 species as pathogenic, having a significantly detrimental effect on the seedling roots as compared to the non-inoculated control. Fifteen species were pathogenic in both the seed and seedling assays. This study provides a comprehensive characterization of oomycete species present in soybean seedling roots in the major production areas in the U.S. and Ontario, Canada, and provides a basis for disease management and breeding programs.

Introduction

Soybean (*Glycine max* (L.) Merr.) is second only to corn (*Zea mays*) in the United States in importance for feed and industrial uses. Poor crop establishment and plant stand due to seed and seedling diseases greatly reduces the soybean crop yield potential in many areas. In 2009, soybean yield loss as a result of seedling diseases in the U.S. was estimated to be 1.51 million tons (Koenning and Wrather 2010). Many factors may influence plant stand and root health, such as seed quality, edaphic and environmental conditions (e.g. soil type, soil moisture, precipitation, and temperature), soil microorganisms, and especially diseases (Broders et al. 2009). Soilborne seed and root diseases are attributed to many pathogens including *Fusarium* and *Rhizoctonia* from the kingdom Fungi and the oomycetes *Pythium* and *Phytophthora* from the kingdom Stramenopila (Kaufmann and Gerdemann 1958, Anderson 1987, Rizvi and Yang 1996). In the U.S., there has been a increase in soybean yield loss caused by the oomycetes *Phytophthora* and *Pythium* (Wrather and Koenning 2009, Koenning and Wrather 2010). This increased incidence of oomycete-related diseases could be due to lack of material resistant to *Pythium* spp., pathotypes of *P. sojae* able to overcome existing *Rps* R genes, changes in precipitation patterns and cultural practices used by growers, such as earlier planting dates and greater rainfall in spring and early summer, in conjunction with minimum tillage practices (Melillo et al. 2014, Dorrance et al. 2016).

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

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

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

Py. ultimum and cation exchange capacity (CEC) correlated with specific species such as *Py. kashmirense, Py. heterothallicum* and *Py. irregulare* increasing their frequency.

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

Materials and methods

Sample collection and isolation. In 2011 and 2012, a survey was conducted across 11 states, covering the primary U.S. soybean production area and Ontario, Canada (Fig. 2.1, Suppl. Table S.2.1). A total of 64 and 61 fields were sampled in 2011 and 2012, respectively. Approximately six fields were sampled per year in each participating state, and those fields were selected based on field history of seedling diseases and plant stand issues. Collaborators followed a standard sampling procedure, which specified collection of 50 symptomatic soybean seedlings from a W-shaped transect across each field. Due to crop rotation practices, diseased soybean fields sampled in 2011 were different from the fields sampled in 2012. Seedling samples from

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

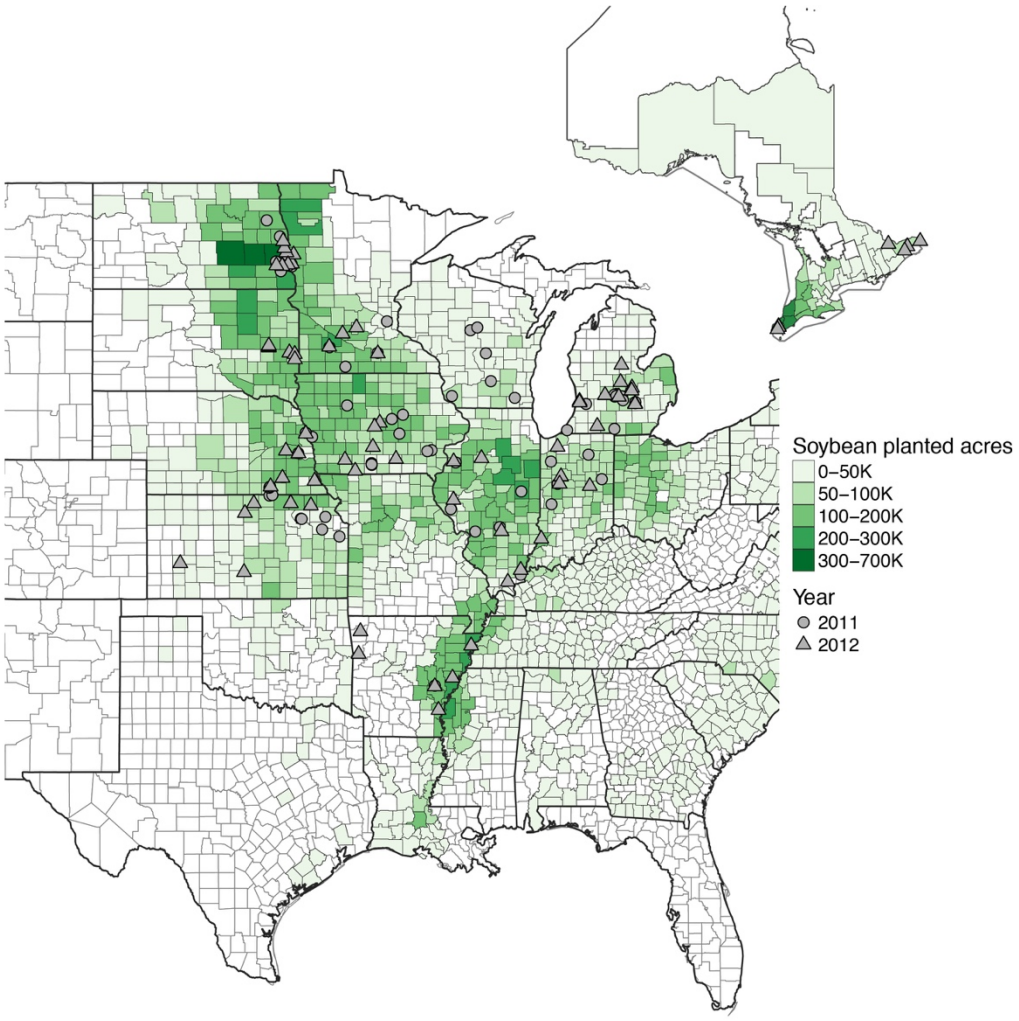

Figure 2.1. Map of sampled soybean fields in 2011 and 2012, and intensity of planted soybean acres demonstrated by color intensity at the county/parish level.

Isolate storage and DNA extraction. Isolates shipped to Michigan State University were transferred to CMA-PARPB medium and 5-mm plugs were taken from fresh cultures and transferred to potato carrot agar slants and hemp seed vials for long-term storage (van der Plaats-Niterink 1981, Erwin and Ribeiro 1996). Three to five 5-mm plugs from fresh cultures were transferred into 50 mL of a 10% V8 broth amended with ampicillin (100 mg/L) in 125 ml Erlenmeyer flasks and incubated for 7 to 10 d 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 µL cetyltrimethylammonium bromide (CTAB) lysis buffer (AutoGen AG00121, AutoGen Inc.) and incubated for 1 h at 65ºC. A phenol-chloroform automated DNA extraction was performed using the AutoGen 850 system (AutoGen Inc., Holliston, MA). DNA was resuspended in 200 µL TE buffer, incubated on an orbital shaker for 1 h, then transferred to 1.5 mL tubes, and stored at -20ºC.

Identification of isolates. 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 1X DreamTaq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM ITS6 and 0.2 µM ITS4, 4 µg/mL of BSA, 1U DreamTaq polymerase (Thermo Scientific, Waltham, MA), and 1 uL DNA. The amplification program consisted of 95ºC for 2 min; 35 cycles of 95ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min; and a final extension at 72ºC for 10 min. Amplicons were purified by adding 5 µL of a mixture of 3U of exonuclease I and 0.5 U of FastAP thermosensitive alkaline phosphatase (Thermo Scientific, Waltham, MA). Samples were incubated for 45 min at 37ºC and enzymes were inactivated by incubation at 85ºC 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 1000 and identity higher than 97% were assigned to a taxonomic designation based on the BLAST output. Sequences were deposited in GenBank under accession codes KU208091 - KU211502.

Seed pathogenicity assay. When available, three isolates of each identified species, that were arbitrarily selected, were evaluated for pathogenicity and virulence on soybean seeds. For a limited number of species, isolates obtained from corn were substituted in order to screen three isolates per species for pathogenicity as indicated in supplemental file 2. A total of 207 isolates representing 84 oomycete species were characterized. A petri dish seed pathogenicity assay was conducted as reported by Broders et al. (2007) with the following modifications: isolates were grown on CMA for 4-7 d, and a 5-mm plug was transferred from this CMA active culture to the center of a 1.5% water agar plate and incubated for 2 days. Seeds of the soybean cultivar 'Sloan' were surface disinfested with a 0.36% sodium hypochlorite solution for 10 min, rinsed with sterile, distilled water three times, and allowed to air dry in a laminar flow hood for 15 min. Ten seeds were placed at the growing edge of the colony. Plates were incubated in the dark for 7 d at 13ºC or 20ºC. These temperatures were based on the average soil temperature at planting in northern and southern U.S. climates (Rojas et al. 2016a). Each isolate was evaluated in 3 replicate plates) at each temperature, and the experiment was conducted three times per temperature.

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

$$\text{DSI} = \frac{\sum(Severity\ rating \times Seeds\ per\ rating)}{(Total\ seeds\ \times\ Highest\ severity\ rating)} \times 100$$

Due to the large number of isolates and replicates, the species were divided into seven sets with each set containing a control without pathogen. A linear mixed model was used to evaluate DSI as a response variable and species as a fixed effect, and nesting isolates within species and experiment as a random effect. Dunnett's contrast was applied to determine species that were significantly different from the control. Temperatures were analyzed independently. In addition, hierarchical clustering was performed to separate species into a cluster using DSI at 13ºC and 20ºC. Statistical analyses were conducted in R version 3.2 (R core team 2015, Vienna, Austria) using packages 'lme4' and 'lsmeans', and graphs were generated with the package 'ggplot2'.

Seedling root rot assay. The same isolates used for the seed pathogenicity assay were also evaluated in a seedling root rot assay. Inoculum was prepared by placing 25 g of long-grain rice and 12 mL of distilled water in 125-mL flasks, followed by autoclaving for 25 min, and cooled overnight (Holmes and Benson 1994). The rice grains were mixed using sterile technique, autoclaved for another 30 min, and cooled overnight. Five 5-mm plugs from 4-7 d old cultures of each isolate were transferred into the rice flasks, and incubated in the dark at room temperature (20-22ºC) for 10-14 d. The rice inoculum was mixed regularly to assure full colonization of rice grains and to loosen and separate grains. Seedling assays were performed in 355 mL capacity paper cups (IC12-J7534, Solo cup, Lake Forest, IL) with four 0.5 cm drainage holes in the bottom. Cups were layered from bottom to top with 50 mL of coarse vermiculite, 150 mL of fine vermiculite, 7 g of colonized rice, 100 mL of fine vermiculite, 6 soybean cv. 'Sloan' seeds, and 100 mL of coarse vermiculite. The vermiculite substrate was initially moistened to water-holding capacity, and thereafter, plants were watered every other day with

tap water. Cups were maintained in a growth chamber (BioChambers, Manitoba, Canada) with a light regime of 14 h light (250 μE $m^{-2} \cdot s^{-1}$) and 10 h dark, at 98% humidity, and 20ºC for 14 d.

Due to the large number of isolates, the isolates were grouped by species and randomly assigned into seven sets that were used as a block. Every isolate had three cup replicates per experiment, and each experiment was conducted three times for every set. Two controls were included within every experiment, a control with non-inoculated autoclaved rice and a non-rice control to account for any effects of the rice on the seedlings. At the completion of the experiment, plant roots were washed with tap water to remove debris for evaluation. Five washed plants were scanned to determine root area and root length and placed in a drying oven at 50ºC for 48-72 h to establish dry weight of roots and shoots. Re-isolations were made as described below.

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

The identity of the isolates was confirmed by single strand conformation polymorphism (SSCP) (Kong et al. 2004, Kong et al. 2005). In order to have a positive confirmation, colony PCR was conducted on the isolate inoculated and the isolate recovered from infected root tissue using primers ITS6 and ITS7 (Kong et al. 2004). Briefly, a small

fragment of mycelia was taken from the plate, placed into 100 µL of sterile distilled water, boiled for 5 min in a heat block at 95ºC, and 1 µL of this boil prep was used for PCR. Amplification was completed as described previously using primers ITS6 and ITS7. The PCR products were used for the SSCP analysis, following methods described by Kong et al. (2004). To ease scoring of gels, denatured PCR products from each original and recovered isolate were run side by side in the polyacrylamide gel. Isolation and SSCP confirmation were conducted for each experiment, thus each isolate had three re-isolation attempts.

Root area and root length image analysis. Images of roots from the seedling root rot assay were obtained with a flatbed scanner (Epson Perfection 4870 Photo Pro; Epson America, Inc., Long Beach, CA) at a resolution of 300 dpi and saved as JPEG files. Every image included a photographic reference scale to calibrate measurements from pixels to cm. All images were analyzed with Assess 2.0 (APS, St. Paul, MN), using HSI color space (hue values between 0 and 121) to limit the selection to just root tissue for determination of root area and length using a calibrated scale.

Data analysis for root measurements. A multivariate analysis of variance (MANOVA) was performed to evaluate seedling variables measured: root dry weight, shoot dry weight, shoot:root weight ratio, root area, and root length. Prior to analysis, the variables were log10 transformed to improve normality and scaled and centered to aid analysis. An initial exploration of all the response variables was conducted using principal component analysis, and the contribution of each variable was examined. Based on contribution, a MANOVA test was utilized to examine differences among the 84 oomycete species characterized, using root dry

weight, root area, and root length as response variables. Species was treated as a fixed effect while isolates and experiment were treated as random effects. To verify significance, univariate analyses were conducted for each of the correspondent response variables using the same model. Dunnett's test was used to determine those species significantly different from the non-rice control. Data were analyzed using R version 3.2 (R core team 2015, Vienna, Austria) with packages 'FactoMineR', 'nlme', 'MASS' and 'lsmeans', and graphs were generated with the package 'ggplot2'. All data and R scripts used in the analyses shown here are deposited on github (https://github.com/Chilverslab/Rojas_Survey_Phytopath_2016) and citable (Rojas et al. 2016b).

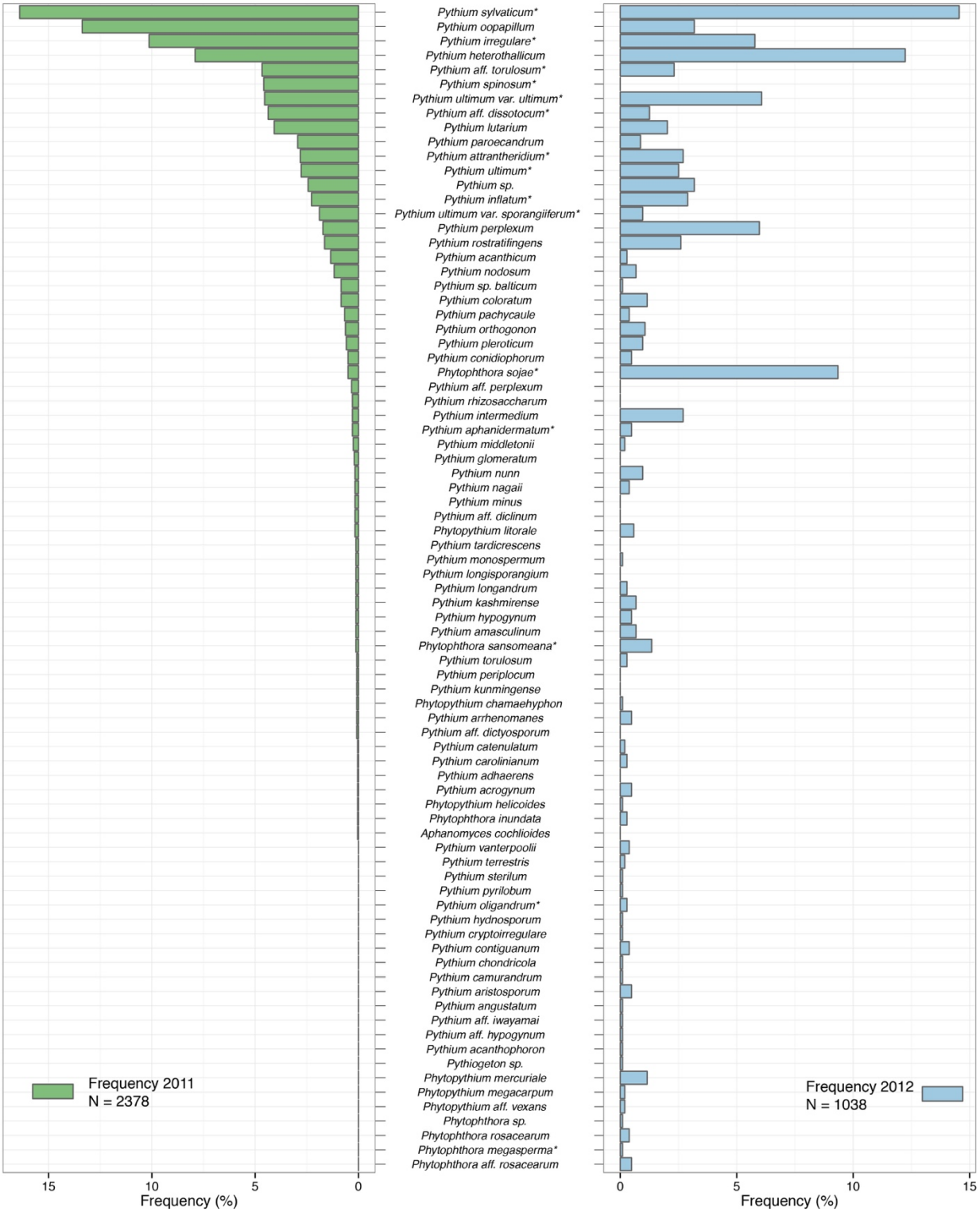

Figure 2.2. Frequency at which different Oomycete species were recovered from diseased soybean seedlings in 2011 and 2012. (*) Species previously reported as associated with soybean in the fungal-host database (http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm).

Results

Sample collection. A total of 3,418 oomycete isolates were collected during the two-year study, 2,380 isolates in 2011 and 1,038 isolates in 2012. A total of 84 oomycete species were identified (Fig. 2.2) using the ITS region of the rDNA. The genus *Pythium* was the most dominant across the samples, followed by *Phytophthora, Phytopythium,* and *Aphanomyces*. In 2011, the 12 most abundant species recovered, which comprised more than 78% of total isolates, were *Py. sylvaticum* (16.3%), *Py. oopapillum* (13.3%), *Py. irregulare* (10.1%), *Py. heterothallicum* (7.9%), *Py.* aff. *torulosum* (4.7%), *Py. spinosum* (4.6%), *Py. ultimum* var. *ultimum* (4.5%), *Py.* aff. *dissotocum* (4.4%), *Py. lutarium* (4.1%), *Py. paroecandrum* (2.9%), *Py. attrantheridium* (2.8%), and *Py. ultimum* (2.8%) (Fig. 2). In 2012, there was a shift in the frequency of species isolated, however, there were similarities between the two years. Seven of the 12 most abundant species, which comprise more than 70% of isolates recovered, in 2012 were also within the top 12 species recovered in 2011. The most abundant species in 2012 were *Py. sylvaticum* (14.5%), *Py. heterothallicum* (12.2%), *Phytophthora sojae* (9.3%), *Py. ultimum* var. *ultimum* (6.1%), *Py. perplexum* (6.0%), *Py. irregulare* (5.8%), *Py. oopapillum* (3.2%), *Py. inflatum* (2.9%), *Py. attrantheridium* (2.7%), *Py. intermedium* (2.7%), *P. rostratifingens* (2.6%) and *Py. ultimum* (2.5%), (Fig. 2). Other genera recovered from soybean seedlings that were outside the scope of this study included members of the fungal genera *Mortierella, Mucor, Gongronella, Rhizoctonia,* and the mycoparasite, *Laetisaria*.

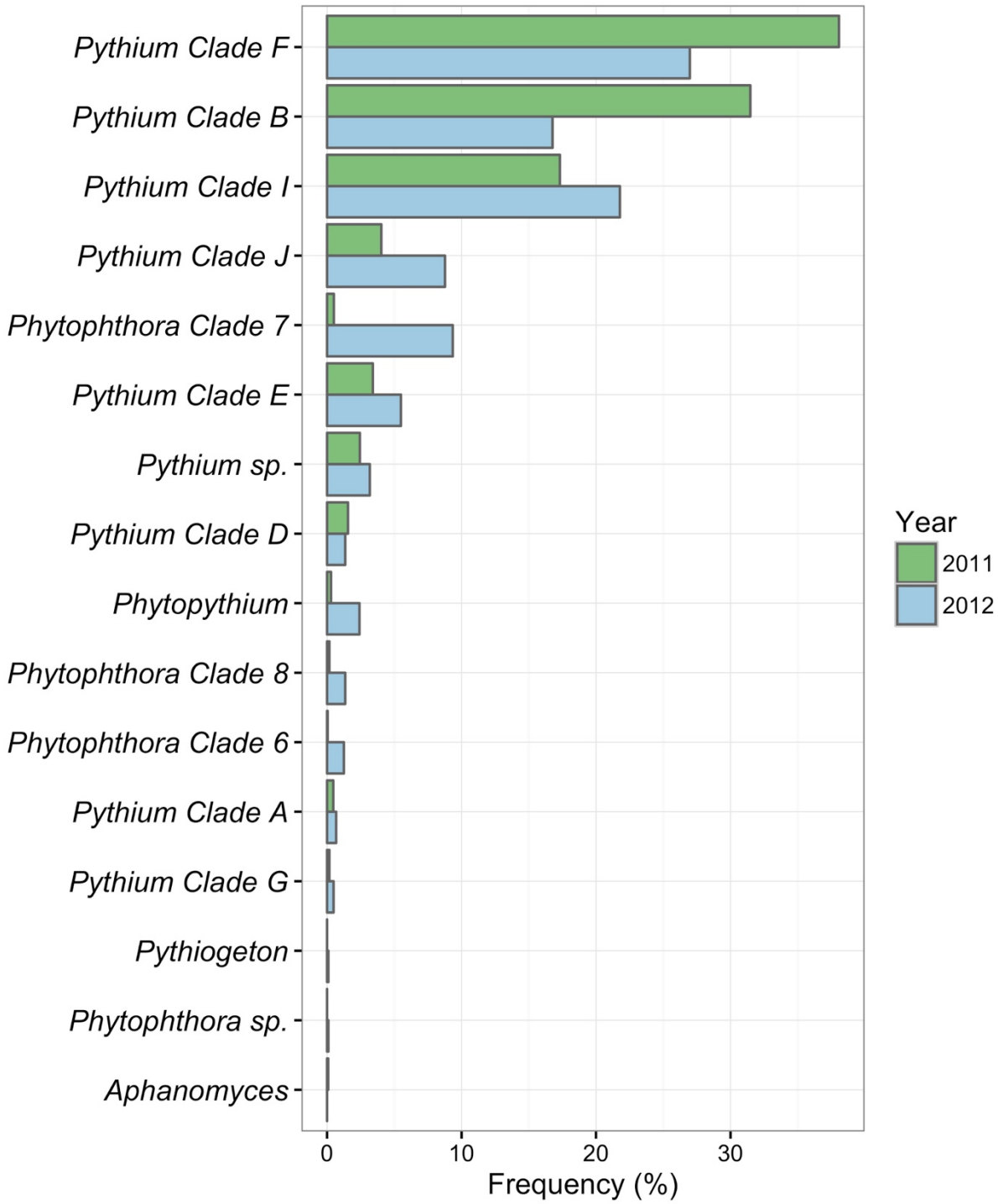

Figure 2.3. Frequency of the oomycete species summarized by clade for 2011 and 2012. Oomycete genera outside of *Pythium* and *Phytophthora* were summarized by genus. Those species designated as spp. are not well resolved based only on the ITS sequence.

Among the 84 species isolated in this study, only 13 species were previously reported to be associated with soybean in the fungal-host database (ARS, http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm), including the well-known pathogen *P. sojae*. The isolation frequency of *P. sojae* at the early plant growth stage sampled was 0.5% in 2011. A second semi-selective medium was included in 2012, in attempt to increase the recovery of *P. sojae* and other *Phytophthora* species. In 2012, the recovery of *Phytophthora* spp. increased to 12% when different methods (different media and numbers of plants per medium) were used. The frequency of *Phytophthora* spp. increased, but still remained low in comparison to genus *Pythium*. The low recovery could be attributed to recovery of fast-growing species, such as *Pythium* and *Mortierella*, that interfere with the isolation of *Phytophthora* spp. (Tsao and Guy 1977).

Summarizing the data by clade (Fig. 2.3), *Pythium* clades F (36% in 2011 and 26% in 2012) and B (23% in 2011 and 13% in 2012) were the most abundant clades isolated during this study. These clades contain known pathogenic species such as *Py. sylvaticum* and *Py. irregulare* in clade F, and *Py. oopapillum* and *Py. torulosum* in clade B. With respect to *Phytophthora*, clade 7 was present at a frequency of 1% in 2011 and 4% in 2012, and clade 8 at <1% in 2011 and 3% in 2012. *Phytophthora* clades 7 and 8 contain the species *P. sojae* and *P. sansomeana*, respectively. The recently recognized genus *Phytopythium* was recovered at a lower frequency with respect to the other related genera and was detected at just 1% and 3% in 2011 and 2012, respectively (Fig. 2.2). There were 91 isolates designated as *Pythium* spp. that were not resolved to species level, but these are currently under further evaluation.

Seed pathogenicity. The main goal of this assay was to determine which species were pathogenic to soybean (i.e. resulting in significant seed rot compared to a non-inoculated control) at temperatures representative of planting conditions in the northern and southern U.S. The seed rot assay at 20ºC identified 11 species that were pathogenic, with disease severity index scores ranging from 80 to a maximum score of 100 (Table 2.1, Table S.2.2). Among the species observed, *Py. aphanidermatum, Py. ultimum* sensu lato, and *Py. cryptoirregulare* were the most virulent and caused severe seed rot. In addition to *Pythium* spp., *P. sansomeana, P. drechsleri* and *Phytopythium helicoides* were also pathogenic on soybean seed, colonizing the seed and causing significantly reduced germination at 20ºC.

Evaluation of seed rot at 13ºC identified 13 *Pythium* spp. as pathogenic, with disease severity indexes ranging from 78 to a maximum score of 100 (Table 2.1, Table S.2.2). However, none of the *Phytophthora* or *Phytopythium* spp. were identified as being pathogenic at this temperature, typical of planting conditions in the Midwest. Only seven *Pythium* spp. were pathogenic at both temperatures, with *Py. ultimum* sensu lato and *Py. cryptoirregulare* being the most virulent. However, there were shifts in virulence observed among oomycete species at the different temperatures. For instance, *P. sansomeana, P. drechsleri* and *Phytopythium helicoides* showed less virulence at 13ºC than 20ºC (Fig. 2.4, Table 2.1), while *Py. sylvaticum, Py. terrestris* and *Py. paroecandrum* appeared more virulent at 13ºC but not at 20ºC.

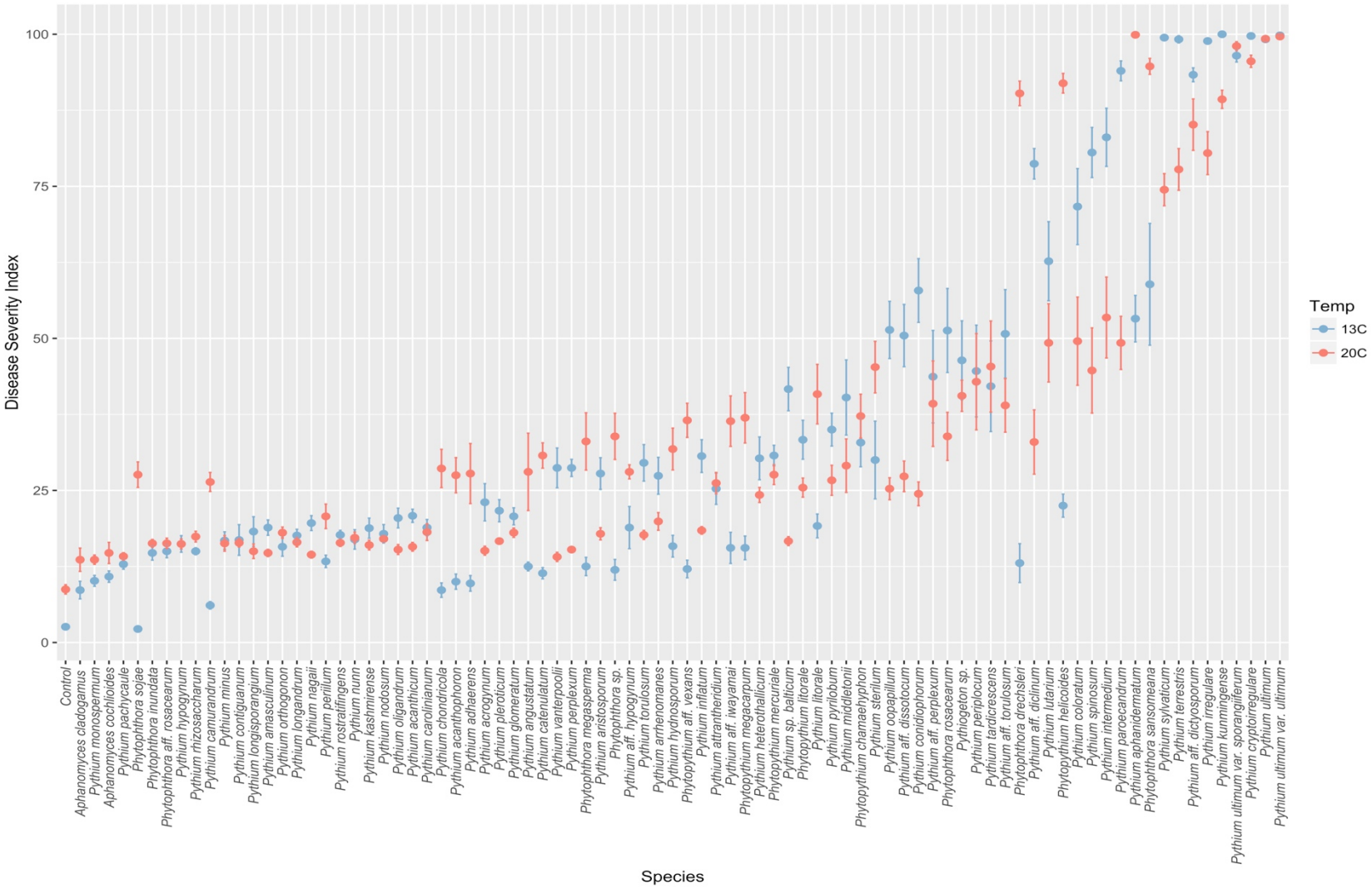

Figure 2.4. Mean disease severity index of 84 oomycete species screened in a seed rot assay at 13ºC and 20ºC. Bars represent standard error and disease severity index values from 0 = non-pathogenic to 100 = highly pathogenic

Table 2.1. Mean disease severity index (DSI) of soybean cv. `Sloan` seeds in response to multiple oomycete species as compared to the non-inoculated control at 13ºC or 20ºC. Only species with significant differences from the control at either temperature are represented (Complete table, see Table S.2.2). A seed rot assay was used to determine pathogenicity of oomycete species using a disease severity scale of 0-4 to rate individual seeds. Data were transformed to disease severity index (0=non-pathogenic; 100=highly virulent).

Species[a]	N[b]	Disease index (%) 13ºC			Disease index (%) 20ºC		
		Mean	SE[c]	*P*-value[d]	Mean	SE	*P*-value
Phytophthora drechsleri	1	13.06	±3.19	NS	90.28	±2.02	(0.049)
Phytophthora sansomeana	2	58.89	±10.01	NS	94.72	±1.31	(0.004)
Phytopythium helicoides	2	22.50	±1.90	NS	91.94	±1.61	(0.004)
Pythium aff. *diclinum*	3	78.70	±2.50	(0.032)	32.96	±5.28	NS
Pythium aff. *dictyosporum*	2	93.33	±1.14	(0.006)	85.14	±4.22	(0.017)
Pythium aphanidermatum	3	53.24	±3.82	NS[e]	99.91	±0.09	(<0.001)
Pythium cryptoirregulare	1	99.72	±0.28	(0.018)	95.56	±1.00	(0.021)
Pythium intermedium	3	83.06	±4.78	(0.016)	53.43	±6.64	NS
Pythium irregulare	3	98.89	±0.45	(0.001)	80.46	±3.53	(0.027)
Pythium kunmingense	2	100.00	±0.00	(0.002)	89.31	±1.50	(0.007)
Pythium paroecandrum	3	93.98	±1.64	(0.002)	49.26	±4.37	NS
Pythium spinosum	3	80.56	±4.12	(0.023)	44.72	±7.00	NS
Pythium sylvaticum	3	99.44	±0.24	(0.001)	74.44	±2.64	NS
Pythium terrestris	1	99.17	±0.59	(0.021)	77.78	±3.42	NS
Pythium ultimum	3	99.17	±0.30	(0.001)	99.26	±0.32	(<0.001)
Pythium ultimum var. *sporangiiferum*	3	96.48	±1.04	(0.001)	98.06	±0.72	(0.001)
Pythium ultimum var. *ultimum*	3	99.81	±0.13	(0.001)	99.63	±0.22	(<0.001)
Control		2.58	±0.31	-	8.73	±0.77	-

[a] 84 species were tested at both temperatures
[b] Number of isolates tested per species
[c] Standard error
[d] *P*-value based on Dunnett's test, significantly different from the non-rice control ($\alpha = 0.05$)
[e] NS = No significant

Due to the large range of virulence responses, disease severity indices at 13ºC and 20ºC were compared using a hierarchical clustering to group oomycete species, which resulted in three defined clusters (Fig. S.2.1). Cluster A represents all species that did not have a negative effect on seed germination. Cluster B contains species with virulence that were not significantly

different from the control, but still caused reduced seed health expressed as disease severity index. Cluster C includes highly virulent species that were significantly different from the control, and two species that were not significantly different from the control, *Py. lutarium* and *Py. coloratum*, which had disease severity index scores at 13ºC of 62.7 and 49.3, and at 20ºC 71.7 and 49.6, respectively (Fig. S.2.1B).

Seedling root rot assay. Five parameters were measured to determine which species were detrimental to growth of soybean seedlings: root dry weight, shoot dry weight, shoot:root ratio, root area, and root length. Using a principal component analysis, the five parameters were evaluated for their contribution in the discrimination of the different species. All of the parameters measured showed differences between the inoculated treatments and the non-inoculated controls (Fig. S.2.2 and S.2.3). The analysis showed that root area, root length and weight per root had the greatest contribution in separating the species in component 1 (PCA1, Fig. S.2.3), explaining 67.7% of the variability observed in the data. Shoot dry weight and shoot:root ratio contributed only 13% and 18% of the variability, respectively. Therefore, shoot dry weight and shoot:root ratio were not used in further analyses. The other three parameters had high correlation values (weight per root r^2=0.955, p=<0.001; root length r^2= 0.934, p=<0.001; root area r^2=0.921, p=<0.001) with the first dimension of the PCA, while the shoot dry weight correlation was lower (r^2=0.730).

Based on the PCA results, a MANOVA analysis was conducted using the three parameters: root area, root length, and weight per root (Fig. S.2.4). Dunnett's test identified 43 oomycete species as significantly different from the non-rice control. In addition, the non-inoculated control was not significantly different from the non-rice control. These parameters

had a negative effect on the combined parameters measured on the soybean seedlings (Table 2.2). In order to determine the contribution of each variable and further explore the results obtained in the MANOVA analysis, univariate analyses were performed on the three parameters: root dry weight, root length, and root area. The univariate analysis for root area showed similar results to the MANOVA analysis, resulting in 43 species with a significant effect on seedlings when compared to the non-rice control. Twenty-one oomycete species were determined to have a significant detrimental effect on seedlings across the multivariate and univariate analyses (Table 2.2, Fig. S.2.4).

Of the 21 pathogenic species across the multivariate and univariate analyses, the *Phytophthora* spp., *P. sojae, P. sansomeana* and *P. drechsleri* were the most virulent causing significant root reduction or death of radicles. The remaining pathogenic species belonged in the genus *Pythium,* and most species were within clade F, (*Py. cryptoirregulare, Py. irregulare, Py. sylvaticum, Py. attrantheridium, Py. intermedium,* and *Py. kunmingense*) and clade B (*Py.* aff. *dissotocum, Py.* aff. *torulosum, Py.* aff. *diclinum, Py.* aff. *dictyosporum, Py. lutarium* and *Py. oopapillum*). Clades with lower number of species found during this study were also designated as pathogenic based on our analysis, like *Pythium* clade I (*Py. heterothallicum, Py. ultimum* sensu lato), clade D (*Py. periplocum*) and *Aphanomyces cladogamus*. All of the *Phytophthora, Pythium,* and *Aphanomyces* isolates evaluated caused a considerable reduction in root development, and in some cases, death of the radicle as well (Fig. S.2.4 and S.2.5). Of the 43 species that were significantly different from the non-rice control in the MANOVA analysis, there were 22 species that were significant in only two or one of the univariate analyses, comprising members of the *Pythium* clades B, F, I and E; as well as different species of *Phytopythium* and *Aphanomyces* (Table 2.2 and Fig. S.2.4).

Ta**ble 2.2.** Forty-three oomycete species highly aggressive on soybean cv. `Sloan` in the seedling root rot assay measured as root area, root length and dry weight per root. Only species with significant differences from the non-rice control are represented (Complete table, see Table S.2.3). Data were analyzed by multivariate analysis of variance (MANOVA) combining all the parameters: root area, root length and weight per root; and followed by univariate analysis for each of the measured parameters. Plants were grown at 20ºC in a growth chamber for 2 weeks.

Species	N[a]	MANOVA *P*-value[b]	Root area (cm^2)[c]			Root length (cm)[c]			Dry weight per root (mg)[d]		
			Mean	SE[e]	*P*-value[f]	Mean	SE	*P*-value	Mean	SE	*P*-value
Aphanomyces cladogamus	1	<0.001	2.33	±0.50	(<0.001)	31.65	±7.18	(0.002)	28.67	±4.85	(0.044)
Aphanomyces cochlioides	1	0.019	4.38	±0.60	(0.019)	53.98	±9.33	(1.000)	38.00	±6.00	(1.000)
Phytophthora drechsleri	1	<0.001	2.26	±0.58	(<0.001)	21.70	±5.61	(<0.001)	16.44	±2.86	(<0.001)
Phytophthora rosacearum	3	<0.001	4.97	±0.63	(<0.001)	58.23	±8.62	(0.031)	42.00	±2.38	(1.000)
Phytophthora sansomeana	2	<0.001	0.26	±0.06	(<0.001)	2.25	±0.56	(<0.001)	2.33	±0.52	(<0.001)
Phytophthora sojae	3	<0.001	2.15	±0.39	(<0.001)	28.18	±5.32	(<0.001)	20.93	±3.65	(<0.001)
Phytopythium aff. *vexans*	2	0.001	4.81	±0.44	(0.001)	54.68	±4.39	(0.388)	38.33	±1.96	(0.587)
Phytopythium chamaehyphon	3	<0.001	4.64	±0.35	(<0.001)	54.82	±3.55	(0.163)	42.07	±2.07	(1.000)
Phytopythium helicoides	2	<0.001	3.79	±0.32	(<0.001)	41.13	±2.73	(0.016)	36.11	±1.70	(0.251)
Phytopythium litorale	3	0.002	5.08	±0.35	(0.002)	63.17	±4.45	(0.677)	53.48	±1.73	(1.000)
Phytopythium mercuriale	3	0.005	5.29	±0.36	(0.005)	65.89	±4.23	(1.000)	54.82	±1.76	(1.000)
Pythium aff. *diclinum*	3	<0.001	1.77	±0.43	(<0.001)	18.60	±4.56	(<0.001)	28.74	±4.58	(<0.001)
Pythium aff. *dictyosporum*	2	<0.001	2.19	±0.50	(<0.001)	22.22	±5.38	(<0.001)	18.78	±4.11	(<0.001)
Pythium aff. *dissotocum*	3	<0.001	2.72	±0.30	(<0.001)	38.13	±4.28	(<0.001)	27.93	±2.55	(<0.001)
Pythium aff. *torulosum*	3	<0.001	2.89	±0.35	(<0.001)	38.98	±5.00	(<0.001)	28.22	±2.54	(<0.001)
Pythium aphanidermatum	3	<0.001	4.07	±0.35	(<0.001)	51.26	±4.60	(0.016)	56.74	±2.91	(1.000)

Table 2.2 (cont'd)

Pythium attrantheridium	3	<0.001	2.05	±0.25	(<0.001)	27.77	±3.46	(<0.001)	23.26	±2.37	(<0.001)
Pythium coloratum	3	<0.001	4.32	±0.52	(<0.001)	51.89	±6.57	(0.003)	56.59	±3.76	(1.000)
Pythium conidiophorum	3	0.002	5.52	±0.62	(0.002)	64.87	±6.97	(0.343)	59.48	±3.86	(1.000)
Pythium contiguanum	3	0.019	5.44	±0.47	(0.019)	66.42	±4.92	(1.000)	62.07	±2.86	(1.000)
Pythium cryptoirregulare	1	<0.001	1.74	±0.22	(<0.001)	17.28	±2.34	(<0.001)	19.56	±2.38	(<0.001)
Pythium heterothallicum	3	<0.001	2.43	±0.25	(<0.001)	34.06	±3.96	(<0.001)	27.56	±2.35	(<0.001)
Pythium hypogynum	3	0.002	5.16	±0.38	(0.002)	62.81	±4.85	(0.339)	53.19	±2.23	(1.000)
Pythium intermedium	3	<0.001	3.41	±0.63	(<0.001)	42.13	±7.78	(<0.001)	41.85	±5.69	(<0.001)
Pythium irregulare	3	<0.001	1.62	±0.29	(<0.001)	19.66	±3.98	(<0.001)	20.96	±2.33	(<0.001)
Pythium kunmingense	2	<0.001	1.30	±0.11	(<0.001)	10.64	±1.17	(<0.001)	13.67	±1.51	(<0.001)
Pythium litorale	1	0.023	4.60	±0.62	(0.023)	53.38	±6.66	(1.000)	38.00	±3.99	(1.000)
Pythium longandrum	3	0.049	5.58	±0.40	(0.049)	67.10	±4.51	(1.000)	59.26	±2.70	(1.000)
Pythium longisporangium	3	0.004	5.84	±0.58	(0.004)	61.18	±5.41	(0.139)	44.67	±3.26	(0.894)
Pythium lutarium	3	<0.001	2.54	±0.33	(<0.001)	33.43	±4.88	(<0.001)	26.15	±2.54	(<0.001)
Pythium minus	3	0.001	5.08	±0.37	(0.001)	58.63	±3.78	(0.451)	48.44	±2.73	(1.000)
Pythium nagaii	3	0.026	5.60	±0.32	(0.026)	71.57	±3.60	(1.000)	52.89	±2.11	(1.000)
Pythium nunn	3	0.039	5.76	±0.41	(0.039)	69.77	±4.60	(1.000)	61.56	±2.41	(1.000)
Pythium oopapillum	3	<0.001	2.67	±0.37	(<0.001)	36.52	±4.98	(<0.001)	27.93	±2.97	(<0.001)
Pythium periilum	3	0.002	5.54	±0.56	(0.002)	59.44	±5.38	(0.186)	49.56	±4.09	(1.000)
Pythium periplocum	3	<0.001	5.00	±0.72	(<0.001)	52.74	±7.38	(<0.001)	38.49	±4.39	(0.003)
Pythium sylvaticum	3	<0.001	2.00	±0.24	(<0.001)	26.12	±3.42	(<0.001)	28.67	±3.03	(<0.001)
Pythium tardicrescens	3	<0.001	4.75	±0.63	(<0.001)	57.75	±7.89	(0.001)	43.70	±4.01	(0.104)
Pythium terrestris	1	<0.001	2.38	±0.37	(<0.001)	23.48	±4.14	(<0.001)	36.22	±4.58	(1.000)

Table 2.2 (cont'd)

Pythium ultimum	3	<0.001	0.18	±0.04	(<0.001)	1.54	±0.43	(<0.001)	5.11	±1.40	(<0.001)
Pythium ultimum var. *sporangiiferum*	3	<0.001	1.34	±0.24	(<0.001)	15.20	±3.17	(<0.001)	19.20	±2.50	(<0.001)
Pythium ultimum var. *ultimum*	3	<0.001	0.48	±0.07	(<0.001)	4.23	±0.74	(<0.001)	8.26	±1.25	(<0.001)
Pythium vanterpoolii	3	0.022	6.02	±0.57	(0.022)	70.14	±7.03	(1.000)	47.70	±2.31	(1.000)
Control		0.339	7.5	±0.28	(0.339)	92.33	±3.15	(1.000)	59.14	±1.71	(1.000)
Non-rice control		NA[g]	10.09	±0.34	NA	111.72	±3.12	NA	68.32	±2.13	NA

[a] Number of isolates tested per species
[b] *P*-value base on multivariate analysis of variance (MANOVA), significantly different from the non-rice control ($\alpha = 0.05$)
[c] Root area and length were determined by using ASSESS 2.0 (APS, St. Paul, MN)
[d] Dry weight per root was established after drying plants at 50ºC for 48-72 h.
[e] Standard error
[f] *P*-value for univariate analysis based on Dunnett's test, significantly different from the non-rice control ($\alpha = 0.05$)
[g] NA = Not applicable

The remaining 41 oomycete species did not have a significant effect on seedlings based on root area, root length, and dry weight per root (Fig. S.2.4 and Table S.2.3). Interestingly, *Pythium* Clade B that contains most of the pathogenic species, also included non-pathogenic species (i.e. *Py. inflatum, Py. catenulatum, Py. angustatum*). In addition to clade B, other clades were also represented including *Pythium* clade E (i.e. *Py. acrogynum, Py. middletonii, Py. pleroticum*), clade J (*Py. perplexum, Py. nodosum, Py. orthogonon*), and clade D (i.e. *Py. amasculinum, Py. oligandrum Py. acanthicum*) (Fig. 2.5). Among the less frequent non-pathogenic species, *Pythium* clade F and A were represented with three species each; and clade I and G with one species each. Apart from *Pythium* spp., three species from *Phytophthora* clade 6 were non-pathogenic, followed by two *Phytopythium* spp., and one species of the genus *Pythiogeton*.

Koch's postulates were completed by re-isolation from inoculated seedlings and identification of isolates via SSCP. All of the species designated as pathogenic on the seedlings were isolated and confirmed by SSCP (Table S.2.3), having two or more successful isolation events. Of the non-pathogenic species, most of the species used in the seedling cup assay were recovered at least once, except *Phytophthora megasperma*, *Py. adhaerens* and *Py. chondricola,* which we failed to re-isolate.

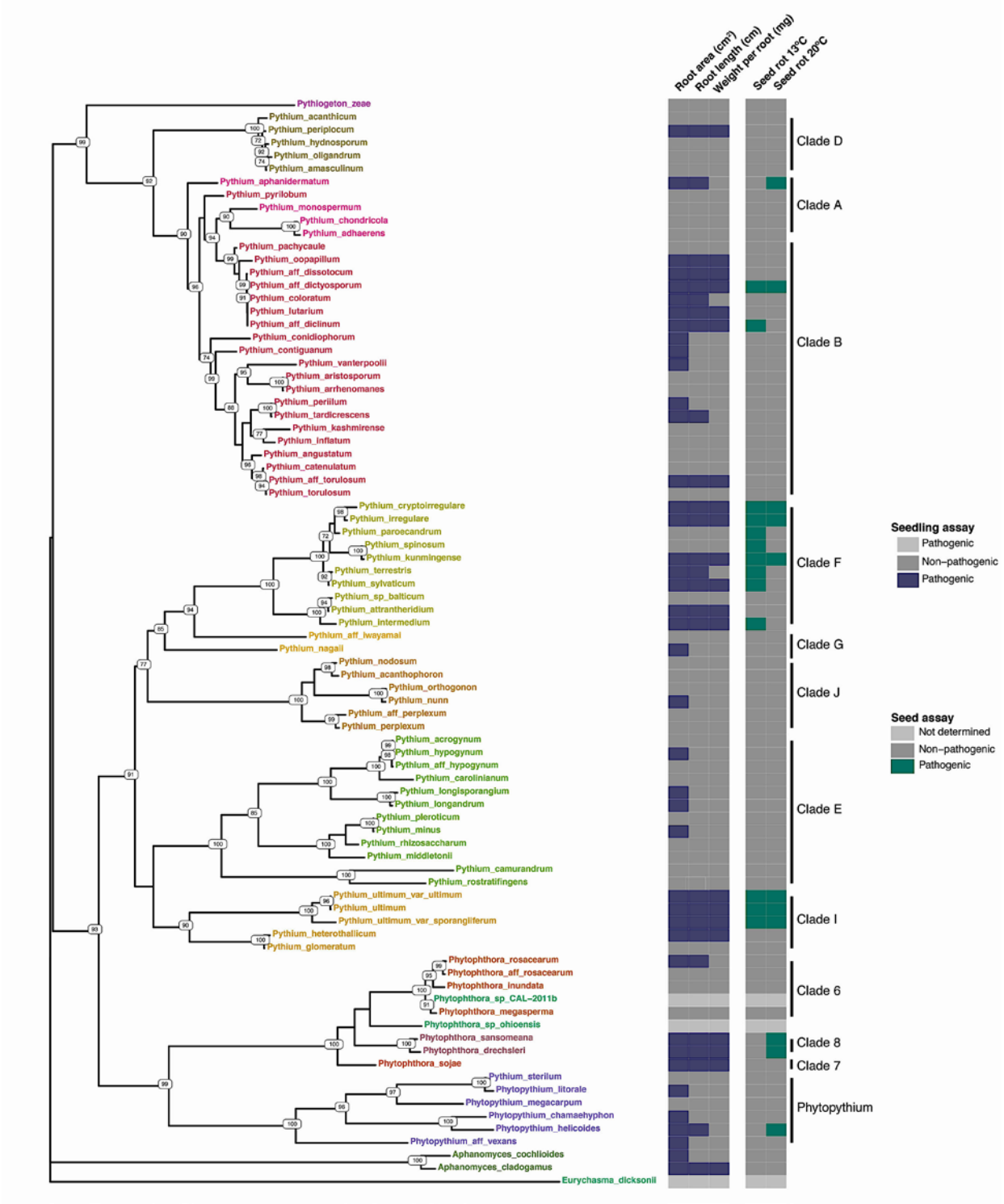

Figure 2.5. Maximum likelihood phylogeny of the ITS sequences of the rDNA for oomycete species found during the survey. Numbers on the branches indicated bootstrap values for 1,000 replicates (> 70). Phylogenetic distribution of pathogenicity traits mapped to taxa represented in the tree. Tip colors indicate members of different clades. Parameters in light gray represent taxa not isolated in the study.

Comparison of seed and seedling root rot assays. Among the species evaluated, *Py. paroecandrum* and *Py. spinosum* were the only species that caused seed rot and did not cause significant damage to plants in the seedling assay. The remaining species determined to be pathogenic with the seed rot assay were also identified as pathogenic with the seedling assay (Fig. 2.5). Among the species pathogenic on seed and seedling *Py. terrestris, Py. aphanidermatum* and *Phytopythium helicoides* were the only species that were not significant for root weight univariate analysis, the rest of species were significant for all analyses. Taking pathogenicity into account, the prevalence of these species was evaluated by state using the data from the survey (Fig. 2.6). The species *Py. sylvaticum, Py. heterothallicum, Py. ultimum* sensu lato, *Py. oopapillum* and *Py.* aff. *dissotocum* were pathogenic and also prevalent across most states sampled during the survey (Fig. 6). Among the non-pathogenic species under the conditions of this study, *Py. perplexum, Py. rostratifingens* and *Py. inflatum* were the most prevalent across the sampled states.

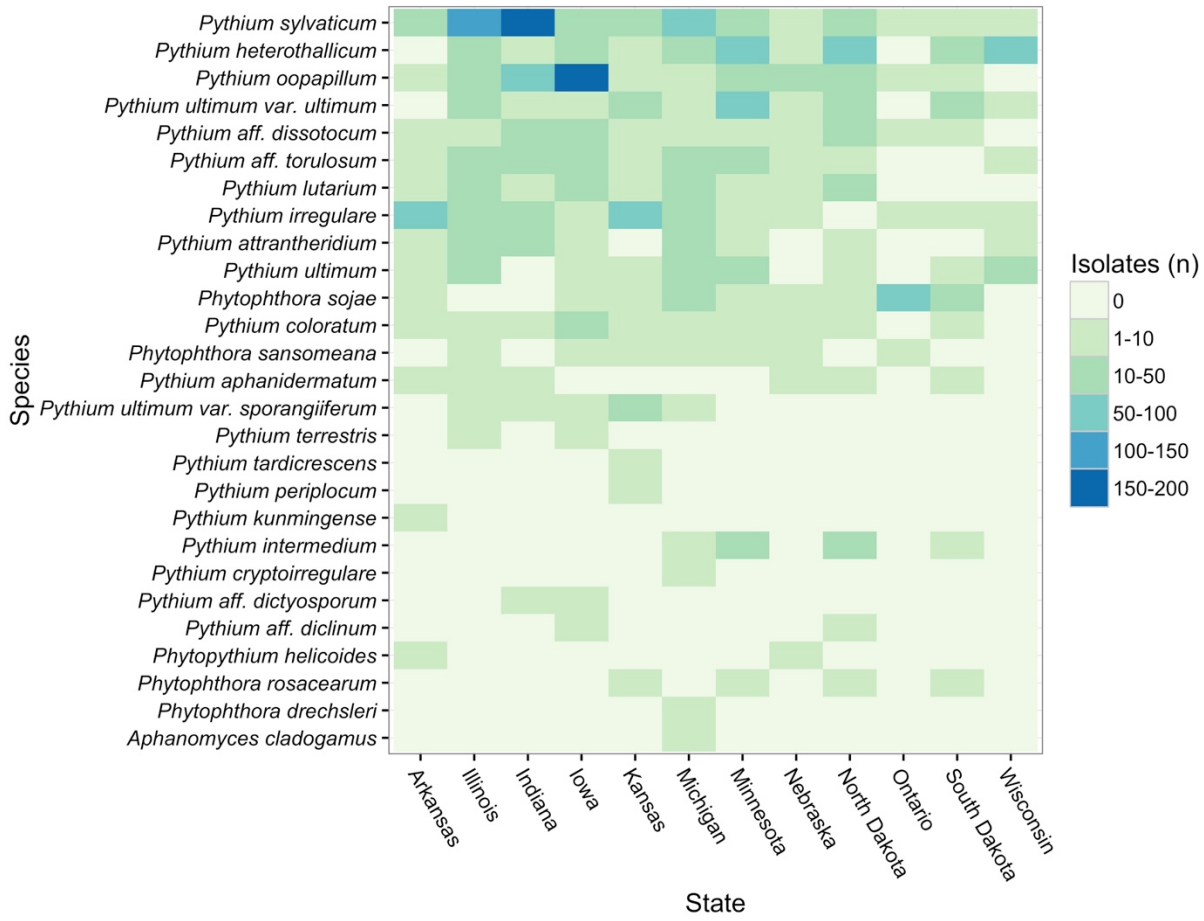

Figure 2.6. Prevalence of pathogenic oomycete species, designated based on seedling assay data, across the states sampled during the current study. Color gradient indicates number of isolates per species collected per state during 2011 and 2012.

Discussion

The current study was undertaken to acquire a greater understanding of the oomycete communities associated with and potentially involved in soybean seedling diseases across the major U.S. soybean producing states and Ontario, Canada. A total of 84 oomycete species were identified out of 3,416 isolates collected primarily from diseased soybean seedlings over the years 2011 and 2012. The 84 species belonged to the genera *Pythium* (94.85%), *Phytophthora* (4.15%), *Phytopythium* (0.91%), *Aphanomyces* (0.06%) and *Pythiogeton* (0.03%). Of the 84 species, 43 were determined to be pathogenic to seeds or seedlings, with the majority of isolates

being pathogenic to both seeds and seedlings. The majority of the isolates recovered were pathogenic on soybean belonging to *Pythium* clades F, B and I, which are known to contain the majority of pathogenic *Pythium* species (Lévesque and De Cock 2004).

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

One of the goals of the second year was to increase the number of *Phytophthora* spp. recovered, therefore, a second medium was included to improve recovery. The medium was amended with hymexazol to inhibit *Pythium* spp., however it is known that it can also affect some *Phytophthora* spp. (Jeffers 1986). In general, the V8-RPBH medium reduced the recovery of *Pythium* spp., but a small percent of isolates were still recovered from most *Pythium* clades (Fig. S.2.6). Aiming to increase the recovery of *Phytophthora* spp., samples were plated into two different media that could also affect the number of oomycetes recovered, in addition to the

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

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

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

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

species in a susceptible cultivar of soybean, identifying 43 species as pathogenic with different levels of virulence. Twenty-one species had a detrimental effect in reducing all parameters measured, and the remaining 22 species reduced either one or two of the parameters compared to the non-rice control (Fig. 2.5). Most of the species identified as seedling pathogens were also characterized as seed pathogens. Only *Py. paroecandrum* and *Py. spinosum* caused seed rot at 13ºC but did not cause root rot on seedlings. The remaining 41 of the 84 oomycete species did not significantly increase root rot compared to the control and were designated as non-pathogenic.

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

It has been suggested that plants infected with *Pythium* spp. have reduced vigor (Pieczarka and Abawi 1978, Gilbert 2002, Paulitz et al. 2002). The reduced vigor is often observed as stunted plants, necrotic root lesions, and leaf yellowing (Kirkpatrick et al. 2006). Therefore, measuring various root parameters is an approach to characterize and parse the effects of different species on the root system of soybean plants. The use of two controls, one with non-inoculated rice and one of a non-rice control, were intended to rule out any negative effects of

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

Several of the species reported as pathogens in the fungal-host database (ARS-USDA) and reported here were prevalent in most of the states surveyed including *Py. sylvaticum, Py. heterothallicum* and *Py. oopapillum* (Fig. 2.6). The species were present in most states, but their abundance varied across the different fields. Other pathogenic species were less prevalent, but

still present in low numbers in more than four states, such is the case with *Py. ultimum, Py.* aff. *dissotoccum* and *Py.* aff. *torulosum*. In regards to *Phytophthora* spp., both *P. sojae* and *P. sansomeana* were recovered in low numbers in most states, however this could be an artifact of sampling method and timing.

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

curated sequences, and if possible, sequencing both barcodes typically increases the confidence of the species designation (Kang et al. 2010, Robideau et al. 2011).

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

APPENDIX

Table S.2.1. GPS coordinates for fields sampled for diseased soybean seedlings and subsequent oomycete isolations during the survey in 2011 and 2012.

Field	State	Year	Latitude	Longitude
ARSO_1	Arkansas	2011	34.5	-91.4
IASO_1	Iowa	2011	41.3	-91.5
IASO_10	Iowa	2011	41.3	-91.7
IASO_2	Iowa	2011	41.7	-92.7
IASO_3	Iowa	2011	42.2	-93.0
IASO_4	Iowa	2011	41.7	-96.0
IASO_5	Iowa	2011	40.9	-93.8
IASO_6	Iowa	2011	40.9	-93.8
IASO_7	Iowa	2011	40.9	-93.8
IASO_8	Iowa	2011	42.6	-94.7
IASO_9	Iowa	2011	42.3	-92.6
ILSO_1	Illinois	2011	40.1	-88.2
ILSO_2	Illinois	2011	40.9	-90.7
ILSO_3	Illinois	2011	37.7	-88.3
ILSO_4	Illinois	2011	39.6	-90.8
ILSO_5	Illinois	2011	39.1	-89.0
ILSO_6	Illinois	2011	38.9	-89.9
INSO_1	Indiana	2011	40.3	-86.9
INSO_2	Indiana	2011	39.7	-87.1
INSO_3	Indiana	2011	40.4	-85.3
INSO_4	Indiana	2011	41.2	-85.7
INSO_5	Indiana	2011	41.0	-87.1
KSSO_1	Kansas	2011	40.0	-97.5
KSSO_2	Kansas	2011	40.0	-97.4
KSSO_3	Kansas	2011	39.3	-95.5
KSSO_4	Kansas	2011	39.3	-96.4
KSSO_5	Kansas	2011	39.3	-96.3
KSSO_6	Kansas	2011	39.0	-95.6
KSSO_7	Kansas	2011	38.8	-94.9
MISO_1	Michigan	2011	41.8	-86.5
MISO_10	Michigan	2011	42.9	-84.7
MISO_11	Michigan	2011	42.9	-84.7
MISO_12	Michigan	2011	42.9	-84.8
MISO_13	Michigan	2011	42.9	-84.8
MISO_2	Michigan	2011	42.6	-85.8
MISO_3	Michigan	2011	42.7	-84.0
MISO_4	Michigan	2011	42.7	-84.0
MISO_5	Michigan	2011	42.8	-84.5
MISO_6	Michigan	2011	42.9	-84.5
MISO_7	Michigan	2011	41.9	-84.8
MISO_8	Michigan	2011	42.7	-84.5
MISO_9	Michigan	2011	42.9	-84.7
MNSO_1	Minnesota	2011	44.1	-93.5
MNSO_2	Minnesota	2011	43.7	-94.7
MNSO_3	Minnesota	2011	46.6	-96.7

Table S.2.1 (cont'd)

MNSO_4	Minnesota	2011	46.6	-96.7
MNSO_5	Minnesota	2011	44.2	-95.3
MNSO_6	Minnesota	2011	45.0	-93.2
NDSO_1	North Dakota	2011	47.0	-96.9
NDSO_2	North Dakota	2011	47.5	-97.1
NDSO_3	North Dakota	2011	48.0	-97.6
NDSO_4	North Dakota	2011	46.6	-96.8
NDSO_5	North Dakota	2011	46.5	-97.1
NDSO_6	North Dakota	2011	46.8	-97.3
NESO_1	Nebraska	2011	41.2	-96.5
NESO_2	Nebraska	2011	40.2	-97.5
NESO_3	Nebraska	2011	40.2	-97.5
NESO_4	Nebraska	2011	41.8	-96.2
WISO_1	Wisconsin	2011	42.8	-90.8
WISO_2	Wisconsin	2011	42.8	-88.5
WISO_3	Wisconsin	2011	43.3	-89.4
WISO_4	Wisconsin	2011	44.1	-89.5
WISO_5	Wisconsin	2011	44.8	-90.1
WISO_6	Wisconsin	2011	44.9	-89.9
ARSO2_1	Arkansas	2012	33.8	-91.3
ARSO2_2	Arkansas	2012	34.5	-91.4
ARSO2_3	Arkansas	2012	34.7	-90.8
ARSO2_4	Arkansas	2012	35.7	-90.1
ARSO2_5	Arkansas	2012	35.4	-94.2
ARSO2_6	Arkansas	2012	36.1	-94.2
IASO2_1	Iowa	2012	40.7	-94.4
IASO2_2	Iowa	2012	-	-
IASO2_3	Iowa	2012	42.1	-93.5
IASO2_4	Iowa	2012	42.0	-93.7
IASO2_5	Iowa	2012	41.0	-94.7
IASO2_6	Iowa	2012	41.1	-92.9
IASO2_7	Iowa	2012	41.4	-93.7
ILSO2_1	Illinois	2012	37.5	-88.7
ILSO2_2	Illinois	2012	37.8	-88.3
ILSO2_3	Illinois	2012	39.0	-89.0
ILSO2_4	Illinois	2012	41.1	-89.7
ILSO2_5	Illinois	2012	39.9	-90.7
ILSO2_6	Illinois	2012	40.9	-90.7
INSO2_1	Indiana	2012	40.3	-85.7
INSO2_2	Indiana	2012	40.5	-86.8
INSO2_3	Indiana	2012	38.7	-87.5
INSO2_4	Indiana	2012	40.5	-86.8
INSO2_5	Indiana	2012	41.4	-86.9
INSO2_6	Indiana	2012	40.3	-86.8
KSSO2_1	Kansas	2012	39.5	-98.4
KSSO2_2	Kansas	2012	39.7	-96.7
KSSO2_3	Kansas	2012	37.8	-98.5
KSSO2_4	Kansas	2012	38.0	-100.8

Table S.2.1 (cont'd)

KSSO2_5	Kansas	2012	39.7	-98.1
KSSO2_6	Kansas	2012	39.7	-96.0
MISO2_1	Michigan	2012	43.3	-84.6
MISO2_2	Michigan	2012	42.7	-84.0
MISO2_3	Michigan	2012	42.7	-86.1
MISO2_4	Michigan	2012	42.9	-85.1
MISO2_5	Michigan	2012	43.1	-84.1
MISO2_6	Michigan	2012	43.0	-84.2
MISO2_7	Michigan	2012	43.7	-84.5
MNSO2_1	Minnesota	2012	44.1	-93.5
MNSO2_2	Minnesota	2012	44.7	-94.8
MNSO2_3	Minnesota	2012	44.3	-95.3
MNSO2_4	Minnesota	2012	44.9	-94.3
MNSO2_5	Minnesota	2012	46.7	-96.7
MNSO2_6	Minnesota	2012	47.0	-96.6
NDSO2_1	North Dakota	2012	47.0	-96.9
NDSO2_2	North Dakota	2012	47.2	-97.0
NDSO2_3	North Dakota	2012	47.3	-97.0
NDSO2_4	North Dakota	2012	46.6	-96.8
NDSO2_5	North Dakota	2012	46.6	-97.1
NDSO2_6	North Dakota	2012	46.6	-97.3
NESO2_1	Nebraska	2012	40.5	-97.1
NESO2_2	Nebraska	2012	41.3	-96.9
NESO2_3	Nebraska	2012	40.4	-95.9
NESO2_4	Nebraska	2012	40.2	-97.5
NESO2_5	Nebraska	2012	41.7	-96.2
NESO2_6	Nebraska	2012	41.2	-96.5
ONSO2_1	Ontario	2012	45.4	-76.3
ONSO2_2	Ontario	2012	45.3	-75.2
ONSO2_3	Ontario	2012	42.2	-82.7
ONSO2_4	Ontario	2012	42.3	-82.7
ONSO2_5	Ontario	2012	45.1	-75.4
ONSO2_6	Ontario	2012	-	-
ONSO2_7	Ontario	2012	42.2	-82.7
ONSO2_8	Ontario	2012	42.2	-82.8
SDSO2_1	South Dakota	2012	44.3	-97.6
SDSO2_2	South Dakota	2012	44.3	-97.6
SDSO2_3	South Dakota	2012	44.3	-97.6
SDSO2_4	South Dakota	2012	44.1	-96.8
SDSO2_5	South Dakota	2012	44.1	-96.6
SDSO2_6	South Dakota	2012	43.9	-96.6

Table S.2.2. Mean disease severity index (%) of soybean seeds cv. `Sloan` tested with the 84 oomycete species at 13ºC and 20ºC. A seed rot assay was used to determine pathogenicity using a 0-4 scale to rate individual seeds. Data were transformed to disease severity index (0 = non-pathogenic; 100 = highly virulent). P-values only showed for species significantly different from the control.

Species	N[a]	Disease index (%) 13ºC			Disease index (%) 20ºC		
		Mean	SE[b]	*P*-value[c]	Mean	SE	*P*-value
Aphanomyces cladogamus	1	8.61	±1.45		13.61	±1.91	
Aphanomyces cochlioides	1	10.83	±0.93		14.72	±1.74	
Phytophthora aff. *rosacearum*	3	15.00	±1.06		16.30	±0.86	
Phytophthora drechsleri	1	13.06	±3.19		90.28	±2.02	(0.049)
Phytophthora inundata	3	14.72	±1.18		16.30	±0.65	
Phytophthora megasperma	1	12.50	±1.50		33.06	±4.71	
Phytophthora rosacearum	3	51.30	±6.90		33.89	±3.95	
Phytophthora sansomea	2	58.89	±10.01		94.72	±1.31	(0.004)
Phytophthora sojae	3	2.22	±0.28		27.59	±2.09	
Phytophthora sp.	1	11.94	±1.71		33.89	±3.80	
Phytopythium aff. *vexans*	2	12.08	±1.45		36.53	±2.80	
Phytopythium chamaehyphon	3	32.87	±3.98		37.22	±3.60	
Phytopythium helicoides	2	22.50	±1.90		91.94	±1.61	(0.004)
Phytopythium litorale	3	33.33	±3.20		25.46	±1.57	
Phytopythium megacarpum	1	15.56	±1.94		36.94	±4.14	
Phytopythium mercuriale	3	30.74	±1.68		27.59	±1.63	
Pythiogeton sp.	1	46.39	±6.50		40.56	±2.56	
Pythium acanthicum	3	20.83	±1.08		15.74	±0.72	
Pythium acanthophoron	1	10.00	±1.25		27.50	±2.89	
Pythium acrogynum	3	23.06	±3.06		15.09	±0.74	
Pythium adhaerens	1	9.72	±1.28		27.78	±4.94	
Pythium aff. *diclinum*	3	78.70	±2.50	(0.032)	32.96	±5.28	
Pythium aff. *dictyosporum*	2	93.33	±1.14	(0.006)	85.14	±4.22	(0.017)
Pythium aff. *dissotocum*	3	50.46	±5.12		27.31	±2.51	
Pythium aff. *hypogynum*	1	18.89	±3.46		28.06	±1.16	
Pythium aff. *iwayamai*	1	15.56	±2.56		36.39	±4.15	
Pythium aff. *perplexum*	3	43.70	±7.60		39.26	±7.03	
Pythium aff. *torulosum*	3	50.74	±7.27		38.98	±4.40	
Pythium amasculinum	3	18.89	±1.26		14.72	±0.59	
Pythium angustatum	1	12.50	±0.72		28.06	±6.36	
Pythium aphanidermatum	3	53.24	±3.82		99.91	±0.09	(<0.001)
Pythium aristosporum	3	27.78	±2.60		17.87	±1.00	
Pythium arrhenomanes	3	27.41	±3.03		19.91	±1.45	
Pythium attrantheridium	3	25.28	±2.57		26.20	±1.75	
Pythium camurandrum	1	6.11	±0.61		26.39	±1.57	

Table S.2.2 (cont'd)

Pythium carolinianum	3	18.89	±1.33		18.15	±1.37	
Pythium catenulatum	3	11.39	±0.92		30.74	±2.08	
Pythium chondricola	1	8.61	±1.19		28.61	±3.15	
Pythium coloratum	3	71.67	±6.24		49.54	±7.25	
Pythium conidiophorum	3	57.87	±5.24		24.44	±1.95	
Pythium contiguanum	3	16.85	±2.52		16.39	±0.68	
Pythium cryptoirregulare	1	99.72	±0.28	(0.018)	95.56	±1.00	(0.021)
Pythium glomeratum	3	20.74	±1.40		18.06	±0.77	
Pythium heterothallicum	3	30.28	±3.52		24.26	±1.24	
Pythium hydnosporum	2	15.83	±1.79		31.81	±3.43	
Pythium hypogynum	3	16.20	±1.36		16.20	±0.82	
Pythium inflatum	3	30.65	±2.69		18.43	±0.61	
Pythium intermedium	3	83.06	±4.78	(0.016)	53.43	±6.64	
Pythium irregulare	3	98.89	±0.45	(0.001)	80.46	±3.53	(0.027)
Pythium kashmirense	3	18.80	±1.65		16.02	±0.79	
Pythium kunmingense	2	100.00	±0.00	(0.002)	89.31	±1.50	(0.007)
Pythium litorale	1	19.17	±1.95		40.83	±4.89	
Pythium longandrum	3	17.59	±1.02		16.48	±0.76	
Pythium longisporangium	3	18.24	±2.43		15.00	±1.19	
Pythium lutarium	3	62.69	±6.50		49.26	±6.44	
Pythium middletonii	3	40.28	±6.18		29.07	±4.40	
Pythium minus	3	16.76	±1.44		16.30	±1.28	
Pythium monospermum	2	10.14	±0.89		13.61	±0.76	
Pythium nagaii	3	19.63	±1.22		14.44	±0.56	
Pythium nodosum	3	17.87	±1.51		17.04	±0.50	
Pythium nunn	3	16.94	±1.60		17.22	±0.93	
Pythium oligandrum	3	20.46	±1.62		15.28	±0.82	
Pythium oopapillum	3	51.39	±4.70		25.28	±1.81	
Pythium orthogonon	3	15.74	±1.56		18.06	±0.93	
Pythium pachycaule	3	12.87	±0.82		14.17	±0.60	
Pythium paroecandrum	3	93.98	±1.64	(0.002)	49.26	±4.37	
Pythium periilum	3	13.33	±1.02		20.74	±2.01	
Pythium periplocum	3	44.63	±7.54		42.87	±7.91	
Pythium perplexum	3	28.70	±1.41		15.28	±0.49	
Pythium pleroticum	3	21.67	±1.81		16.67	±0.50	
Pythium pyrilobum	1	35.00	±2.70		26.67	±2.47	
Pythium rhizosaccharum	3	15.00	±0.57		17.41	±0.88	
Pythium rostratifingens	3	17.69	±0.75		16.39	±0.59	
Pythium sp. *balticum*	3	41.67	±3.57		16.67	±0.73	
Pythium spinosum	3	80.56	±4.12	(0.023)	44.72	±7.00	
Pythium sterilum	1	30.00	±6.39		45.28	±4.24	
Pythium sylvaticum	3	99.44	±0.24	(0.001)	74.44	±2.64	(0.083)
Pythium tardicrescens	3	42.13	±7.45		45.37	±7.49	
Pythium terrestris	1	99.17	±0.59	(0.021)	77.78	±3.42	
Pythium torulosum	3	29.54	±3.00		17.69	±0.74	

Table S.2.2 (cont'd)

Pythium ultimum	3	99.17	±0.30	(0.001)	99.26	±0.32	(<0.001)
Pythium ultimum var. *sporangiiferum*	3	96.48	±1.04	(0.001)	98.06	±0.72	(0.001)
Pythium ultimum var. *ultimum*	3	99.81	±0.13	(0.001)	99.63	±0.22	(<0.001)
Pythium vanterpoolii	3	28.70	±3.27		14.07	±0.76	
Control		2.58	±0.31		8.73	±0.77	

[a] Number of isolates tested by species
[b] SE = Standard error
[c] Significance level, if not stated equal to 1.0

Table S.2.3. Mean weight per root (mg), root area (cm2), root length, and mean weight per shoot (mg) of soybean cv. `Sloan` seedlings challenged with each of the 84 oomycete species. P-values for multivariate analysis of variance (MANOVA) and univariate analyses are presented for the three parameters included in the statistical model. P-values, based on the variables compared to the non-rice control.

Species	Isolates[a]	Isolation[b]	MANOVA P-value[c]	Weight per root (mg)			Root area (cm^2)			Root length (cm)			Weight per shoot	
				Mean	SE[d]	P-value[e]	Mean	SE	P-value	Mean	SE	P-value	Mean	SE
Control				59.14	1.71		7.5	0.28		92.33	3.15		169.05	2.66
Non-rice control				68.32	2.13		10.09	0.34		111.72	3.12		181.27	3.15
Aphanomyces cladogamus	1	+	(<0.001)	28.67	4.85	(0.044)	2.33	0.5	(<0.001)	31.65	7.18	(0.002)	140.89	3.45
Aphanomyces cochlioides	1	+	(0.019)	38	6	(1.000)	4.38	0.61	(0.019)	53.98	9.33	(1.000)	150.44	4.44
Phytophthora aff. rosacearum	3	+++	(0.601)	51.33	2.4	(1.000)	6.82	0.47	(0.601)	79.79	5.4	(1.000)	153.04	3.94
Phytophthora drechsleri	1	++	(<0.001)	16.44	2.86	(<0.001)	2.26	0.58	(<0.001)	21.7	5.61	(<0.001)	126.89	5.62
Phytophthora inundata	3	++	(1.000)	65.93	2.92	(1.000)	6.87	0.48	(1.000)	76.58	4.59	(1.000)	156.3	4.14
Phytophthora megasperma	1	-	(0.222)	40	5.09	(1.000)	5.48	0.79	(0.222)	64.65	10.98	(1.000)	150.89	8.16
Phytophthora rosacearum	3	+++	(<0.001)	42	2.38	(1.000)	4.97	0.63	(<0.001)	58.23	8.62	(0.031)	142.37	4.63
Phytophthora sansomea	2	+++	(<0.001)	2.33	0.52	(<0.001)	0.26	0.06	(<0.001)	2.25	0.56	(<0.001)	107	9.44
Phytophthora sojae	3	+++	(<0.001)	20.93	3.65	(<0.001)	2.15	0.39	(<0.001)	28.18	5.32	(<0.001)	138.15	5.37
Phytophthora sp.	1	+	(1.000)	44.22	3.79	(1.000)	7.27	0.57	(1.000)	79.82	7.61	(1.000)	151.11	16.39
Phytopythium aff. vexans	2	++	(0.001)	38.33	1.96	(0.587)	4.81	0.44	(0.001)	54.68	4.39	(0.388)	141.34	3.36

Table S.2.3 (cont'd)

Phytopythium chamaehyphon	3	+++	(<0.001)	42.07	2.07	(1.000)	4.64	0.35	(<0.001)	54.82	3.55	(0.163)	146.96	3.54
Phytopythium helicoides	2	++	(<0.001)	36.11	1.7	(0.251)	3.79	0.32	(<0.001)	41.12	2.73	(0.016)	141.44	4.91
Phytopythium litorale	3	+++	(0.002)	53.48	1.73	(1.000)	5.08	0.35	(0.002)	63.17	4.45	(0.677)	148.3	5.6
Phytopythium megacarpum	1	++	(0.226)	36.22	2.78	(1.000)	5.33	0.57	(0.226)	59.49	6.11	(1.000)	145.33	5.71
Phytopythium mercuriale	3	+++	(0.005)	54.81	1.76	(1.000)	5.29	0.36	(0.005)	65.89	4.23	(1.000)	143.93	5.46
Pythiogeton sp.	1	+	(0.057)	36.44	3.05	(1.000)	4.84	0.61	(0.057)	51.84	3.91	(1.000)	149.56	4.25
Pythium acanthicum	3	++	(1.000)	68.96	3.59	(1.000)	10.09	0.66	(1.000)	121.3	7.04	(1.000)	174	4.46
Pythium acanthophoron	1	+	(1.000)	55.11	5.96	(1.000)	8.96	0.88	(1.000)	93.52	9.01	(1.000)	159.33	8.14
Pythium acrogynum	3	+++	(0.050)	64	2.37	(1.000)	5.34	0.32	(0.050)	59.9	3.59	(1.000)	158.37	3.93
Pythium adhaerens	1	-	(0.646)	39.78	8.13	(0.518)	7.09	1.52	(0.646)	70.18	13.63	(1.000)	156.67	10.69
Pythium aff. diclinum	3	+++	(<0.001)	28.74	4.58	(<0.001)	1.77	0.43	(<0.001)	18.6	4.56	(<0.001)	111.19	7.59
Pythium aff. dictyosporum	2	+++	(<0.001)	18.78	4.11	(<0.001)	2.19	0.5	(<0.001)	22.22	5.38	(<0.001)	120.22	6.22
Pythium aff. dissotocum	3	++	(<0.001)	27.93	2.55	(<0.001)	2.71	0.3	(<0.001)	38.13	4.28	(<0.001)	148.74	3.33
Pythium aff. hypogynum	1	++	(0.472)	44.89	5.49	(1.000)	5.63	0.57	(0.472)	59.06	5.35	(1.000)	140.44	13.98
Pythium aff. iwayamai	1	+	(0.482)	38.44	2.98	(1.000)	5.57	0.51	(0.482)	63.91	3.51	(1.000)	138.67	5.27
Pythium aff. perplexum	3	+++	(0.071)	54.67	2.97	(1.000)	6.39	0.57	(0.071)	78.25	7.07	(1.000)	144.89	7.8
Pythium aff. torulosum	3	++	(<0.001)	28.22	2.54	(<0.001)	2.89	0.35	(<0.001)	38.98	5	(<0.001)	140.07	5.44
Pythium amasculinum	3	+	(0.296)	66.59	2.52	(1.000)	6.21	0.34	(0.296)	75.35	3.65	(1.000)	166.96	4.78

Table S.2.3 (cont'd)

Pythium angustatum	1	+	(1.000)	52.89	4.04	(1.000)	8.59	0.64	(1.000)	96.25	8.29	(1.000)	160.44	4.2
Pythium aphanidermatum	3	+++	(<0.001)	56.74	2.91	(1.000)	4.07	0.35	(<0.001)	51.26	4.6	(0.016)	150	4.68
Pythium aristosporum	3	+++	(0.529)	62.44	2.45	(1.000)	6.47	0.33	(0.529)	77.33	3.36	(1.000)	155.85	5.09
Pythium arrhenomanes	3	+++	(0.088)	55.63	2.52	(1.000)	6.15	0.47	(0.088)	73.89	5.52	(1.000)	151.19	5.96
Pythium attrantheridium	3	++	(<0.001)	23.26	2.37	(<0.001)	2.05	0.25	(<0.001)	27.77	3.46	(<0.001)	142.22	4.23
Pythium camurandrum	1	++	(1.000)	47.56	5.03	(1.000)	7.18	0.8	(1.000)	79.66	7.76	(1.000)	145.11	8.79
Pythium carolinianum	3	+++	(0.460)	54.22	2.72	(1.000)	6.85	0.59	(0.460)	74.15	5.95	(1.000)	155.93	3.26
Pythium catenulatum	3	++	(1.000)	50.37	2.47	(1.000)	7.21	0.46	(1.000)	83.36	5.26	(1.000)	159.41	3.64
Pythium chondricola	1	-	(1.000)	49.56	5.16	(1.000)	7.26	0.9	(1.000)	81.76	8.58	(1.000)	154.44	7.78
Pythium coloratum	3	+++	(<0.001)	56.59	3.76	(1.000)	4.68	0.54	(<0.001)	56.52	6.81	(0.003)	160.3	5.88
Pythium conidiophorum	3	+++	(0.002)	59.48	3.86	(1.000)	5.52	0.62	(0.002)	64.87	6.97	(0.343)	166.22	5.91
Pythium contiguanum	3	++	(0.019)	62.07	2.86	(1.000)	5.44	0.47	(0.019)	66.42	4.91	(1.000)	159.11	4.35
Pythium cryptoirregulare	1	++	(<0.001)	19.56	2.38	(<0.001)	1.74	0.21	(<0.001)	17.28	2.34	(<0.001)	132	5.66
Pythium glomeratum	3	+++	(0.588)	59.85	2.89	(1.000)	6.43	0.47	(0.588)	75.85	6.11	(1.000)	161.41	5.02
Pythium heterothallicum	3	++	(<0.001)	27.56	2.35	(<0.001)	2.43	0.25	(<0.001)	34.06	3.96	(<0.001)	149.93	3.35
Pythium hydnosporum	2	+	(1.000)	50.44	2.8	(1.000)	8.36	0.5	(1.000)	92.17	4.83	(1.000)	155.67	5.32
Pythium hypogynum	3	+++	(0.002)	53.19	2.23	(1.000)	5.16	0.38	(0.002)	62.81	4.85	(0.339)	144.59	6.26

Table S.2.3 (cont'd)

Pythium inflatum	3	+++	(1.000)	64.37	3.04	(1.000)	7.31	0.45	(1.000)	93.47	5.04	(1.000)	176.81	3.63
Pythium intermedium	3	+++	(<0.001)	41.85	5.69	(<0.001)	3.41	0.63	(<0.001)	42.13	7.78	(<0.001)	132.81	9.48
Pythium irregulare	3	++	(<0.001)	20.96	2.33	(<0.001)	1.62	0.29	(<0.001)	19.66	3.98	(<0.001)	137.85	3.5
Pythium kashmirense	3	+++	(0.388)	58.89	2.37	(1.000)	6.39	0.34	(0.388)	78.92	3.8	(1.000)	155.63	5.47
Pythium kunmingense	2	+++	(<0.001)	13.67	1.51	(<0.001)	1.3	0.11	(<0.001)	10.64	1.17	(<0.001)	123.78	4.77
Pythium litorale	1	+	(0.023)	38	3.99	(1.000)	4.6	0.62	(0.023)	53.38	6.66	(1.000)	152	6.7
Pythium longandrum	3	+++	(0.049)	59.26	2.7	(1.000)	5.58	0.4	(0.049)	67.1	4.51	(1.000)	155.48	4.05
Pythium longisporangium	3	+++	(0.004)	44.67	3.26	(0.894)	5.84	0.58	(0.004)	61.18	5.41	(0.139)	150.81	3.37
Pythium lutarium	3	+++	(<0.001)	26.15	2.54	(<0.001)	2.53	0.33	(<0.001)	33.43	4.88	(<0.001)	143.93	4.07
Pythium middletonii	3	+++	(0.065)	57.33	1.72	(1.000)	5.9	0.37	(0.065)	70.71	4.53	(1.000)	150	4.67
Pythium minus	3	+++	(0.001)	48.44	2.73	(1.000)	5.07	0.36	(0.001)	58.63	3.78	(0.451)	152.15	2.83
Pythium monospermum	2	++	(1.000)	64.56	3.39	(1.000)	7.12	0.58	(1.000)	80.03	7.67	(1.000)	164.67	5.04
Pythium nagaii	3	+++	(0.026)	52.89	2.11	(1.000)	5.6	0.32	(0.026)	71.57	3.6	(1.000)	150.3	7.17
Pythium nodosum	3	+++	(1.000)	72	3.61	(1.000)	9.95	0.7	(1.000)	117.86	7.1	(1.000)	176.89	5.31
Pythium nunn	3	+++	(0.039)	61.56	2.41	(1.000)	5.76	0.41	(0.039)	69.77	4.6	(1.000)	166	4.09
Pythium oligandrum	3	+++	(1.000)	63.11	2.83	(1.000)	6.79	0.45	(1.000)	78.24	3.92	(1.000)	150.07	3.02
Pythium oopapillum	3	+++	(<0.001)	27.93	2.97	(<0.001)	2.67	0.37	(<0.001)	36.52	4.98	(<0.001)	148.67	3.99
Pythium orthogonon	3	+++	(1.000)	64.81	2.8	(1.000)	6.64	0.27	(1.000)	79.67	3.17	(1.000)	170.67	5.01
Pythium pachycaule	3	+	(1.000)	71.19	3.83	(1.000)	8.09	0.48	(1.000)	99.19	6.13	(1.000)	175.26	5.88

Table S.2.3 (cont'd)

Pythium paroecandrum	3	+++	(1.000)	67.78	2.77	(1.000)	7.53	0.41	(1.000)	95.39	4.67	(1.000)	169.11	3.46
Pythium periilum	3	+++	(0.002)	49.56	4.09	(1.000)	5.54	0.56	(0.002)	59.44	5.38	(0.186)	152.81	3.19
Pythium periplocum	3	+++	(<0.001)	38.49	4.39	(0.003)	5	0.72	(<0.001)	52.74	7.38	(<0.001)	141.19	4.7
Pythium perplexum	3	+++	(1.000)	76.44	3.52	(1.000)	10.06	0.66	(1.000)	120.46	6.61	(1.000)	185.7	5.87
Pythium pleroticum	3	+++	(0.451)	66.52	1.52	(1.000)	6.31	0.31	(0.451)	77.05	3.78	(1.000)	173.04	4.18
Pythium pyrilobum	1	+	(0.852)	42.67	3.42	(1.000)	6.14	0.85	(0.852)	66.3	6.81	(1.000)	151.78	7.97
Pythium rhizosaccharum	3	+++	(1.000)	65.33	2.41	(1.000)	6.39	0.38	(1.000)	73.24	3.74	(1.000)	164.37	3.68
Pythium rostratifingens	3	+++	(1.000)	69.7	3.24	(1.000)	8.62	0.55	(1.000)	107.14	6.33	(1.000)	180.07	5.42
Pythium sp. balticum	3	+++	(0.301)	63.85	2.17	(1.000)	6.23	0.33	(0.301)	75.86	3.66	(1.000)	161.93	4.51
Pythium spinosum	3	+++	(0.052)	65.7	4.22	(1.000)	6.75	0.76	(0.052)	83	8.95	(1.000)	170.96	5.2
Pythium sterilum	1	+	(1.000)	44.22	4.31	(1.000)	6.36	0.76	(1.000)	67.29	4.69	(1.000)	157.11	9.26
Pythium sylvaticum	3	+++	(<0.001)	28.67	3.03	(<0.001)	2	0.24	(<0.001)	26.12	3.42	(<0.001)	137.7	3.78
Pythium tardicrescens	3	+++	(<0.001)	43.7	4.01	(0.104)	4.75	0.63	(<0.001)	57.75	7.89	(0.001)	134.37	6.04
Pythium terrestris	1	+	(<0.001)	36.22	4.58	(1.000)	2.38	0.37	(<0.001)	23.48	4.14	(<0.001)	135.33	5.94
Pythium torulosum	3	+++	(1.000)	68.44	3.06	(1.000)	6.62	0.5	(1.000)	74.91	4.86	(1.000)	168.52	5.04
Pythium ultimum	3	+++	(<0.001)	5.11	1.4	(<0.001)	0.18	0.04	(<0.001)	1.54	0.43	(<0.001)	86	4.83
Pythium ultimum var. sporangiiferum	3	+++	(<0.001)	19.2	2.5	(<0.001)	1.34	0.24	(<0.001)	15.2	3.17	(<0.001)	135.85	3.87

Table S.2.3 (cont'd)

Pythium ultimum var. ultimum	3	+++	(<0.001)	8.26	1.25	(<0.001)	0.48	0.07	(<0.001)	4.23	0.74	(<0.001)	117.04	4.11
Pythium vanterpoolii	3	+++	(0.022)	47.7	2.31	(1.000)	6.02	0.57	(0.022)	70.14	7.03	(1.000)	146.89	3.37

[a] Isolates tested per species

[b] Isolation events: +++ = six or more isolation events; ++ = between 3 to 6 isolation events; + = between 1 to 3 isolation events; - = no isolation events.

[c] *P*-value for multivariate analysis of variance (MANOVA)

[d] SE = Standard error

[e] *P*-value for univariate analyses

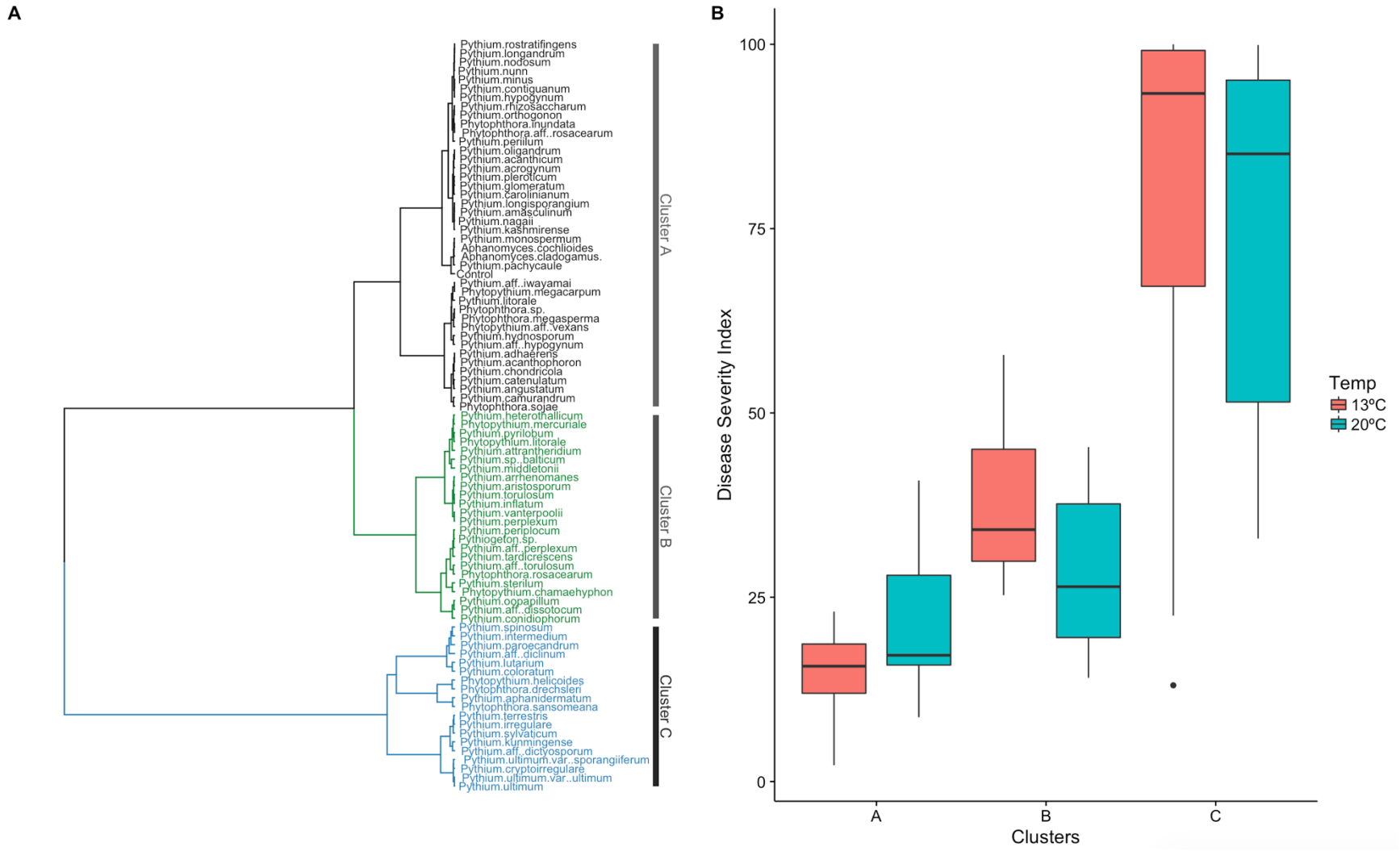

Figure S.2.1. **(A)** Hierarchical analysis of the disease severity index of 84 oomycete species to establish three clusters related to pathogenicity on soybean and **(B)** boxplot of disease severity index by clusters at 13°C and 20°C as evaluated in a seed rot assay.

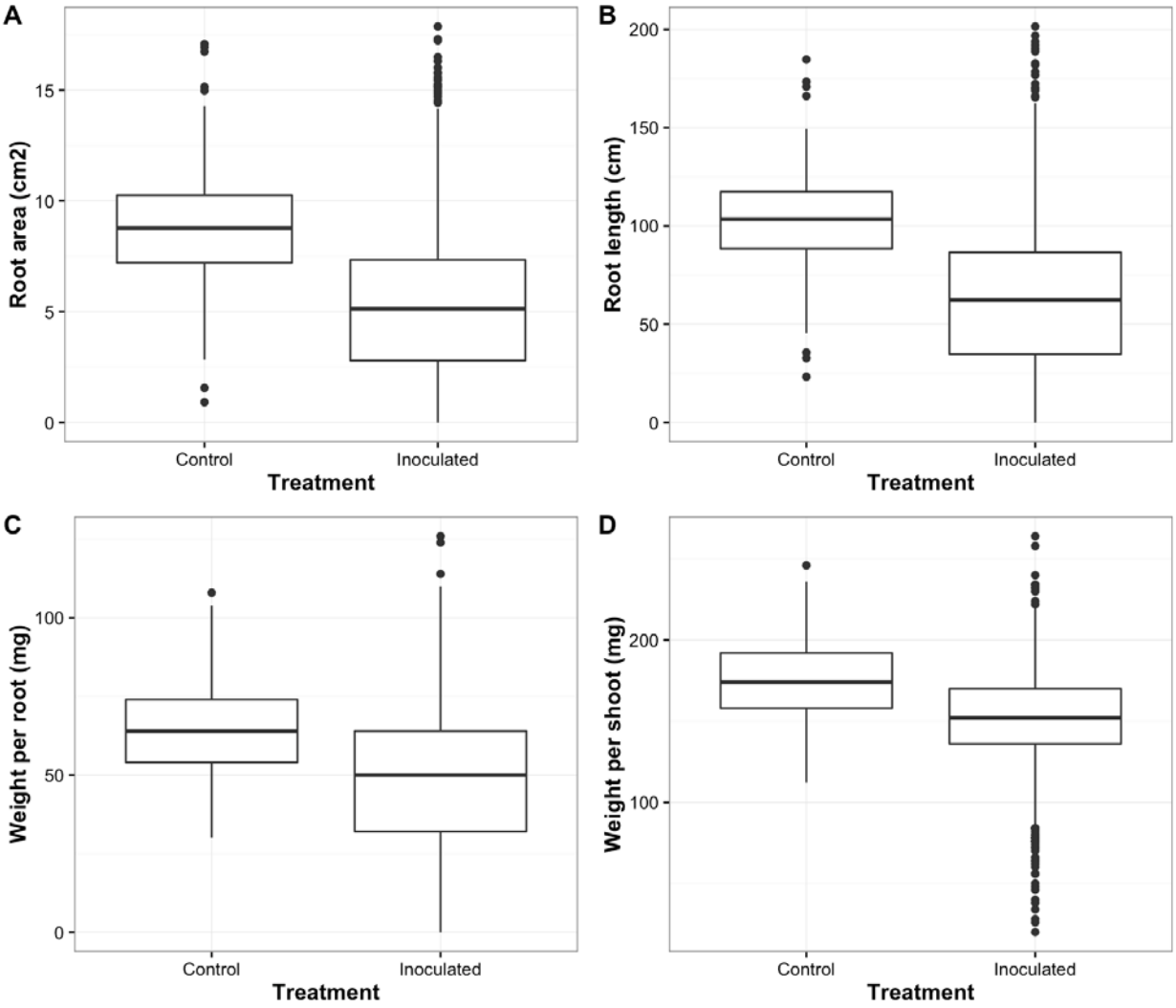

Figure S.2.2. Comparison of the non-inoculated controls and inoculated seedlings with the 84 oomycete species combined for the four parameters measured: (A) root area (cm2), (B) root length (cm), (C) weight per root (mg), and (D) weight per shoot (mg). Boxplot represent distribution of data, the line represents the median for each group and dots indicates outliers.

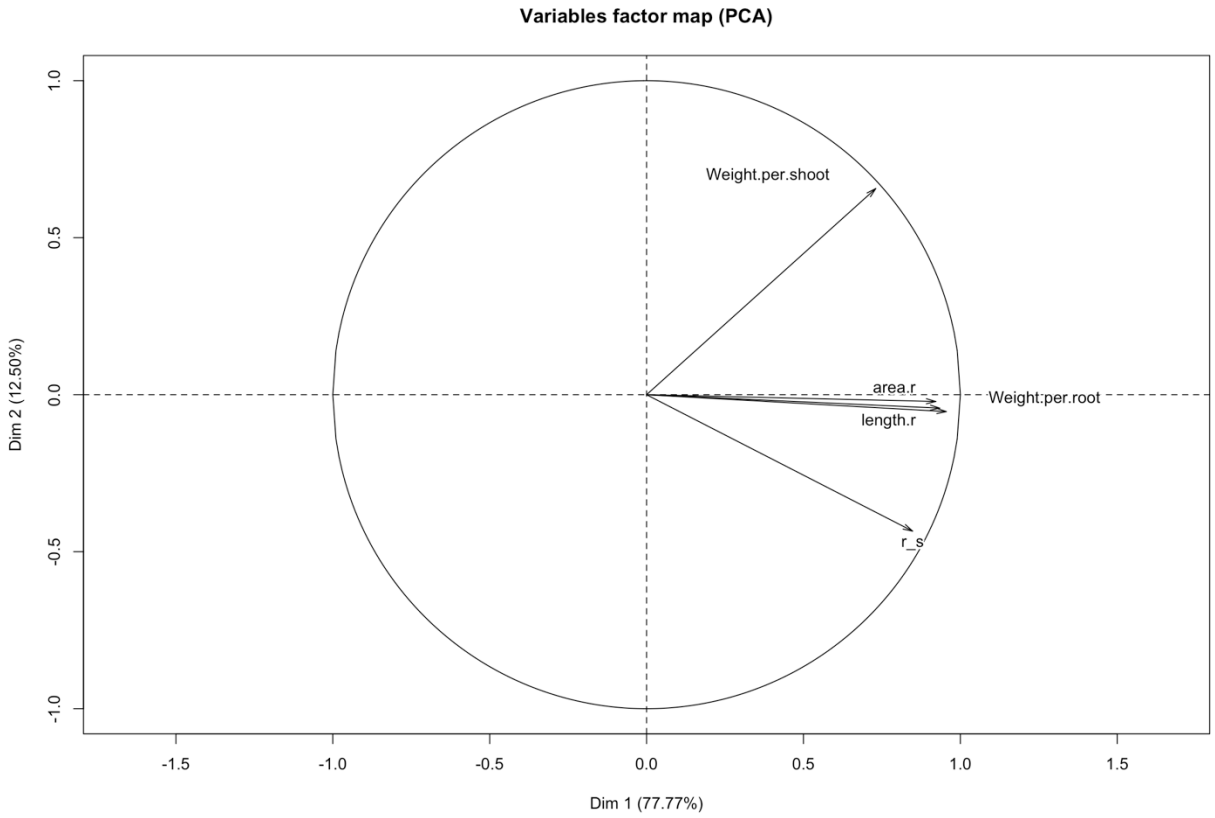

Figure S.2.3. Principal Component Analysis (PCA) on seedling parameters measured to determine correlation and contribution for the evaluation pathogenic/non-pathogenic oomycete species. The values on parenthesis indicate the percent of variance explained by the respective axis. The length and the direction of the vectors indicate the contribution of each parameter to the corresponding variance.

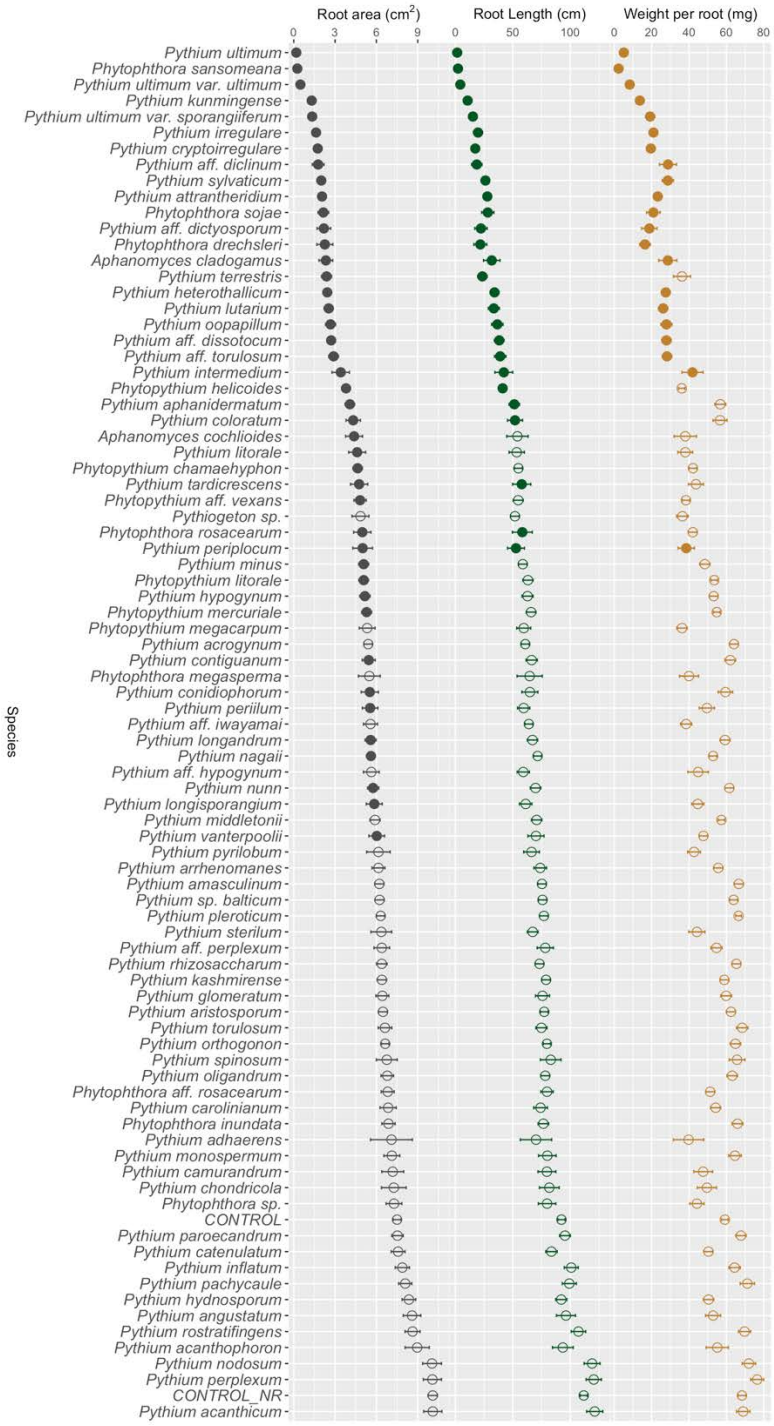

Figure S.2.4. Mean soybean root area (cm2), mean root length (cm), and mean weight per root (mg) after being challenged by 84 oomycete species using a soybean seedling root rot assay. Bars represent standard error and darker points represent species significantly different from the non-rice control ($P < 0.05$).

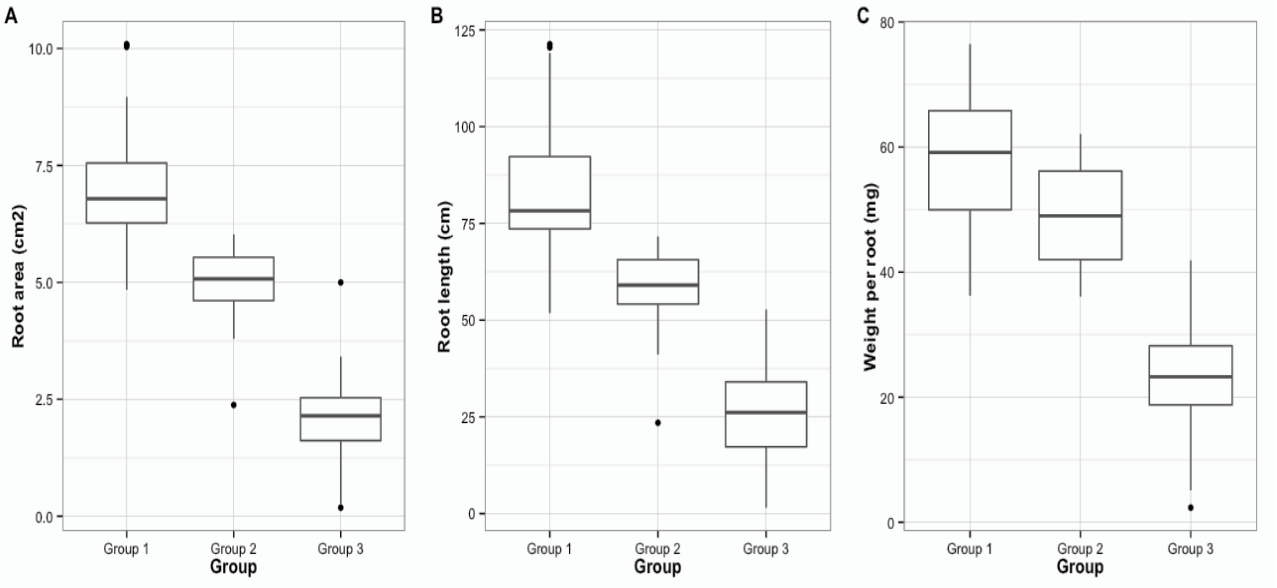

Figure S.2.5. Distribution of parameters analyzed: (A) root area (cm2), (B) root length (cm), and (C) weight per root (mg), on three groups based on the significance of the MANOVA and univariate analysis. Group 1 (non-significant for all analyses), group 2 (significant for MANOVA, but not for all univariate analyses), group 3 (significant for both MANOVA and univariate analyses). Boxplot represent distribution of data, the line represents the median for each group and dots indicates outliers.

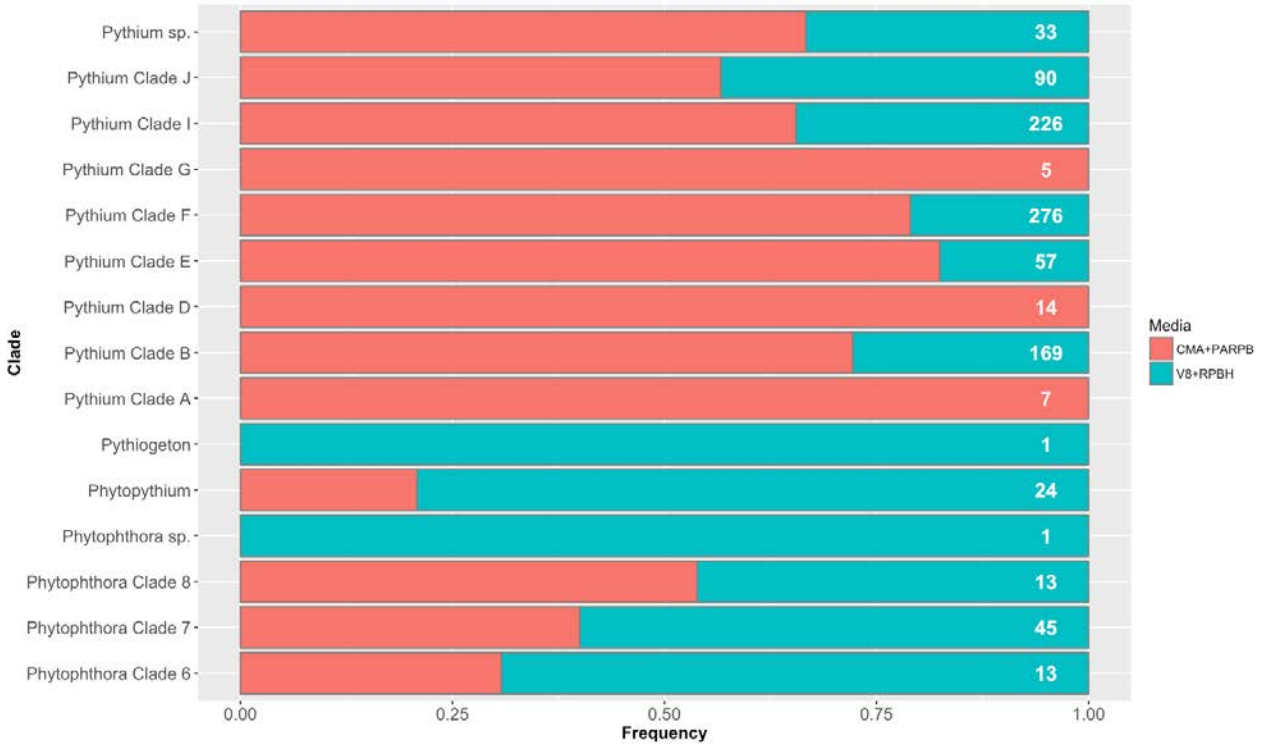

Figure S.2.6. Effect of media on the frequency of recovery of oomycete species summarized by clade in 2012. Numbers at the end of the bar represent number of total isolates per clade.

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Chapter 3

Oomycete species associated with soybean seedlings in North America – Part II: Diversity and ecology in relation to environmental and edaphic factors

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ABSTRACT

Soybean (*Glycine max* (L.) Merr.) is produced across a vast swath of North America, with the greatest concentration in the Midwest. Root rot diseases and damping-off are a major concern for production, and the primary causal agents include oomycetes and fungi. In this study, we focused on examination of oomycete species distribution in this soybean production system and how environmental and soil (edaphic) factors correlate to oomycete community composition at early plant growth stages. Using a culture-based approach, a total of 3,418 oomycete isolates were collected from 11 major soybean producing states and most were identified to genus and species using the ITS region of the rDNA. *Pythium* was the predominant genus isolated and investigated in this study. An ecology approach was taken to understand the diversity and distribution of oomycete species across geographical locations of soybean production. Metadata associated with field sample locations were collected using geographical information systems (GIS). Operational taxonomic units (OTUs) were used in this study to investigate diversity by location, with OTUs being defined as isolate sequences with 97% identity to one another. The mean number of OTUs ranged from 2.5 to 14 per field at the state level. Most OTUs in this study, classified as *Pythium* clades, were present in each field in every state, but major differences were observed in the relative abundance of each clade, which resulted in clustering of states in close proximity. Since there was similar community composition (presence/absence) but differences in OTU abundance by state the ordination analysis did not show strong patterns of aggregation. Incorporation of 37 environmental and edaphic factors using vector fitting and Mantel test, identified 15 factors that correlate with the community composition in this survey. Further investigation using redundancy analysis (RDA) identified latitude, longitude, precipitation and temperature as factors that contribute to the

variability observed in community composition. Soil parameters such as, clay content and electrical conductivity also affected distribution of oomycete species. The present study suggests that oomycete species composition across geographical locations of soybean production is affected by a combination of environmental and edaphic conditions. This knowledge provides the basis to understand the ecology and distribution of oomycete species, especially those able to cause diseases in soybean, providing cues to develop management strategies.

Introduction

Soybean (*Glycine max* (L.) Merr.) is a major crop in North America with a reported 85 million production acres in the U.S., yielding an estimated value of 40 billion dollars annually (American Soybean Association 2015). The prevalence of root rot diseases in soybean production in the U.S. and Ontario, Canada has resulted in increased production costs and reduced yields due to reduced plant stands, which can require replanting of entire fields. Soybean seeds and seed treatments constitute 48% of the growers input cost, which represents a total cost of 7.5 billion U.S. dollars annually (American Soybean Association 2015). Among the most common causes of soybean root rot diseases and damping-off are oomycete species, the most prevalent of which are members of the genus *Pythium* and *Phytophthora* (Dorrance et al. 2003, Broders et al. 2007). However, the extent of the oomycete community composition and species distribution associated with soybean roots are not well known in the U.S. Successful disease management relies on a thorough understanding of the pathosystem. In this regard, there is a crucial need to characterize oomycete species distribution, community composition, and the role of biotic and abiotic factors have on these communities.

The genus *Pythium* is typically linked with early season diseases, such as seedling root rot and damping-off, and multiple species have been often implicated (Zhang et al. 1998, Zhang and Yang 2000). *Phytophthora sojae* is also widely recognized as a major soybean pathogen causing root and stem rot (Tyler 2007). There has been an exponential increase in the reporting of new oomycete species in the last 20 years, as a result of the sequencing of genes, mainly internal transcribed spacer of rDNA (ITS) and cytochrome oxidase subunit I (COI) (Lévesque 2011). The use of these DNA markers have enabled the identification of new or overlooked causal agents of disease. Using these markers as tools, surveys of oomycete species composition and description of new species allows for a greater understanding of oomycete communities, including identification of pathogens, host ranges, and environmental conditions that influence the composition of species in pathogen communities.

The influence of environment on the diversity and distribution of plant pathogens is not a new concept. However, it is important to consider different approaches, such as the use of ecological methods to gain a better understanding of pathosystems for improved disease management. Most of the studies on oomycete diversity have focused on the genus *Phytophthora*, due to the threat that it represents to natural and agricultural ecosystems (Hansen et al. 2012, Scibetta et al. 2012, Parke et al. 2014, Knaus et al. 2015, Nagel et al. 2015). Parke et al. (2014) studied the *Phytophthora* spp. community assembly in a landscape nursery setting to improve disease management by identifying critical points and practices that may increase species abundance of *Phytophthora*, hence increasing the risk for disease. The diversity of *Phytophthora* spp. was catalogued in different components of the production system including irrigation water, potting mix, and the field environment. A critical production point identified was the soil and gravel, which served as the main source for *Phytophthora* spp., potentially

increasing the introduction of pathogenic species into the nursery (Parke et al. 2014). This systematic approach provides a good example of the application of community-level analysis in improving disease management. Nelson and Karp (2013) utilized molecular techniques to study the diversity of oomycete species associated with the invasive grass species *Phragmites australis* (Cav.) Trin. ex Steudel (European common reed) and the related native species. The pathogenicity of the these oomycetes isolates was evaluated by Crocker et al. (2015) on the invasive and native grass species. Addressing the interaction of host and oomycete community present in the soil, Crocker et al. (2015) found that *Pythium* spp. were abundant for both hosts, but *Pythium* spp. when associated with soil in locations invaded by *Phragmites* spp. had increased virulence compared to *Pythium* spp. isolated from soil surrounding only native species.

There have been recent studies focused on the distribution of oomycete species associated with soybean seedling diseases at an intra-state scale. Oomycete species were obtained by isolation from seedlings or by baiting from soil under controlled conditions. The isolates recovered were further characterized by evaluating for pathogenicity or fungicide resistance, thus providing a regional profile of the oomycete species distribution and their traits (Broders et al. 2007, Jiang et al. 2012, Marchand et al. 2014, Zitnick-Anderson and Nelson 2015). To gain a more global understanding of oomycete diversity and community composition, the effect of abiotic factors must also be taken into consideration, since environmental conditions influence the distribution and abundance of species. It has been previously reported that soil (edaphic) properties such as pH ~ 6, low calcium concentration (1.515 μg/g), and low cation exchange capacity (CEC; 13.02 meq/100g) were associated with species diversity in different *Pythium* communities in Ohio, resulting in reduced diversity and higher levels of disease incidence (Broders et al. 2009). Zitnick-Anderson et al. (2014) reported significant models that associated

CEC with specific *Pythium* spp. isolated from soybean fields in North Dakota: *Py. irregulare* and *Py heterothallicum* were associated with increased CEC, while *Py. kashmirense* was associated with decreased CEC. These findings reveal the complexity of the system and the need to implement ecological approaches to understand the distribution and diversity of oomycete species and the prevalence of pathogenic species.

In this study, we present a continental scale study of oomycetes that infect soybean roots using a culture-based approach to gain insight into large-scale patterns of the diversity of these organisms. To identify environmental and edaphic factors, metadata were obtained from geographic coordinates at each sample site. By incorporating associated environmental data, we analyzed the correlation of temperature, soil chemical and physical properties, precipitation, and latitude among others, with the oomycete community composition. We hypothesize that oomycetes species distribution is affected by regional edaphic and environmental conditions, and species are more likely to infect soybean plants under specific environmental conditions, such as cold temperatures and high soil moisture. The goals of the present study were: (i) establish community structure of oomycete species associated with soybean seedling diseases across soybean producing states in the U.S. and Ontario, Canada, (ii) explore the influence of different environmental and edaphic factors on oomycete community structure, and (iii) determine the prevalence and distribution of oomycete species responsible for seed and root rot diseases.

Materials and Methods

Isolation, culture collection and DNA extraction. A survey was conducted across 11 U.S. states and Ontario, Canada by the sampling of 64 and 61 fields in 2011 and 2012, respectively (Fig. S.3.1). Between four and seven fields were sampled per participating state by

collaborators in each of those states, targeting fields with a history of seedling disease or plant stand issues. Collaborators followed a standard sampling procedure, where 50 symptomatic seedlings were collected from a W-shaped transect across each field. In some fields there was an insufficient number of plants with above-ground symptoms to collect 50 symptomatic plants, and in those cases seedlings were randomly sampled. Soil samples were taken from some Ontario fields and isolates were baited from soil using soybean seed, therefore these samples were not included for analyses. Seedlings were transported in coolers and samples were processed within 24-h post collection (Rojas et al. 2016a). Plant processing and isolation were done as reported in Rojas et al. (2016a). Briefly, seedlings were washed under running tap water, and 1-cm root sections with characteristic discoloration associated with root infection were cut and placed onto a semi-selective medium. In 2011, the semi-selective medium was corn meal agar (CMA) amended with PARPB (Jeffers 1986). Due to the low number of *Phytophthora* spp. collected in 2011, both CMA-PARPB and V8 medium amended with RPBH were used in the second year of the survey in an attempt to improve the recovery of *Phytophthora* spp. (Jeffers 1986, Dorrance et al. 2008).

Culture plates were incubated for 7 d at 20ºC, and checked daily for hyphal growth and morphology consistent with oomycetes. Single pure cultures were obtained by hyphal tipping and transfer to fresh CMA-PARPB or V8-RPBH medium. Transfer of 5-mm plugs from fresh isolate cultures onto potato carrot agar slants and hemp seed vials were used for long term storage (van der Plaats-Niterink 1981, Erwin and Ribeiro 1996). In addition, three to five 5-mm plugs from fresh cultures were placed into 50 mL of a 10% V8 broth amended with ampicillin (100 mg/L) and incubated for 7 to 10 day at room temperature without agitation. Oomycete mycelia were harvested from broth cultures, lyophilized overnight, and ground for DNA

extraction. DNA extraction was achieved by adding 100 mg of ground mycelia and 800 µL CTAB lysis buffer into sterile AutoGen tube racks (AutoGen AGPR-S-STAR; AutoGen AG00121, AutoGen Inc.) and incubated for 1 h at 65ºC. A phenol-chloroform automated DNA extraction was performed using the AutoGen 850 system (AutoGen Inc., Holliston, MA). DNA was resuspended in 200 µL TE buffer with incubation on an orbital shaker for 1 h at 65ºC, then transferred to 1.5 mL tubes and stored at -20ºC.

Identification of isolates. Sequences of the internal transcribed spacer 1 and 2 regions of rDNA were obtained by amplification and sequencing with primers ITS6 and ITS4 (Cooke et al. 2000). The PCR mix consisted of 1X DreamTaq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM ITS6 and 0.2 µM ITS4, 4 µg/mL of BSA, 1U DreamTaq polymerase (Thermo Scientific, Waltham, MA), and 1 µL DNA. The thermal cycling program consisted of 95ºC for 2 min; 35 cycles of 95ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min; and a final extension at 72ºC for 10 min. Amplicons were purified by adding 5µL of a mixture of 3U of exonuclease I and 0.5U of FastAP thermosensitive alkaline phosphatase (Thermo Scientific, Waltham, MA), followed by 45 min at 37ºC, and enzymes were inactivated by incubation at 85ºC for 10 min. Amplicons were Sanger sequenced in both directions and consensus sequences obtained for downstream analyses. Sequences were deposited in GenBank under accession codes KU208091 - KU211502 (Rojas et al. 2016a).

Pre-processing and OTU assignment. The ITS sequences were used to conduct a diversity analysis by grouping the sequences into Operational Taxonomic Units (OTUs) using Mothur v1.36 (Schloss et al. 2009) as reported on the Mothur batch file (Rojas et al. 2016b).

Sequences were pre-processed eliminating those with homopolymers of 20 bases or longer. Sequences were reduced to unique sequences and aligned to a reference ITS alignment published by Robideau et al. (2011), allowing for reverse complement to improve and maintain the best match. Sequences were also discarded with an alignment length less than 500 bases. The sequence dataset was further reduced by keeping unique sequences; pre-clustering was performed to reduce sequencing error, allowing a maximum of three nucleotide differences.

In addition, a pre-classification step was conducted using a bootstrap cutoff of 80 out of 100 iterations to eliminate OTUs outside of the target oomycete taxonomy. Distance was calculated on the resulting sequence dataset using a cutoff of 0.1, followed by clustering using the furthest neighbor algorithm. The resulting OTUs were selected based on a distance cutoff of 97% similarity, meaning all sequences in each OTU were within a 3% distance from other sequences, the OTU designation represents "species-like" designation. In addition, OTUs were assigned a taxonomic classification from kingdom to genus, including clade for *Pythium* and *Phytophthora* for further analyses and comparisons. A phylotype analysis was conducted using identification based on local blast searches described in Rojas et al. (2016a) and using a reference dataset provided by Robideau et al. (2011). The phylotype designation uses the taxonomic assignments based on the database rather than a similarity threshold to bin the samples into groups. The results were collapsed into a phylotype abundance table with corresponding taxonomy assignment (kingdom to species level) for downstream analyses. For interpretation it should be noted that it is possible to have multiple OTUs per phylotype (species designation) due to sequence variation. Resulting OTU and phylotype tables were exported in BIOM files for analysis in R version 3.2 (R core team 2015, Vienna, Austria) using the package 'phyloseq' (McMurdie and Holmes 2013).

Community and diversity analysis. Estimates for within-group or field diversity (α diversity) were calculated using the 'vegan' (Oksanen et al. 2013) 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 was summarized by state. Fields across states were evaluated for correlation with latitude and longitude using α diversity measures and Spearman correlation. In order to evaluate the community structure, OTU tables were constructed and normalized as relative abundance to determine among-group diversity (β-diversity) using Bray-Curtis distances to compare communities pairwise. The resulting dissimilarity matrices were used to assess clustering of the communities by state, and to evaluate communities by field using ordination analysis as principal coordinate analysis (PCoA).

Edaphic and environmental parameters of the sampled fields were acquired based on geographic information system (GIS) coordinates. Fields without this information were not included in this analyses. Soil chemical and physical properties were obtained from the National Resources Conservation Service (NRCS) soil database (http://www.nrcs.usda.gov/). Ambient temperature (maximum, minimum, and mean) and precipitation (maximum, minimum, and mean) for different time ranges, including yearly and planting season (April, May and June), were obtained from the PRISM (Parameter-elevation Regressions on Independent Slopes Model) Climate Group (http://www.prism.oregonstate.edu/). Other parameters such as topology and images for land usage were queried from United States Geological Survey (USGS) (http://www.usgs.gov/) and National Agricultural Statistics Service (USDA NASS) (http://www.nass.usda.gov/research/Cropland/SARS1a.htm), respectively. The information obtained was analyzed in conjunction with community structure and diversity data in R using the

packages ‘vegan’ and ‘MASS’. The different environmental and edaphic factors were evaluated for community structure and diversity association by using vector fitting in conjunction with the ordination analysis. Environmental factors were plotted as vectors using the ‘envfit’ function from vegan. The environmental and edaphic parameters were corroborated by use of a Mantel test to confirm the correlation with community composition (Ramette 2007). Parameters that showed association with community structure were tested for correlation graphically using ordination and Spearman correlation.

In order to corroborate the effect of environmental variables on oomycete community structure, environmental variables were tested using distance based redundancy analysis (dbRDA) (Legendre and Anderson 1999). Community data was input as a matrix of Bray-curtis distances and all the explanatory variables were entered into the model and compared to a null model (no explanatory variables), to conduct stepwise selection using the function *ordistep*() in vegan (Oksanen et al. 2013) with 10,000 permutations. The model was further refined using variance factor inflation (VIF) maintaining values < 10. All data and R scripts used in the analyses shown here are deposited on github (https://github.com/Chilverslab/Rojas_Survey_Phytopath_2016) and citable (Rojas et al. 2016b).

Results

OTU and phylotype richness per field across states. During the two-year survey, 125 fields were sampled and 3,418 oomycete isolates were recovered. Although fungi were not the focus of this study, 222 fungal isolates were recovered on the oomycete semi-selective medium and the majority consisted of members within the phylum Zygomycota, followed by the phyla Basidiomycota and Ascomycota. The sequences that were identified as fungal species were

removed prior to downstream analyses. Based on phylotype analysis conducted with a local blast, 3,242 of the oomycete isolates reside in the genus *Pythium,* with the remaining isolates corresponding to the following genera, *Phytophthora* -142 isolates*, Phytopythium* - 31 isolates, *Aphanomyces* -two isolates, and lastly one isolate in the genus *Pythiogeton.* In the OTU analysis, 2,380 sequences from 2011 and 1,038 sequences from 2012 were combined and analyzed using a 97% similarity threshold, which resulted in 216 OTUs, of which 194 corresponded to *Pythium,* 13 *Phytopythium,* 4 *Phytophthora,* 4 *Brevilegnia* and 1 *Aphanomyces*. The average number of observed OTUs ranged from 2.5 to 14 on average per field across the different states (Table 3.1). Arkansas in 2011 had the highest diversity with an average number of 14 OTUs per field, while South Dakota in 2011 and Iowa in 2012 had the lowest levels of diversity with observed OTUs of 2.5 and 2.8, respectively. The Shannon-Wiener index calculated per field showed that diversity ranged from 0.8 to 2.3 across the different states, where more than 50% of the fields had values of around 1.5 (Table 3.1), thus displaying a moderate diversity among fields sampled in the different states. Despite the sampling effort, the recovery of oomycete species in some field and state locations was low, resulting in low species diversity. The Simpson index of diversity favors the dominant or common OTUs in the community and uses a scale from 0 (no diversity) to 1. Approximately 60% of the fields sampled were around 0.5 to 0.8, and by state ranged from 0.3 to 0.9 showing a moderate to high species diversity (Table 3.1). In addition, there were differences in terms of diversity evenness, more than 50% of the sites had an evenness above 0.7, which indicates that a majority of the fields sampled contained close to even distribution of the OTUs observed.

Table 3.1. Oomycete community diversity and evenness by state and year. Data represents state-year average and standard deviation of soybean seedlings sampled by field.

State/Year	Fields sampled	Isolates	Observed OTUs[a]		Shannon-Wiener index		Simpson index		Evenness[e]
			Mean[b]	SD[c]	Mean	SD	Mean	SD	
Arkansas 2011	1	320	14.00	ND[d]	1.19	ND	0.60	ND	0.45
Arkansas 2012	6	75	7.33	±4.10	1.57	±0.80	0.67	±0.33	0.79
Illinois 2011	6	243	9.00	±3.20	1.66	±0.40	0.73	±0.12	0.76
Illinois 2012	6	147	7.17	±1.50	1.62	±0.19	0.74	±0.09	0.82
Indiana 2011	5	398	10.20	±1.80	1.56	±0.14	0.69	±0.06	0.67
Indiana 2012	5	33	4.00	±2.30	1.08	±0.72	0.55	±0.33	0.78
Iowa 2011	9	398	6.89	±3.30	1.09	±0.63	0.48	±0.27	0.57
Iowa 2012	4	19	2.75	±1.00	0.83	±0.21	0.51	±0.10	0.82
Kansas 2011	7	213	7.43	±2.80	1.36	±0.55	0.61	±0.26	0.68
Kansas 2012	6	93	6.67	±2.70	1.59	±0.51	0.73	±0.14	0.84
Michigan 2011	11	190	5.00	±3.20	1.15	±0.69	0.57	±0.30	0.71
Michigan 2012	7	134	8.29	±3.70	1.75	±0.49	0.76	±0.13	0.83
Minnesota 2011	6	185	10.67	±6.20	1.86	±0.54	0.77	±0.10	0.79
Minnesota 2012	6	130	8.17	±4.10	1.76	±0.49	0.78	±0.11	0.84
N Dakota 2011	9	210	9.56	±5.60	1.78	±0.63	0.76	±0.16	0.79
N Dakota 2012	6	162	10.67	±2.90	1.92	±0.28	0.79	±0.07	0.81
Nebraska 2011	4	75	7.75	±3.70	1.65	±0.46	0.75	±0.10	0.81
Nebraska 2012	3	49	6.67	±4.00	1.64	±0.61	0.76	±0.13	0.86
Ontario 2012	1	64	9.00	ND	0.85	ND	0.33	ND	0.39
S Dakota 2011	6	24	2.50	±1.40	0.75	±0.49	0.47	±0.25	0.82
S Dakota 2012	5	114	13.00	±3.80	2.39	±0.29	0.89	±0.04	0.93
Wisconsin 2011	6	51	4.67	±2.00	1.24	±0.53	0.62	±0.21	0.81

[a] OTU = Operational Taxonomic Unit defined at the 97% threshold.

[b] Mean across fields sampled for the corresponding state and year.

[c] Standard deviation for fields sampled for the corresponding state and year.

[d] ND = Not determined

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

Community composition at the state level. Using the taxonomy assignment of the OTUs, these were grouped to the oomycete clade level across the states and years sampled (Fig. 3.1). It was conspicuous that *Pythium* clades F, B and I were the most abundant taxa, however, most clades were present to some level in each state. *Phytophthora* Clade 7, which includes *P. sojae,* was the most abundant for this genus overall and it was highly abundant in the province of Ontario (Fig. 3.1). Ontario was the only region that used a soil baiting method, which resulted in a high number of *P. sojae* isolates, and for that reason, it was excluded from most analyses to avoid confounding results. The among-group diversity (β-diversity) that describes the compositional dissimilarity between oomycete communities of states was calculated using the Bray-Curtis dissimilarity index. The resulting matrix was analyzed by cluster analysis and plotted as a dendrogram to determine the diversity relationship of each state to another state (Fig. 3.2A). Geographically adjacent states had similar community structure and as such grouped together, for example, Michigan, Indiana and Illinois grouped together. The analysis did not reveal strong clustering by year of the communities at the state level.

Spatial and temporal effects on community composition at field level. Using diversity per field as response, the effect of latitude and longitude were investigated to corroborate the role of spatial factors on community composition. A significant but weak positive correlation was observed between latitude and diversity (rho = 0.258, *P*-value = 0.0164, Fig. 3.2B), with greater diversity at higher latitudes. This correlation was also observed when comparing latitude using the Simpson diversity index (rho = 0.239, *P*-value = 0.026) and number of observed OTUs (rho = 0.189, *P*-value = 0.081), the latter being non-significant. In addition, longitude was examined to determine if a relationship existed with diversity. It was observed that

diversity slightly decreased towards the east (Fig. 3.2C), this a weak correlation (rho = -0.224, p-value = 0.037), when compared to latitude. Using an analysis of similarity (anosim) permutation test, fields were grouped by latitude and evaluated for community similarity. The results demonstrated significant differences between field community composition at different latitudes (R statistic = 0.103, *P*-value=0.001) and more similar composition at similar latitudes. By addressing differences within the field communities in each state using anosim, low differentiation of the communities was found within states, suggesting a similar community composition within state (R statistic = 0.226, *P*-value = 0.001), but there was significant differentiation among states, when state was used as a group. In addition, a temporal effect was also evaluated to determine the contribution of year to differences observed across communities at the field level. Using year as a grouping factor to study dissimilarities of the communities resulted in no differentiation between field communities from 2011 and 2012 (R statistic = 0.018, *P*-value = 0.115). In order to corroborate the anosim results and determine the contribution to the variance of the year and state factors using field community composition as a response, a permanova (adonis) test was performed that resulted in significant differences for state and year sampled (Table 3.2), where 20% of the community structure variability is explained by state grouping and 1.5% by year grouping.

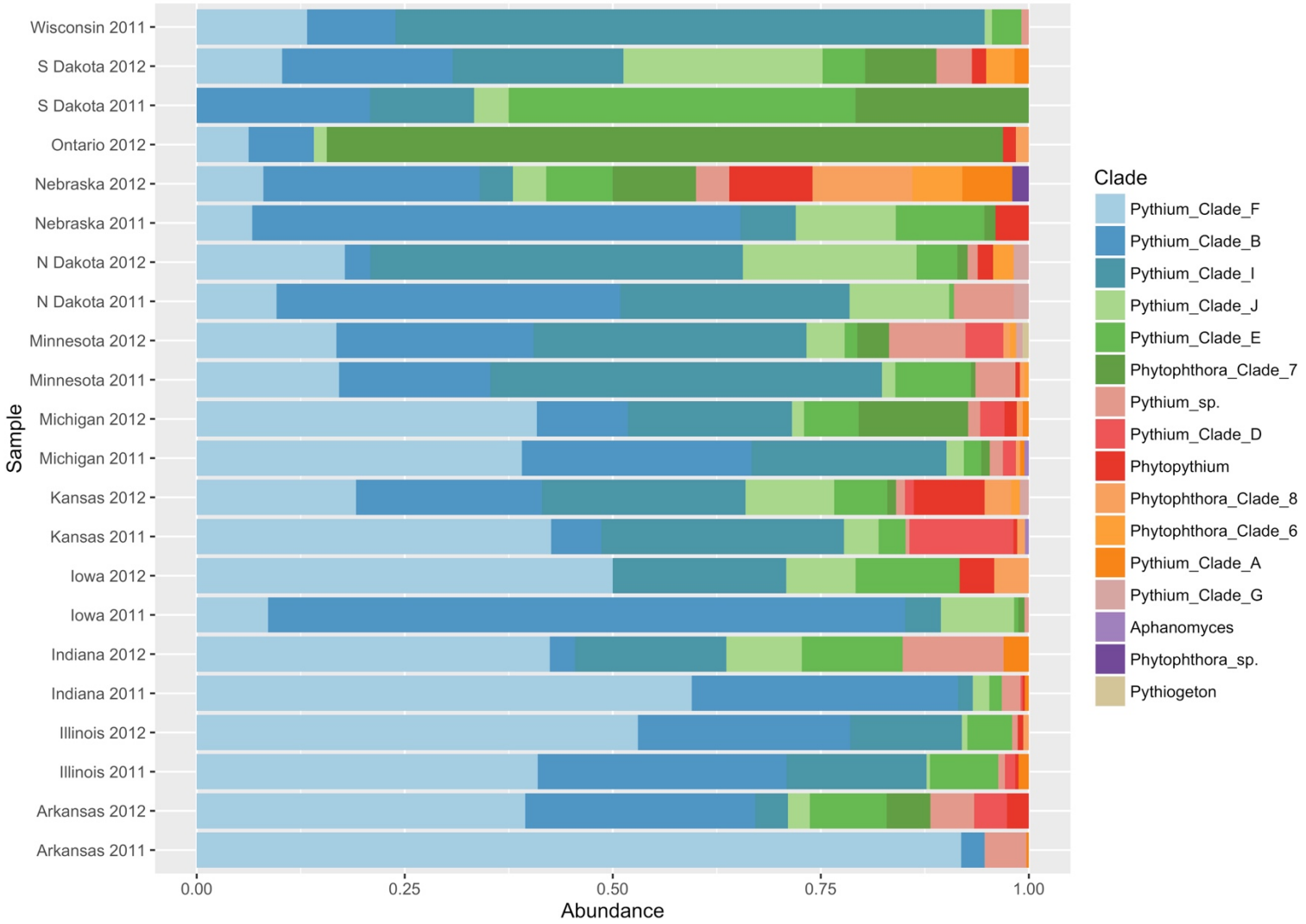


Figure 3.1. Relative abundance of OTUs summarized by clade or genera and state for 2011 and 2012. The clades or genera are color coded according to the legend from top to bottom.

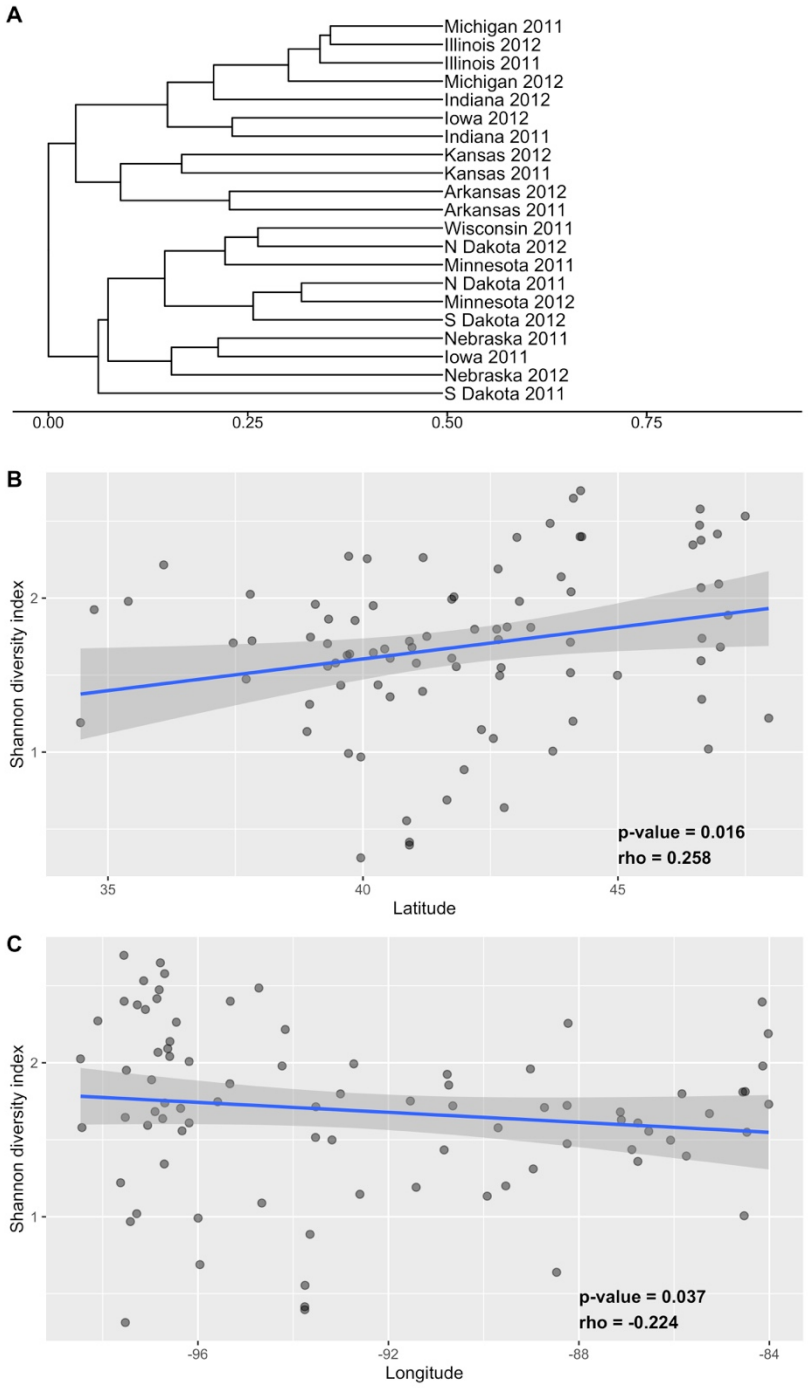


Figure 3.2. Oomycete community structure of species recovered from soybean seedling evaluated by (A) cluster analysis based on Bray-Curtis distance by state surveyed. Dendrogram was constructed using hierarchical clustering with complete linkage; (B) diversity of oomycete communities expressed as Shannon index across the latitudes of the fields sampled; (C) diversity of oomycete communities expressed as Shannon index across longitudes of the fields sampled.

Table 3.2. Evaluation of oomycete community structure (beta diversity) differences across states, years and state-year interaction based on Bray-Curtis distances using ADONIS.

Adonis on Bray-Curtis distances	Df[a]	F statistic	R^{2b}	Pr(>F)[b]
State	11	2.908	0.207	0.001
Year	1	2.346	0.015	0.003
State:Year	9	1.952	0.113	0.001
Residuals	103		0.665	
Total	124		1.000	

[a] Degrees of freedom
[b] R-squared and *P*-value based on 999 permutations

Abiotic effects on community composition at the field level. Further investigation of the among-group diversity (β-diversity) between fields was examined using a PCoA. The first principle coordinate axis explained 12.1% and the second principle coordinate axis accounted for 9.4% of the variability (Fig. 3.3). The PCoA analysis did not yield clear distance separation of samples by state or year. By incorporating environmental and edaphic factors, it was possible to address which factors caused this gradient or continuum of communities based on their correlation with the PCoA axes. A total of 37 different environmental and edaphic factors were tested using the 'envfit' function, which fits environmental vectors into the PCoA ordination plot, which resulted in 24 factors correlated with the ordination (Table 3.1). Temperature and precipitation were among the main environmental factors associated with oomycete community structure using different scales, such as seasonal parameters, annual averages, or 30-year averages (Table 3.3). Apart from temperature and precipitation, soil factors of density, water holding capacity, pH, and CEC were also significant factors. Latitude, longitude, and precipitation showed the most significant correlation of the evaluated factors related to oomycete community structure (Table 3.3).

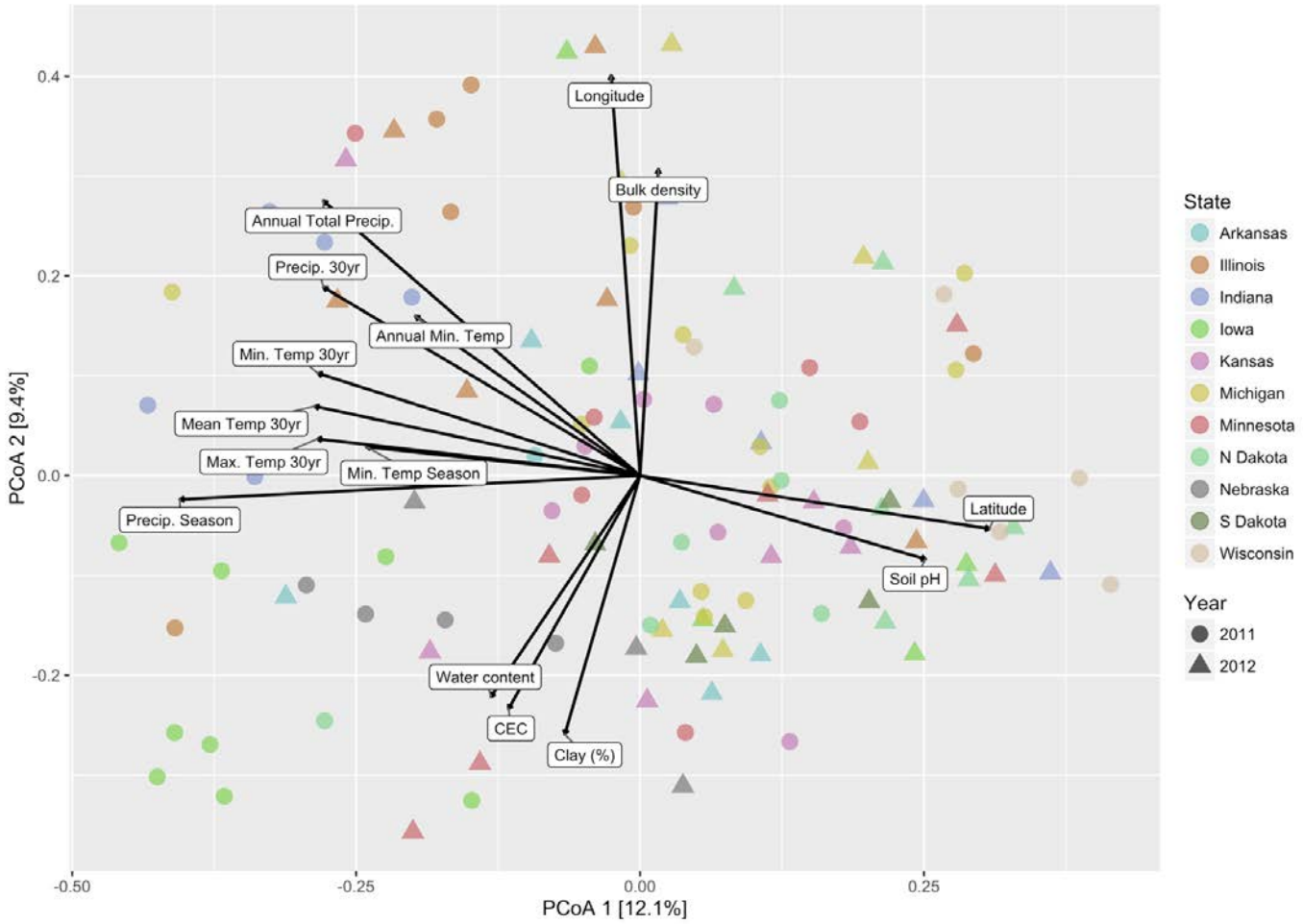


Figure 3.3. Principal coordinate analysis (PCoA) of oomycete communities collected from soybean seedling in the US based on a Bray-Curtis distance computed from taxon counts. Environmental and edaphic factors are plotted as vectors based on correlations with community distance. Only vectors with significant correlations are represented and the length of the vector represents the strength of the correlation. (CEC = Cation Exchange Capacity, Season: corresponds to April, May and June where most planting and or sampling was conducted).

Table 3.3. Significance of factors affecting oomycete communities associated with soybean seedlings, based on using 'envfit' function form vegan.

Environmental/ Edaphic Parameter	Axis.1[a]	Axis.2[b]	'envfit' test		Mantel test	
			R^2	*P*-value	Statistic	*P*-value
Precipitation season (mm)	-0.405	-0.024	0.165	0.001	0.070	0.024
Longitude	-0.025	0.401	0.161	0.001	0.083	0.003
Total Precipitation (mm)	-0.279	0.275	0.154	0.001	0.117	0.006
Precipitation 30 years average (mm)	-0.279	0.189	0.113	0.002	0.103	0.006
Latitude	0.308	-0.053	0.097	0.004	0.115	0.004
Soil bulk density (g/cm^3)	0.016	0.307	0.094	0.004	0.023	0.275
Minimum temperature 30 years average (ºC)	-0.284	0.102	0.091	0.006	0.085	0.022
Mean temperature 30 years average (ºC)	-0.286	0.069	0.087	0.007	0.122	0.001
Maximum temperature 30 years average (ºC)	-0.284	0.037	0.082	0.009	0.153	0.001
Clay content (%)	-0.066	-0.259	0.072	0.019	0.151	0.003
Soil pH	0.251	-0.084	0.070	0.022	0.044	0.106
Cation Exchange Capacity (CEC; meq/100g)	-0.116	-0.234	0.068	0.017	0.080	0.033
Water content	-0.131	-0.222	0.066	0.022	0.143	0.003
Minimum temperature (ºC)	-0.198	0.160	0.065	0.018	0.068	0.073
Minimum temperature season (ºC)	-0.241	0.029	0.059	0.031	0.106	0.006

[a] Axis 1 explains 12.1% of the variability between communities
[b] Axis 2 explains 9.4% of the variability between communities

Factors significant for 'envfit' analysis were also tested using the Mantel test to corroborate results obtained with the vector fitting analysis. Most factors were significant for both tests with the exception of soil bulk density, soil pH, or annual minimum temperature (Table 3.3). The correlations for significant environmental and edaphic parameters were not above 0.16, however many factors were found to be correlated and contribute to the community composition. Environmental parameters, such as temperature and precipitation at different time

scales, and edaphic factors, such as clay content, cation exchange capacity, and soil water content, were among the most correlated factors. These correlations were further evaluated using the correlated PCoA axis with the respective linked factor. Factors with long vectors were examined including seasonal precipitation, seasonal minimum temperature, clay content (%), and soil bulk density (Fig. 3.4). Seasonal precipitation and minimum seasonal temperature were evaluated against community composition similarity represented by the correlated PCoA axis. Additionally, samples were visualized with color by latitude. Both factors, seasonal minimum temperature and seasonal precipitation (April to June), showed a negative correlation, indicating that community composition similarity was higher at locations with low precipitation and low temperature average seasonal values (Precipitation: rho = -0.375; Min. temp. season: rho = -0.316).

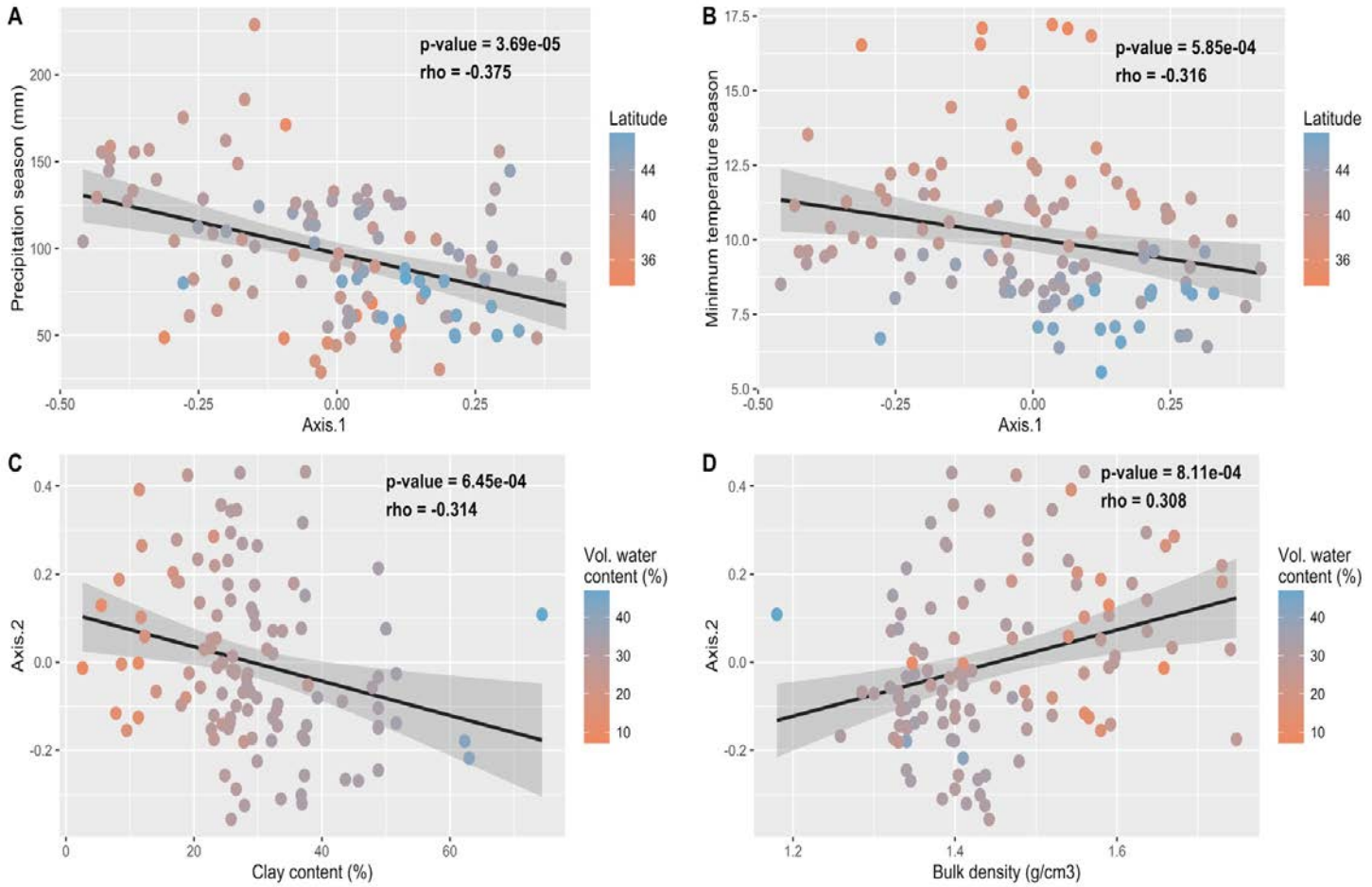


Figure 3.4. Representation of the correlation of among community similarity (PCoA ordination axis) and four environmental factors: (A) seasonal precipitation (mm, from April - June) with samples colored by latitude; (B) seasonal minimum temperature (ºC, April - June) with samples colored by latitude; (C) clay content (%) with samples colored by volumetric water content (%); and (D) bulk density of the soil (gm/cm3) with samples colored by volumetric water content (%). Spearman correlation values (rho) and *P*-value are presented for each comparison. Season refers to the planting and or sampling period which ranged from April to June.

In regards to latitude and longitude, fields sampled in this study at higher latitudes and located to the west of the sampling area received a lower amount of precipitation and recorded lower temperatures, which affected the community composition. In contrast, samples collected from lower latitudes and to the east received a higher amount of precipitation and experienced higher temperatures, causing a distinct community composition (Fig. 3.4A and 3.4B). The edaphic factors also had an effect on community composition across the samples collected. Clay content and soil bulk density were factors that showed a gradient among the community composition (clay content: rho = -0.314; bulk density: rho = 0.308). In addition, Figure 3.4C and 3.4D data points were colored based on water content and related to the two parameters analyzed, clay content and bulk density. In this case, clay content had a negative correlation with community similarity, where community composition differs along the gradient of soil clay content. High clay content soils are different in community structure to soils that have a low clay content, and these differences correlate with water content (Fig. 3.4C). Soil bulk density had a positive correlation with relatedness among communities, meaning that soils with lower bulk density had communities more similar to each other than communities from high vs low bulk density soils. Whereas, soils with a higher density tended to have a reduced water content, resulting in a community composition that was more variable with larger distances between communities (Fig. 3.4D).

Redundancy analysis on community composition at the field level. By using distance-based redundancy analysis, a model selection was conducted by reducing the number of environmental and edaphic variables. The environmental and edaphic variables were redundant and resulted in multicollinearity, thus using a stepwise variable selection, most variables were rejected. The selection of the variables was retested using variance inflation factors to adjust the

model. The selected variables were latitude, longitude, precipitation and temperature during the season, and clay and electrical conductivity. The variables overlapped the variables obtained in the vector fitting analysis on the PCoA and Mantel test, with the exception of electrical conductivity (EC, Table 3.4). The final model resulted in 13% of the variability explained by the environmental variables. The highest contributions to variance are latitude (4.76%), longitude (3.07%) and seasonal precipitation (2.47%), which also contributed the most in the vector fit and Mantel test approach.

Table 3.4. Relationships of the predictor variables determined by distance based redundancy analysis to the oomycete community composition from soybean seedlings. The 34 environmental and edaphic variables were tested against a null model and selected using stepwise selection and variance inflation factors (vif).

Explanatory variables	**variance**	**F**	**Pr(>F)**
Latitude	0.048	5.083	<0.001
Longitude	0.031	3.667	<0.001
Season Precipitation	0.025	3.105	<0.001
Season min. temperature	0.011	1.573	0.033
Clay (%)	0.009	1.374	0.094
Electrical Conducitivity (EC)	0.007	1.490	0.051

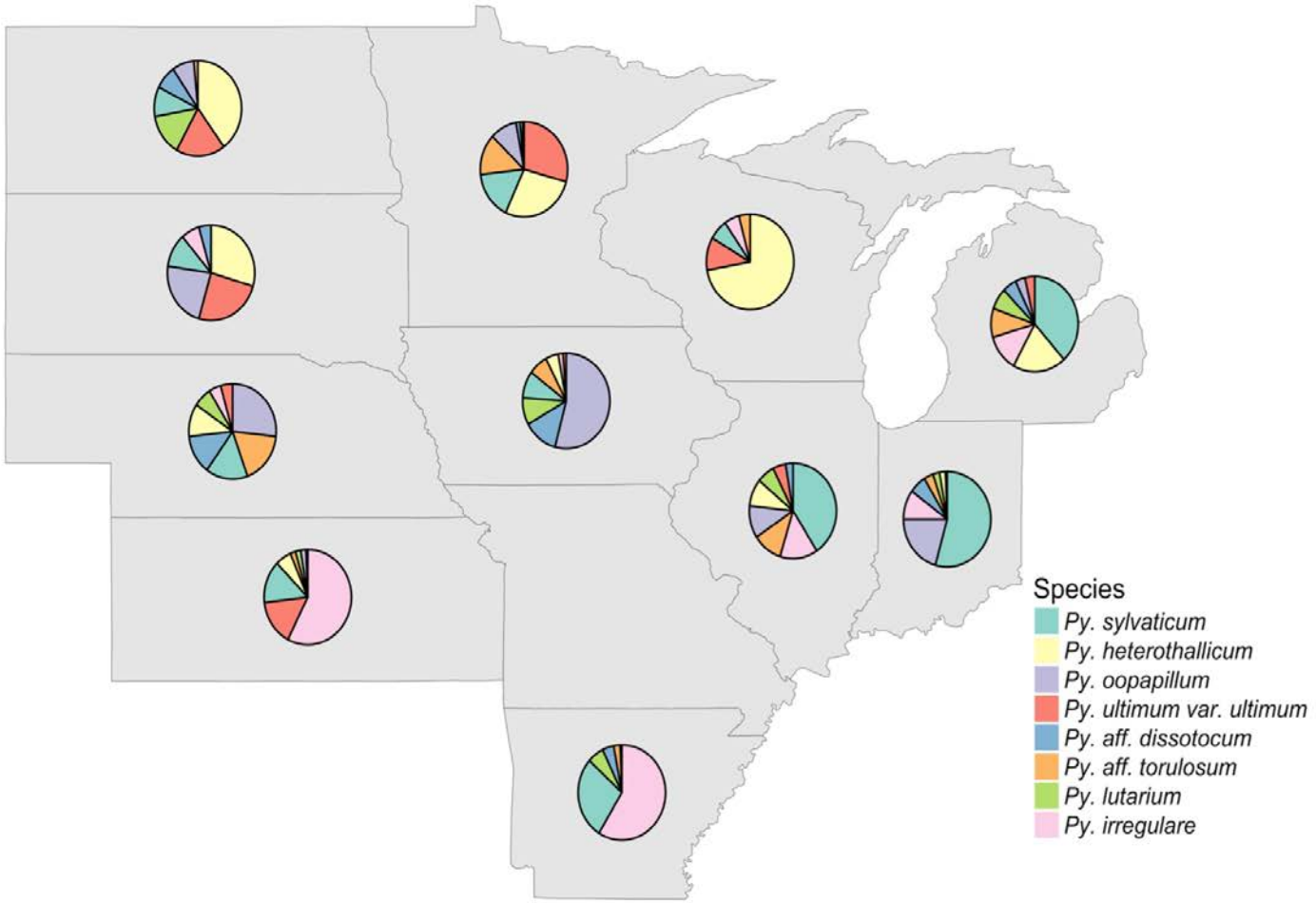


Figure 3.5. Distribution and abundance of the top 8 pathogenic oomycete species across the states sampled in 2011 and 2012.

Relative abundance and distribution of the top pathogenic species. The abundance of the top eight most frequent pathogenic species was examined by state and it was evident that there were dominant species across multiple state regions. For instance, *Py. sylvaticum* was dominant in Michigan, Illinois and Indiana, while states further north were dominated by *Py. heterothallicum* (Fig. 3.5). These northern states were also dominated by *Py. ultimum* var. *ultimum.* States towards the south were dominated by *Py. irregulare,* but the Arkansas community was also dominated by *Py. sylvaticum*. Nebraska, Iowa and South Dakota communities also contained a considerable percentage of *Py. oopapillum* isolates (Fig. 3.5). To address the trends of individual pathogenic species, abundance of each species was examined in the context of the environmental and edaphic factors previously identified as drivers of community composition (Fig. S.3.3). As found in previous studies, some of these parameters could have an effect on their abundance. For instance, *Py. sylvaticum* abundance, represented as log-transformed counts of isolates per field, was low with high values of soil pH, CEC and percent clay content; while its abundance increased with precipitation and temperature (ca. 150 mm and 12ºC). Conversely *Py. heterothallicum* abundance increased with increasing pH, percent clay content and CEC; while the abundance declined with decreasing values of temperature and precipitation.

Discussion

This study is one of a few that has been conducted to examine oomycete diversity and community composition in an agricultural system at a continental scale. A total of 3,418 oomycete isolates were collected from soybean seedling roots in 125 soybean fields (Rojas et al. 2016a). The ITS rDNA sequences were analyzed using two approaches: OTUs at 97% similarity

and phylotype at the species level. The OTU approach resulted in 216 OTUs, where OTUs represent species-like designation, however, intraspecific ITS variability in some species complexes could be higher than the designated species threshold (Schroeder et al. 2013), therefore OTUs could represent similar species. In the phylotype approach, sequences are assigned based on taxonomy to a local curated database and binned using taxonomic designation. Despite the high number of OTUs (n = 216) relative to phylotypes (n = 84), a similar community composition was observed when compared at the clade level. Both OTU and phylotype analyses have different advantages and disadvantages (Schloss and Westcott 2011). Phylotype is limited by the database, whereas OTU analysis does not require a taxonomic delimitation to bin sequences into groups. Operational taxonomic units were used in this study to obtain the best resolution to investigate the ecological diversity of oomycetes associated with soybean.

Diversity analysis showed that most fields displayed a moderate to high diversity, showing up to 14 OTUs on average per field. Species abundance varied across the two years sampled, which was more conspicuous when looking at OTUs grouped at the clade level. In general, most fields had a high evenness, which refers to how evenly represented the different OTUs were in each field sampled. The expanse of the survey showed that at this plant stage *Pythium* spp. are widely present on symptomatic seedlings in fields across the predominant soybean production area of North America. The survey revealed that *Pythium* clades F, B, I and J were dominant in most states and contributed to at least 50% of the community composition. These clades also contain most of the plant pathogenic species (Lévesque and De Cock 2004). The remaining percent of the communities were partitioned into the *Pythium* clades A, D, E, and G, *Phytophthora* clades 6, 7, and 8, and the genera *Phytopythium*, *Aphanomyces* and *Pythiogeton*. This study corroborates state-level studies that found *Pythium* spp. abundantly

present in soybean fields (Rizvi and Yang 1996, Murillo-Williams and Pedersen 2008, Ellis et al. 2012, Zitnick-Anderson and Nelson 2015).

The composition of the communities associated with soybean seedlings was significantly different by state, but states geographically close in proximity exhibited similarity based on clustering. This finding suggests an effect of geographical location on oomycete community composition. Therefore, field community diversity was evaluated as response to latitude and longitude. A diversity gradient was observed by latitude, with diversity increasing as latitude increased, but the linear model did not find a significant correlation for longitude. The result was also supported by the use of anosim, a distribution free method of multivariate analysis. With the anosim test finding a significant effect by latitude (R-statistic = 0.103, *P*-value=0.001), but not longitude (R-statistic = 0.040, *P*-value = 0.099). The present study is limited to regions where soybeans are grown, however this spatial effect is concordant with other systems, especially in fungal groups (Tedersoo et al. 2014). Ectomycorrhizal fungi reached the highest diversity at mid-latitudes ranging between 40 – 60º, while other fungal groups like Ascomycota peaked at latitudes associated with tropical regions. It has been suggested that oomycetes may have a higher diversity in tropical areas, however, this is based on the description of new genera in these areas (Nigrelli and Thines 2013), and requires additional investigation.

The effect of spatial distribution on the differentiation of field communities among states was further evaluated using the permanova (adonis) analysis, which is a non-parametric method to determine sample grouping. The differences among community composition resulted in 20% of the variability explained by state. With respect to temporal variation or the effect of season on community composition, only 1.5% of variability was explained by year in the permanova test, despite environmental conditions that varied between 2011 and 2012, and the use of two semi-

selective medium in 2012. The anosim resulted in non-significant differences between the years. However, looking at composition by year and state explained 11.1% of the variability, suggesting that community composition is affected at a regional scale across the seasons sampled rather than by sampling year. These results suggest that there is a spatial component that contributes to community composition, which is stronger than a seasonal sampling component.

The ordination method of principal coordinate analysis, that uses differences among-field-communities (ß-diversity) revealed that 12% and 9.4% variability is explained not by the presence of different OTUs but from the differences in abundance between states and years. Environmental and edaphic factors were explored to determine the effect on community composition. Of 34 environmental and edaphic parameters tested, 15 showed significant correlations with the among-group oomycete diversity at the field level. A vector fitting approach and Mantel test were conducted to confirm those factors that could explain the variance across community composition. Of the environmental parameters evaluated, temperature and precipitation were correlated with community composition. With respect to edaphic factors, soil bulk density, clay content, pH and CEC were factors that also correlated with community composition. As expected, multiple factors contributed to explain the variability of community composition across fields, these contributions were small, but significant, which is common in ecology studies as many variables may contribute to species abundance and distribution. A further delimitation of the environmental and edaphic variables as predictors of community composition was done using redundancy analysis. The goal was to systematically reduce the variables, reducing collinear factors that explain the community observed and also the contribution of each factor. This method resulted in corroboration of parameters previously detected by vector fitting and Mantel test, resulting in latitude, longitude, seasonal temperature,

seasonal precipitation and clay content as variables that explained the variance observed in community composition. This supports the idea of geographical location and local environment playing a role in the oomycete community composition.

The environmental and edaphic factors identified in this study relate to the biology and ecology of these organisms, for example moisture as affected by precipitation and soil water holding properties is a requirement for oospore germination, sporangia formation and zoospore motility (Martin and Loper 1999). In addition, nutrient availability, like ions, has been demonstrated to have differential effects on particular *Pythium* spp. For example, chlorine availability can decrease inoculum density of *Py. ultimum* while promoting soil colonization by *Py. oligandrum* (Martin and Hancock 1986). Among the different edaphic factors evaluated, electrical conductivity, CEC and soil pH were associated with the oomycete community present. For instance, *Py. heterothallicum* increased in abundance at around pH 7 to 8 and a CEC of 30 – 40 meq/100g. Whereas, *Py. sylvaticum* abundance decreased under the same conditions (Fig. S.3.3 and Fig. S.3.5). It suggests that high values of CEC are correlated with high diversity, since some dominant species will decrease their propagule density favoring other species (Martin and Loper 1999, Broders et al. 2009).

Another factor to consider is the temporal component of sampling, as some species might become active under different environmental conditions or plant stages. Surprisingly, *Phytophthora* spp. were recovered in low abundance despite the use of a semi-selective medium in 2012 that contained hymexazol. Hymexazol is added to semi-selective medium to reduce *Pythium* recovery while increasing *Phytophthora* spp. recovery (Tsao and Guy 1977, Jeffers 1986). *Phytophthora sojae,* recognized as an aggressive pathogen of soybean, was found in 8 of 11 states surveyed, but was recovered at low frequencies. The low abundance of *P. sojae* was

somewhat surprising as it was found to be widely present in fields across the U.S. in a survey which utilized a baiting method from soil samples (Dorrance et al. 2016), and in samples from Ontario in the present study, which also used a baiting method. Therefore, it suggests that this species might have a low propagule density in the soil, and these only become active under specific conditions, such as soil saturation for extended periods of time. The microbial seed bank present in the soil is not represented in the current study, rather it is a subsample of the active community at the time of sampling. It has been widely discussed how dormant spores and other long-term survival structures are present in the soil, and germinate under specific conditions, such as soil saturation, cool or warm temperature depending on the species or when biologically important elements including plant exudates and volatiles are present (Lennon and Jones 2011).

Previous studies have demonstrated that despite the abundance of *Pythium* spp. in natural systems, the community structure of this genus and other oomycetes can be affected by biotic and abiotic factors (Arcate et al. 2006, Nelson and Karp 2013). Overall, we found evidence that spatial effects contribute mainly to community composition at the field level across different states, however, the autocorrelation of spatial effects with environmental data can also contribute to community composition. In fact, temperature and precipitation were also designated as variables that contributed to explain the variance observed across field community composition. Therefore, local conditions seem to correlate with the community composition at each field within each state, where precipitation and temperature at planting will determine the species actively germinating and infecting soybean seedlings. It is important to clarify that the variables correlated in the present study predict 16% of the variability, which means that there are more parameters involved affecting the community structure of oomycetes in soybean fields. These factors could include seed treatments, host genetics and even agricultural practices, hence, some

species could be promoted by particular conditions at the field scale. In addition, community composition at the state scale was also affected by fluctuation from year to year. For instance, 2011 had more frequent precipitation with mild temperatures, whereas 2012 had dry conditions and higher temperatures at the state level (Fig. S.3.2). These fluctuations were not evident at a continental scale between seasons, rather at the state level between seasons as evaluated with anosim and adonis. The regional climate from year to year could promote or suppress the germination and proliferation of different oomycete species, affecting the geographical niches. For example, it was noted that *Py. oopapillum* was abundant under the high precipitation and cool temperatures experienced at the start of the 2011 growing season, but was far less abundant in the warmer drier season of 2012. The effect of temperature on virulence and possible abundance of this species was reflected in a seed rot assay where *Py. oopapillum* was far more virulent at 13°C than at 20°C (Rojas et al. 2016a).

Changes in agricultural practices over the last few decades such as reduced rotations, early planting and minimum- or no-till coupled with changes in precipitation have promoted conditions that favor oomycete seedling disease (Workneh et al. 1999, Wrather and Koenning 2009, Koenning and Wrather 2010, Melillo et al. 2014). To this end, the primary goal of this research was to use an ecology approach to address the diversity of oomycetes associated with soybean seedling diseases and characterize the effect of environment and edaphic factors on their distribution and abundance. The results indicate that communities were dominated by *Pythium* clades F, B and I, which contain a large percentage of the plant pathogenic species (Lévesque and De Cock 2004). The differences in abundance of the OTUs across different states were correlated initially with a spatial effect, dictated mainly by latitude, and in lower contribution by longitude. As a result of this, environmental factors intrinsically related with geographical

location, like temperature and precipitation, contributed to oomycete community composition at the field scale. These conditions demonstrated an effect on the abundance of the top pathogenic species, suggesting an effect on the overall community composition and assembly at each field. This information provides a basis to understand the composition of oomycete communities in soybean fields, however further research is needed to characterize other factors that contribute to the community assembly, including agricultural practices. It will be necessary to conduct controlled experiments to refine the effect of these environmental conditions on the community and the species at the field scale. Current efforts are underway to use amplicon-based community analysis to characterize the communities present in the soil of these fields. The use of amplicon and metagenomics approaches will allow for additional understanding of the diversity of the microbial seed bank present and the ecology of these ecosystems (Lindahl et al. 2013, Sapkota and Nicolaisen 2015, Song et al. 2015). The information provided on community composition will help us develop models to reduce and manage the abundance of species as related to disease on soybean and possibly other crops. Future studies should address the influence of cultivar genotype, crop rotation, management inputs, soil fertility practices, and tillage practices on oomycete communities and root diseases.

APPENDIX

Table S.3.1. Significance and correlation of environmental and edaphic factors using 'envfit' function form vegan that affect oomycete community associated with soybean seedlings.

Environmental/ Edaphic Parameter	Axis.1	Axis.2	'envfit' analysis		Mantel test	
			R^2	*P*-value	Statistic	*P*-value
Longitude	-0.025	0.401	0.161	0.001*	0.083	0.003*
Precipitation	-0.279	0.275	0.154	0.001*	0.117	0.006*
Precipitation Season 2012	-0.101	-0.349	0.132	0.001*	0.014	0.327
Precipitation Season 2012	-0.303	0.278	0.169	0.001*	0.076	0.041*
Precipitation Season	-0.405	-0.024	0.165	0.001*	0.07	0.024*
Average precipitation 30 years	-0.279	0.189	0.113	0.002*	0.103	0.006*
Latitude	0.308	-0.053	0.097	0.004*	0.115	0.004*
Bulk density	0.016	0.307	0.094	0.004*	0.023	0.275
Minimum temp. season 2011	-0.29	0.109	0.096	0.005*	0.103	0.021*
Mena temp. season 2011	-0.296	0.061	0.091	0.005*	0.142	0.001*
Minimum temp. 30 year average	-0.284	0.102	0.091	0.006*	0.085	0.022*
Maximum temp. season 2012	-0.296	-0.047	0.09	0.006*	0.158	0.001*
Mean temp. 30 year average	-0.286	0.069	0.087	0.007*	0.122	0.001*
Mean temp. season 2012	-0.295	-0.057	0.09	0.007*	0.155	0.001*
Maximum temp. season 2011	-0.289	0.019	0.084	0.007*	0.173	0.001*
Minimum temp. season 2012	-0.283	-0.066	0.084	0.008*	0.144	0.002*
Maximum temp. 30 year average	-0.284	0.037	0.082	0.009*	0.153	0.001*
Cation Exchange Capacity	-0.116	-0.234	0.068	0.017*	0.08	0.033*
Minimum temp. year	-0.198	0.16	0.065	0.018*	0.068	0.073
Clay content (%)	-0.066	-0.259	0.072	0.019*	0.151	0.003*
Soil pH	0.251	-0.084	0.07	0.022*	0.044	0.106
Water content	-0.131	-0.222	0.066	0.022*	0.143	0.003*
Minimum temp. season	-0.241	0.029	0.059	0.031*	0.106	0.006*
Sand content (%)	0.153	0.167	0.051	0.051	0.059	0.087
Electrical Conductivity	0.196	-0.119	0.053	0.052	-0.011	0.553
Annual Mean Temp.	-0.188	0.093	0.044	0.066	0.112	0.006*
Organic matter (%)	0.181	-0.077	0.038	0.113	-0.017	0.642
Silt content (%)	-0.19	-0.031	0.037	0.119	0.035	0.174
Mean temp. season	-0.188	0.013	0.036	0.121	0.121	0.005*
Annual maximum temp.	-0.171	0.028	0.03	0.176	0.137	0.002*
Effective Cation-Exchange Capacity	-0.088	0.138	0.027	0.24	0.011	0.424

Table S.3.1 (cont'd)

Maximum temp. season	-0.14	0.001	0.02	0.343	0.119	0.002*
Slope average	-0.053	0.096	0.012	0.509	-0.023	0.698
Shape area	-0.103	-0.011	0.011	0.535	-0.008	0.559
Shape length	-0.079	-0.051	0.009	0.643	-0.055	0.879
Available water capacity	-0.034	-0.077	0.007	0.68	0.02	0.334
Aspect	0.003	0.055	0.003	0.822	0.032	0.128

* Significant environmental and edaphic parameters

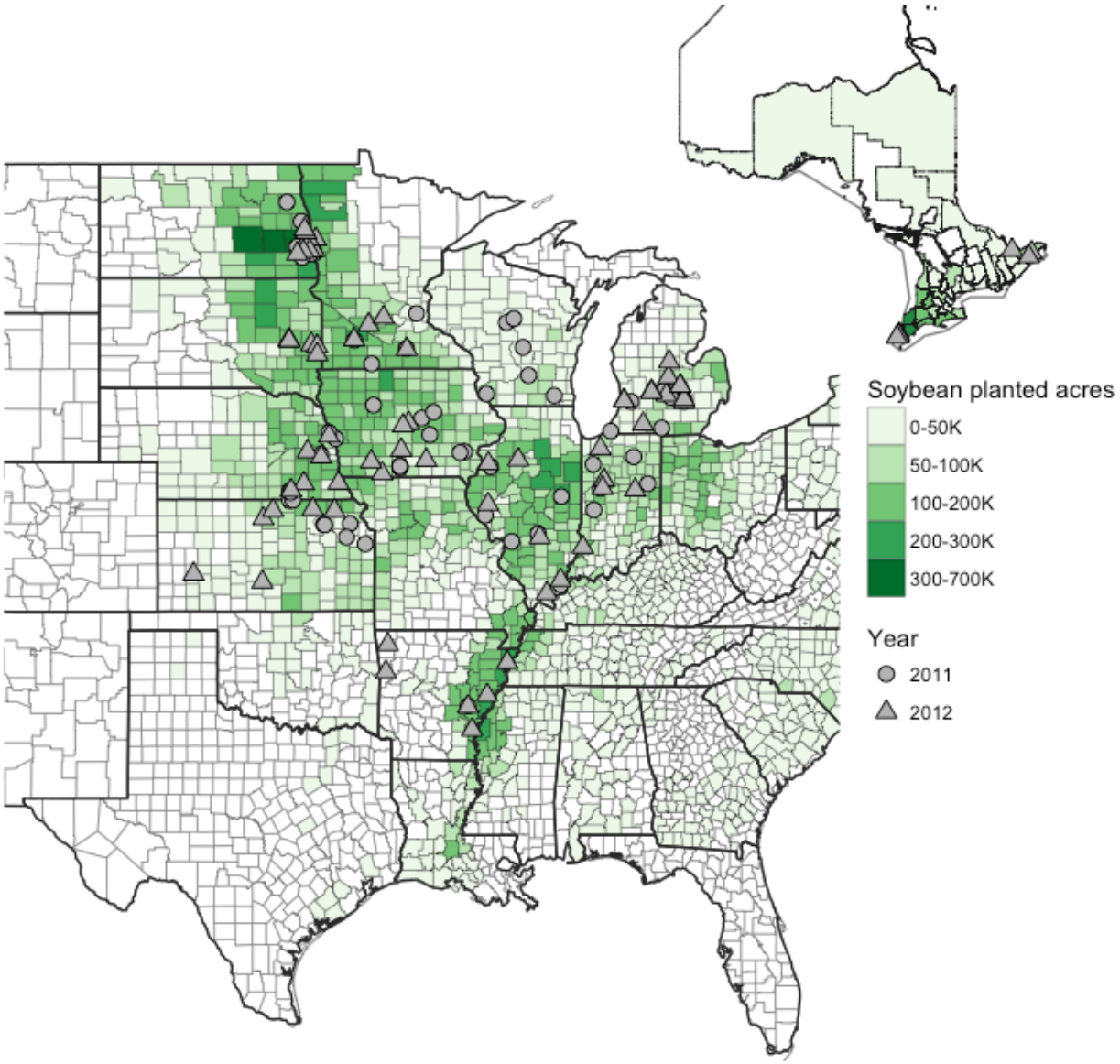


Figure S.3.1. Map of sampled soybean fields in 2011 and 2012, and intensity of planted soybean acres demonstrated by color intensity at the county/parish level.

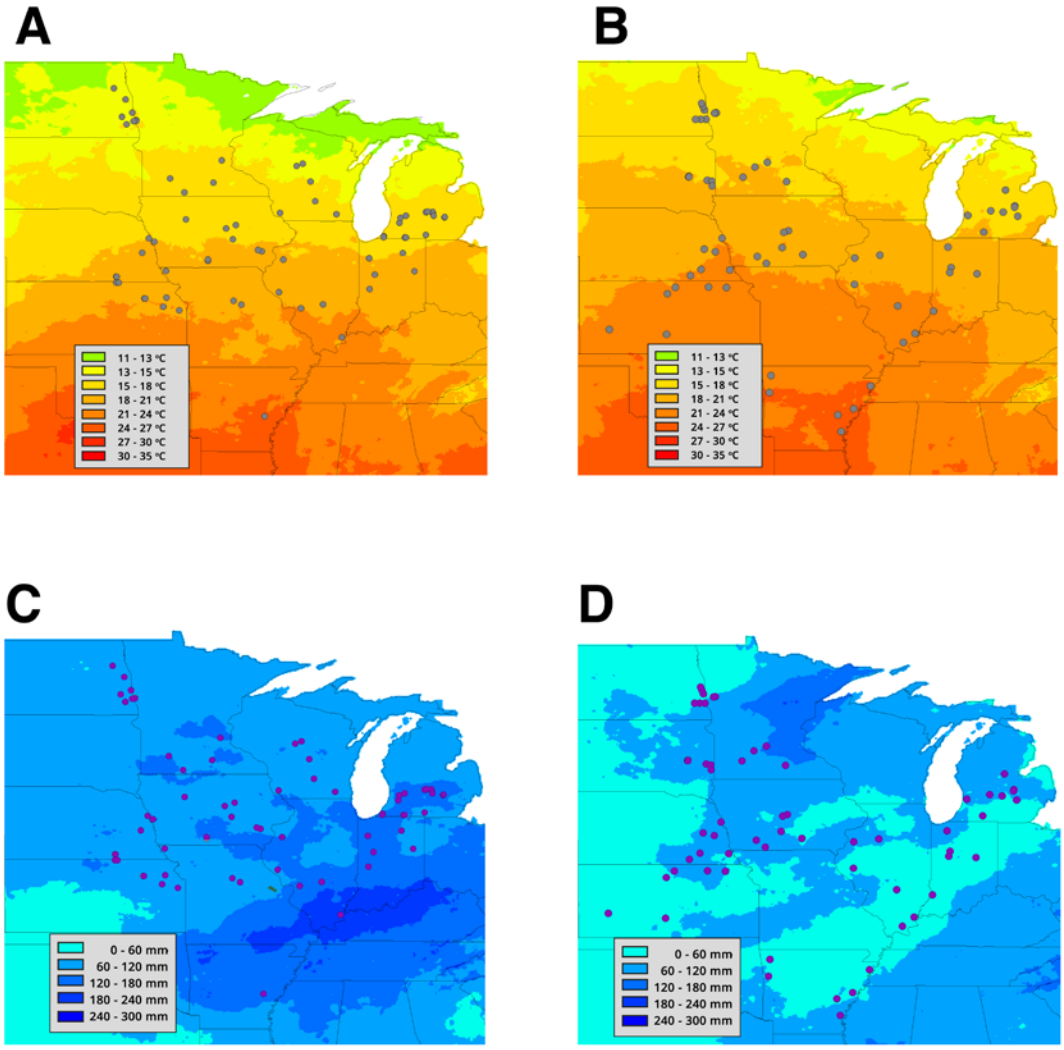


Figure S.3.2. Climate conditions across soybean fields sampled in the US from May to June (A) mean temperature 2011, (B) mean temperature 2012, (C) mean precipitation 2011, and (D) mean precipitation 2012.

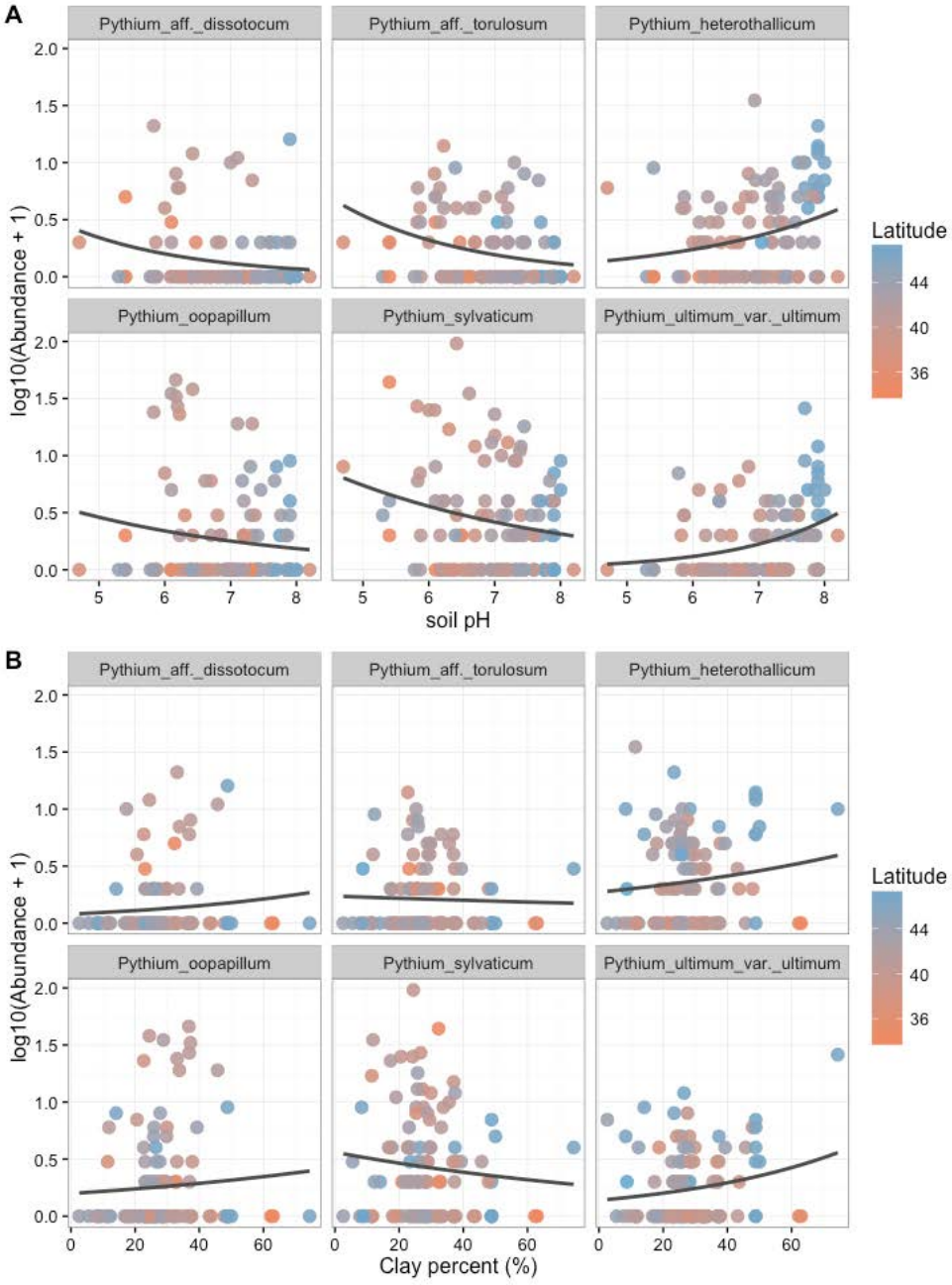


Figure S.3.3. Abundance of designated pathogenic oomycete species *Py. sylvaticum, Py. heterothallicum, Py. oopapillum, Py. ultimum* var. *ultimum*, *Py.* aff. *dissotocum* and *Py.* aff. *torulosum* by different environmental factors. (a) soil pH, (b) clay percent (%), (c) seasonal precipitation (April – June), (d) season minimum temperature (April – June) and, (e) cation exchange capacity (CEC). Trends displayed are based on negative binomial distribution.

Figure S.3.3 (cont'd)

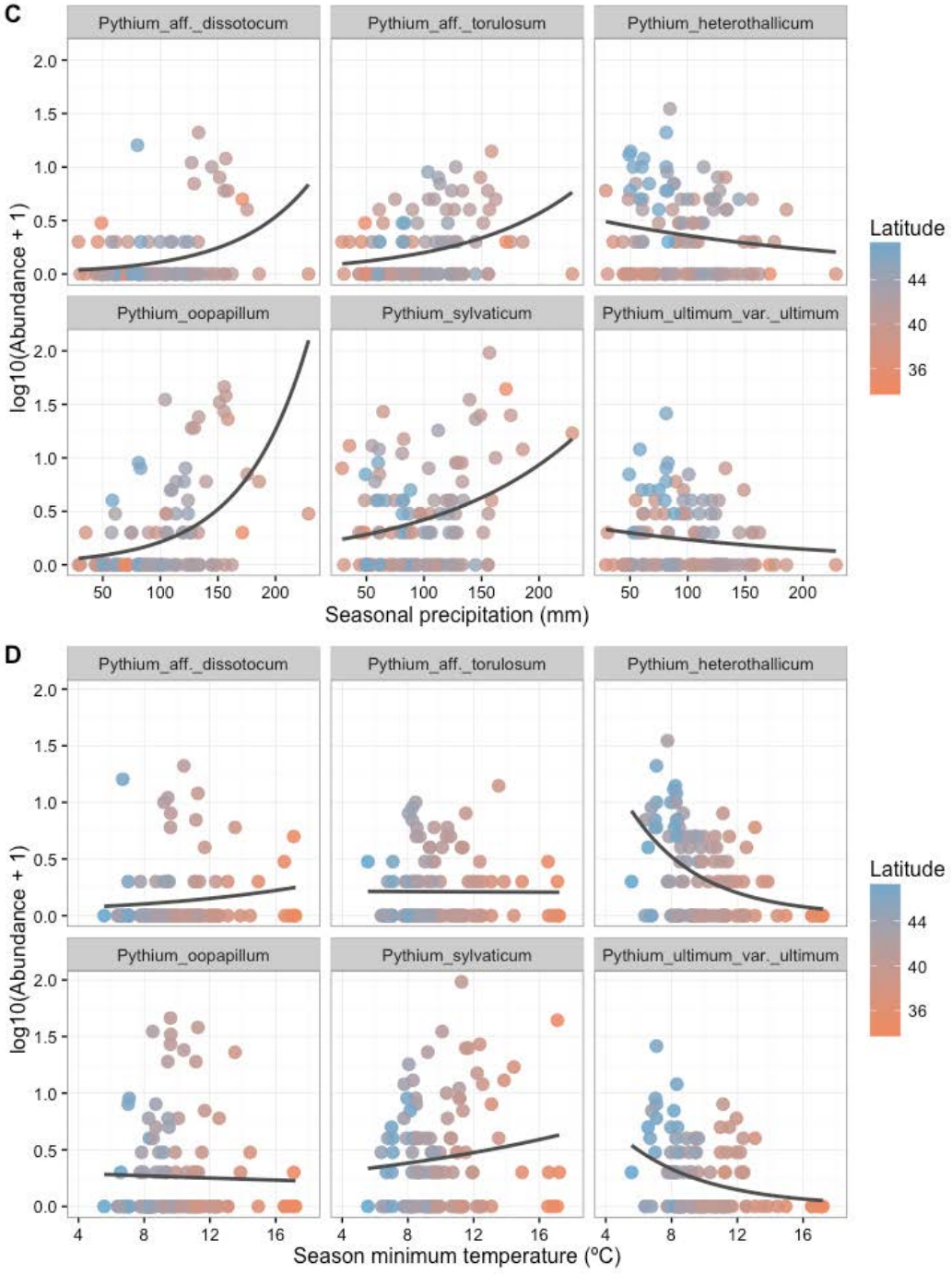
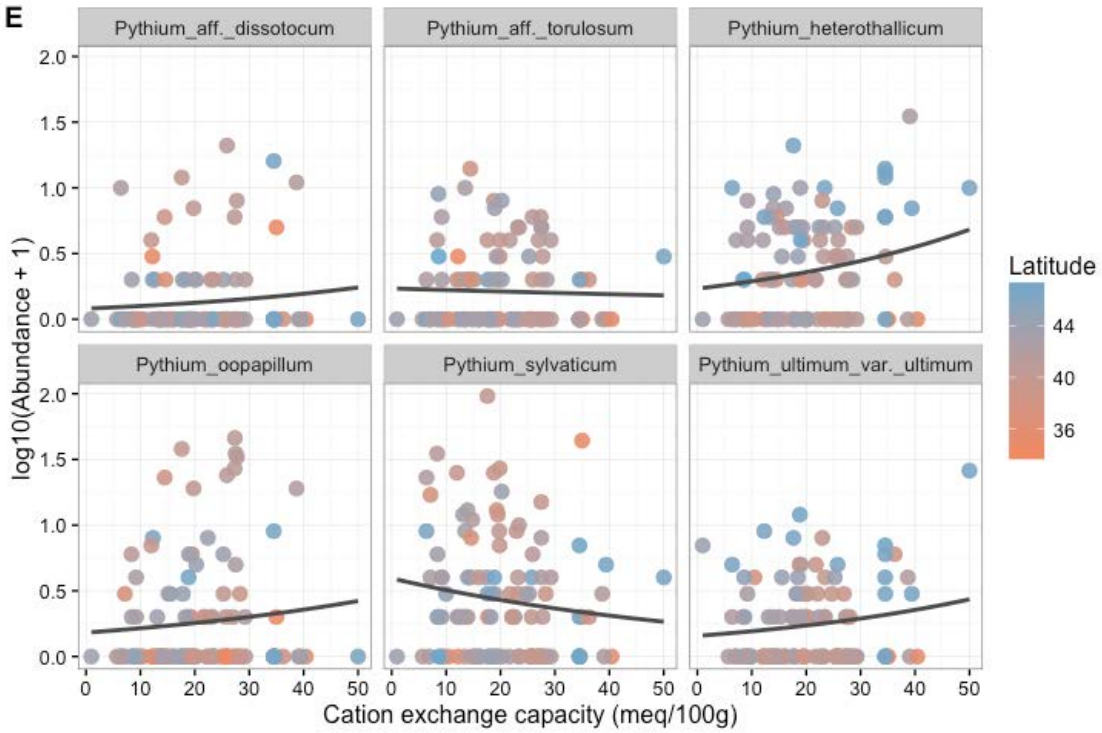


Figure S.3.3 (cont'd)



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Chapter 4

Development and application of qPCR and RPA genus and species-specific detection of *Phytophthora sojae* and *Phytophthora sansomeana* root rot pathogens of soybean

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ABSTRACT

Phytophthora root rot of soybean, caused by *Phytophthora sojae* is one of the most important diseases in the Midwest US, causing losses of up to 1.2 million tons per year. Disease may also be caused by *P. sansomeana*, however the prevalence and damage caused by this species is not well known, partly due to limitations of current diagnostic tools. Efficient, accurate and sensitive detection of pathogens is crucial for management, thus multiplex qPCR and isothermal (RPA: Recombinase Polymerase Amplification) assays were developed using a hierarchical approach. The assays consist of a genus-specific probe and two species-specific probes that target a *atp*9-*nad*9 mitochondrial region that is highly specific for the genus *Phytophthora*. The qPCR approach multiplexes the three probes and a plant internal control. The RPA assays run each probe independently, obtaining a result in as little as 20 mins. The multi-copy mitochondrial genome provides sensitivity with sufficient variability to discern among different *Phytophthora* spp. The assays were highly specific when tested against a panel of 96 *Phytophthora* spp. and range of *Pythium* spp. The consistent detection level of the assay is 100 fg for the qPCR assay and 10 pg for the RPA assay. The assays were validated on symptomatic plants collected from Michigan (USA) and Ontario (Canada) during the 2013 field season, showing correlation with isolation. In 2014, the assays were validated with samples from nine soybean producing states in the U.S. The assays are valuable diagnostic tools for detection of *Phytophthora* spp. affecting soybean.

Introduction

Phytophthora root and stem rot of soybean is one of the most prevalent and widely distributed soybean diseases, causing reduced yield and worldwide losses of ca. 2.3 million metric tons per year (Erwin and Ribeiro 1996, Wrather et al. 2010). *Phytophthora sojae*, the main causal agent of this damaging disease, was initially reported in the mid-1950s in the Midwest region of the United States (Kaufmann and Gerdemann 1958), and has since become a major concern for soybean production causing annual losses of approximately 1.2 million metric tons in the U.S. (Wrather et al. 2010). *Phytophthora sojae* is an oomycete pathogen that survives in the soil as oospores. Under optimal conditions, oospores germinate and infect seeds and roots causing seed rot and damping-off of seedlings. *Phytophthora sojae* may also cause root and stem rot that results in wilting and plant death. While the typical brown to purple water-soaked lesions on the stem appear mid-late season on infected plants, early season infection may also result in an uneven plant stand and possibly necessitate replanting (Dorrance et al. 2009, Bienapfl et al. 2011).

Disease symptoms at early plant development stages are not distinguishable from those caused by other soil-borne pathogens, such as *Pythium*, *Rhizoctonia*, and *Fusarium* spp. (Rizvi and Yang 1996), such as root rot, wilting and seedling damping-off. Without the presence of the stem lesion that occurs mid-season, the diagnosis of *Phytophthora* root rot requires laborious isolation and identification of the causal agent. However, isolation of the pathogen at early stages is often challenging and lengthy due to the slow-growth of *P. sojae* and secondary colonization by other fungi and oomycetes on the plant tissue that outgrow *P. sojae* on the medium. Confounding diagnosis further, a new oomycete species was reported as a soybean root pathogen in the early 1990s, but not formerly described until 2009 as *Phytophthora sansomeana*

(Hamm and Hansen 1981, Reeser et al. 1991, Malvick and Grunden 2004, Hansen et al. 2009). *Phytophthora sansomeana* is a homothallic pathogen that causes damping-off of soybean, but also infects a broad host range such as corn, Douglas-fir, alfalfa and some weed species (Malvick and Grunden 2004, Hansen et al. 2009, Zelaya-Molina et al. 2010). In contrast to *P. sansomeana*, *P. sojae* has a very narrow host range with soybean as the main host (Erwin and Ribeiro 1996) and is classified in clade 7b along with *P. cinnamomi, P. vignae* and *P. niederhauserii,* which is different from *P. sansomeana* in clade 8a grouping with *P. cryptogea, P. drechsleri,* and *P. medicaningis* (O'Brien et al. 2009, Martin et al. 2014). In addition, *P. sansomeana* has not been associated with cultivar specificity, unlike *P. sojae,* where pathotypes have been established based on cultivars with specific resistance genes (Hansen et al. 2012). The similar symptoms caused by other *Phytophthora* spp. and other oomycetes in soybean seedlings complicates diagnostics, reinforcing the need for an accurate and sensitive assay to diagnose these causal agents.

Detection of *Phytophthora* spp. for diagnostic purposes in the field and/or laboratory has focused on the use of enzyme-linked immunosorbant assays (ELISA), for an initial and rapid assessment of diseased plant samples. The ELISA detection assay from Agdia Inc. employs polyclonal antibody against *Phytophthora* spp. with limited sensitivity (O'Brien et al. 2009). ELISA sensitivity threshold for different *Phytophthora* spp. was reported at 0.1 ng of freeze dried mycelia (Bowman et al. 2007). ELISA is less specific than PCR, due to cross-reactivity with other oomycetes, such as *Pythium*, a common root rot seedling pathogen of soybean (O'Brien et al. 2009, Wrather et al. 2010, Agdia 2016). In addition to ELISA, conventional PCR and qPCR are two of the main approaches broadly used to diagnose and quantify plant pathogens. Wang et al. (2006) developed a *P. sojae* conventional PCR assay targeting the

internal transcribed spacer (ITS) of the rDNA. The same primer set for *P. sojae* was also adapted in a SYBR Green assay for pathogen quantification with a claimed detection level of 1 fg of genomic DNA, however the assay showed limited specificity when challenged with multiple *Phytophthora* spp. (Wang et al. 2006, Bienapfl et al. 2011). Bienapfl et al. (2011) also developed a second assay for conventional PCR and qPCR. This assay targeted the ITS region of the rDNA, with a sensitivity of 10 pg in conventional PCR and 1 pg when used in a SYBR Green qPCR assay. Catal et al. (2013) used the same assay, but reported a detection level of 10 fg. However, specificity of the assay was only challenged with a limited number of *Phytophthora* spp., including some species from Clade 7.

The availability of isothermal DNA amplification techniques has opened a new field for the development and application of diagnostics for plant pathogens (Kubota et al. 2008, Fukuta et al. 2013, Fukuta et al. 2014, Yan et al. 2014, Li et al. 2015, Miles et al. 2015, Hansen et al. 2016). The isothermal techniques present a new framework, where rapid and simple detection of pathogens can be made since the reaction is incubated at constant temperature, thereby simplifying required instrumentation. As a consequence of the minimal requirements, isothermal diagnostic methods could be conducted directly in the field. An example of this is loop-mediated isothermal amplification (LAMP), which is mediated by a set of four primers which form hairpin-like structures which facilitate amplification (Yan et al. 2014). Dai et al. (2012) developed a *P. sojae* LAMP assay that targets a transposon-like element in the promoter region of the virulence gene *Avr3a,* which is a unique element of the *P. sojae* genome. The specificity of this LAMP assay was tested against 10 *Phytophthora* spp. and *Pythium ultimum* and the sensitivity of this assay on pure genomic DNA was established at 20 pg. To the best of our knowledge, no assays, either qPCR or isothermal, have been developed to detect *P. sansomeana*.

The recent development of a hierarchical approach of *Phytophthora* genus-specific and species-specific qPCR assays based on mitochondrial genes by Bilodeau et al. (2014) provides a novel system for diagnostics. This approach utilized two loci, one for the purposes of amplifying all *Phytophthora* spp. (*trnM-trnP-trnM*), and the other one capable of genus and species-specific detection (*atp*9-*nad*9). This resulted in the development and validation of two genus specific assays and species-specific TaqMan probes for 13 *Phytophthora* spp. plus the *P. citricola* complex and the same approach can be further utilized to design unique probes for many *Phytophthora* spp. Miles et al. (2015) adapted the marker system to work with an isothermal technique known as recombinase polymerase amplification (RPA, TwistDx Ltd., Cambridge, UK), which produces results in little as 15 min. The RPA is an isothermal-based technique where annealing and amplification occur at the same temperature (any temperature between 39-42ºC), eliminating temperature-based specificity. The process starts by forming a primer-enzyme complex, which consists of a recombinase and primers (optimal size of 30-35 bp), that recognize homologous regions on the target DNA, resulting in the formation of a replication loop on the double stranded DNA. This process is aided by the binding of single-strand binding proteins, leading to the amplification by DNA polymerase, which also recognizes and binds the homologous region. Fluorometric RPA assays, like the ones developed in Miles et al. (2015), utilize highly modified probes during this amplification which are partially cleaved at the abasic site analog tetrahydrofuran (THF) by an endonuclease type VI, releasing the fluorophore and displacing the 3' side of the probe that contained a C3 spacer block. Therefore, the 5' end of the probe becomes a primer also used during the amplification process (Piepenburg et al. 2006, Yan et al. 2014). The RPA assay is similar to conventional PCR achieving exponential endpoint amplification. However, RPA assays are more tolerant of contaminants present than

conventional PCR assays, making them more robust on crude samples. Furthermore, these assays typically occur within a 20 min timeframe as opposed to 2 hours for a typical PCR assay. Miles et al. (2015), developed a genus-specific assay for *Phytophthora* spp. and two species-specific assays (*P. ramorum* and *P. kernoviae*), showing the flexibility of this system for designing assays for *Phytophthora* spp.

To improve diagnostic assays available for *P. sojae* and *P. sansomeana* we built upon the approaches described by Bilodeau et al. (2014) and Miles et al. (2015). The objectives of this research were to: (1) develop a robust, sensitive and specific multiplex qPCR assay for *P. sojae* and *P. sansomeana* at species-specific level; (2) design a species-specific RPA assay for both *P. sojae* and *P. sansomeana*; (3) evaluate the cross-platform transferability of the assays and (4) validate the field application of assays with paired plant and soil samples collected in 2013 and 2014.

Materials and Methods

***Phytophthora sojae* and *P. sansomeana* isolates.** Isolates were obtained from diseased soybean seedlings using a semi-selective medium CMA -PARPB (Jeffers 1986) as part of a 12-state survey (2011 and 2012) in order to identify oomycete species causing soybean seedling diseases (Rojas et al. 2016). Additional strains for both species were obtained from the World *Phytophthora* Genetic Resource Collection (University of California, Riverside), Embrapa Trigo (Passo Fundo, RS, Brazil) and Dr. Everett Hansen at Oregon State University to account for genetic variability across geographical locations and hosts (Table S.4.1). Isolates were grown and maintained on semi-selective CMA-PARPB medium. Mycelia were grown in clarified V8-juice broth (100 mL of clarified V8-juice, 1 g of $CaCO_3$, and 900 mL of water) for 5 days, then

harvested and lyophilized for DNA extraction. Freeze-dried mycelia were ground and 40 mg of ground mycelia were used for DNA extraction with DNeasy Plant Mini Kit (QIAGEN, Valencia, California). DNA concentration and quality were evaluated with Nanodrop ND-1000 (Thermo-Fisher Scientific, Waltham, MA, USA).

Real-time PCR probe design for *P. sojae* and *P. sansomeana*. The mitochondrial locus *atp9-nad9* was used to design the species-specific probes due to the interspecific polymorphisms across a range of taxa (Bilodeau et al. 2014). The locus *atp9-nad9* was amplified and sequenced using the primers Nad9-F and Nad9-R (Table 4.1) described by Bilodeau et al. (2014) for all the *P. sojae* and *P. sansomeana* isolates. The target locus was amplified in a 25 µL reaction volume containing 1X DreamTaq Buffer (Thermo Fisher Scientific, Waltham, MA), 200 µM dNTPs (Life Technologies), 0.2 µM of Nad9-F and Nad9-R primers (Table 4.1), 0.1 mg/mL BSA and 1 unit of DreamTaq polymerase (Thermo Fisher Scientific). Thermal cycling consisted of denaturation at 95ºC for 3 min; 35 cycles at 95ºC for 1 min, 61ºC for 1 min and 72ºC for 1 min; and final extension at 72ºC for 10 min. Products were visualized on an 1% agarose gel stained with ethidium bromide. Amplification products were treated with a 3U/reaction of Exonuclease I (ExoI, Thermo Fisher Scientific) and 0.5U/reaction of Shrimp alkaline phosphatase (SAP, Thermo Fisher Scientific) and incubated at 37ºC for 40 min. Treated PCR products were submitted to Macrogen USA for sequencing (MacrogenUSA, Rockville, MD). Each template was sequenced in both directions, and CodonCode Aligner (CodonCode Corporation, Centerville, MA) was used to assemble, edit, and generate the consensus sequences.

Table 4.1. Primers and probes used in this study for sequencing of *atp9-nad9* locus and *P. sojae* and *P. sansomeana* multiplex genus and species specific qPCR.

Primer/Probes	Sequence (5' - 3')	Length (bp)	GC%	Target
Nad9-F[a]	TACAACAAGAATTAATGAGAAC	22	27.3	*atp*9-*nad*9
Nad9-R[a]	GTTAAAATTTGTACTACTAACAT	23	21.7	*atp*9-*nad*9
Primers				
PhyG_ATP9_2FTail[a]	AATAAATCATAACCTTCTTTACAACAAGAATTAATG	36	22.2	*atp*9
PhyG-R6_Tail[a]	AATAAATCATAAATACATAATTCATTTTTATA	32	9.4	*nad*9
Probes				
Phytophthora genus-specific TaqMan probe[b]	[FAM] AAAGCCATC [ZEN] ATTAAACARAATAAAGC [IABkFQ]	26	28.8	*atp*9-*nad*9
P. sojae species-specific TaqMan probe	[HEX] TTGATATAT [ZEN] GAATACAAAGATAGATTTAAGTAAAT [IABkFQ]	35	17.1	*atp*9-*nad*9
P. sansomeana species-specific TaqMan probe	[Quasar670] TATTAGTACTAAYTACTAATATGCATTATTTTTAG [BHQ-2]	35	18.6	*atp*9-*nad*9
Plant Internal Control				
FMPI2b[a]	GCGTGGACCTGGAATGACTA	20	55.0	*cox*I
FMPI3b[a]	AGGTTGTATTAAAGTTTCGATCG	23	34.8	*cox*I
Plant-IC probe[a]	[CalFluorRed610] CTTTTATTATCACTTCCGGTACTGGCAGG [BHQ-2]	29	44.8	*cox*I
Internal Control (Soil)				
PPF[c]	[CalFluorRed610] AAAGTAAGCTTATCGATACCGTCGACCT [BHQ-2]	28	42.9	Internal Control[d]

[a] Primers and probes reported by Bilodeau et al. (2014)
[b] Modified from Bilodeau et al. (2014)
[c] Adapted from Bilodeau et al. (2012)

Sequences for *P. sojae* and *P. sansomeana* strains were aligned with MUSCLE in Geneious 4.7.6 (Biomatters Ltd., Auckland, New Zealand) and evaluated for polymorphism at the intra-species level for design of species-specific probes. In addition, the probe design was refined by aligning *P. sojae* and *P. sansomeana* sequences against sequences of 91 valid *Phytophthora* spp. and 30 putative *Phytophthora* taxa (Martin et al. 2014). Species-specific probes were developed based on highly polymorphic regions using the following parameters: (1) melting temperature 5ºC higher than the amplification primers; (2) 15 to 36 bp in length with no more than two Gs or Cs in the last five nucleotides from the 3' end; and (3) mismatched nucleotides positioned in the center of the probe to avoid secondary structures. Two hydrolysis probes were designed: the *P. sojae* species-specific TaqMan probe was labeled with HEX at the 5' end, an internal ZEN quencher, and 3' Iowa Black FQ quencher (IDT, Coralville, Iowa); and the *P. sansomeana* species-specific TaqMan probe was labeled with Quasar670 at the 5' end, and 3' Black Hole Quencher-2 (BHQ-2) (Biosearch Technologies, Inc., Novato, CA).

qPCR conditions for *P. sojae* and *P. sansomeana* assay. The qPCR assays were performed on a CFX96 Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA). The qPCR primers employed in this study were adapted from Bilodeau et al. (2014) and the *Phytophthora* genus-specific TaqMan probe labeled with FAM (fluorescein) at the 5' end, an internal ZEN quencher, and 3' end Iowa Black FQ quencher (Table 4.1). In addition, a plant internal control and internal control for soil samples were established. For the plant internal control, primers and probe developed by Bilodeau et al. (2014) were also included in the reaction, the probe was labeled at the 5' end with CalFluor Red 610 and Black Hole Quencher-2 (BHQ-2) at the 3' end. Internal control for soil samples was adapted from Bilodeau et al. (2012), using the *Pythium* plasmid pUC96-4 and primers PPF1F and PPF1R, each of them with binding

sites for the *Phytophthora* genus primers PhyG_ATP9 and PhyG-R6 tailed on, respectively. Internal control was amplified using *Phytophthora* genus primers PhyG_ATP9_2FTail and PhyG-R6_Tail, the product was cleaned with the Exonuclease and phosphatase as described previously, and diluted to 10^{-9} (0.2-0.3 fg/µL, ca. 1500 – 2000 copies). Reactions were performed in a final volume of 20 µL using the Real Master Mix without Rox (5 Prime; Fisher Scientific Company, LLC, Waltham, MA). Reagent volumes per single reaction are shown in Table S.4.2, for plant and soil samples. The thermal cycling conditions were 95ºC for 2 min, 50 cycles at 95ºC for 15 s, and 57ºC for 1 min 30 s.

Evaluation of qPCR sensitivity and specificity. The assay specificity was tested against a panel of 131 *Phytophthora* spp. strains representing all 10 clades, including species closely related species to *P. sojae* and *P. sansomeana*, that comprises 96 valid species and 14 provisional species. The panel also included 3 different subspecies of *P. alni* (subsp. *alni, multiformis* and *uniformis*), 3 phylogenetic groups of *P. cryptogea* (GI, GII and sp. *kelmania* GIII), and 6 phylogenetically distinct species (sp. aff. *brassicae*-1, sp. aff. *brassicae*-2, sp. aff. *colocasiae*-1, sp. aff. *erythroseptica*, sp. aff. *siskiyouensis*, *cinnamomi* var. *robiniae*, and *citricola* clade E). All *Phytophthora* isolates were obtained and are available from the World *Phytophthora* Genetic Resource Collection at the University of California, Riverside (Table S.4.5). In addition, 21 *Pythium* spp., and 1 *Phytopythium* sp. were included in the panel to validate the specificity. DNA was diluted to 1ng/µL and used in the assay.

A ten-fold serial dilution of *P. sojae* (strains IASO_8-13.10 and IASO_3-41.17) and *P. sansomeana* (strains V-KSSO2_3-6 and MICO_3-24) DNA ranging from 10 ng to 1 fg were used as standards to determine sensitivity level, establish amplification efficiency and resolve the limit of detection (LOD). The *P. sojae* and *P. sansomeana* genomic DNA serial dilution was prepared

using 1 ng/µL salmon sperm DNA (Invitrogen, Carlsbad, CA) as a carrier DNA to prevent DNA lost through binding to plasticware or degradation. The DNA for the standard curve was quantified using Quant-iT dsDNA high-sensitivity assay kit (Invitrogen, Carlsbad, CA). For the real-time qPCR assay, PCR efficiency was calculated with the formula: $E = 10^{(-1/slope)} - 1$. The limit of detection was determined following MIQE guidelines (Bustin et al. 2009).

Cross-platform validation of qPCR assays. In order to test the cross-platform transferability of the assay, *P. sojae* and *P. sansomeana* genomic DNA serial dilution standards were assayed independently on three platforms: ABI StepOne Plus (Applied Biosystem, Foster City, CA), Roche LightCycler 96 (Roche Diagnostics, Mannheim, Germany) and Bio-Rad CFX96 (Bio-Rad, Hercules, CA). All tests were conducted under the same reaction and cycling conditions for all platforms to validate the qPCR assay.

Recombinase polymerase amplification (RPA) development for *P. sojae* and *P. sansomeana*. Primers (TrnM-F and TrnM-R) and probe (TrnM-P) for the *Phytophthora* genus-specific assay were developed by Miles et al. (2015) and the sequences are listed in Table 4.2. In order to develop a *P. sojae* and *P. sansomeana* species-specific RPA assay, the alignment of the *atp9-nad9* region was built including multiple strains of both species along with several *Phytophthora* spp. Primers were designed manually based on the *Phytophthora* spp. alignment following the recommendations provided in the TwistAmp exo kit (TwistDx, Babraham, Cambridge, UK). The assay uses a general *Phytophthora* forward primer (Atp9-F) located in the *atp9* region and species-specific reverse primer (Psojae-*nad*9-R or Psan-*nad*9-R) placed in the *atp9-nad9* spacer region (Table 4.2). Detection was based on a *Phytophthora* genus-specific probe, which has the following characteristics: 46-52 bp long, where 30 bases are on the 5', follow by fluorophore (FAM) and quencher (Black Hole Quencher-1, BHQ-1) separated 2-4

bases from each other, and a tetrahydrofuran abasic site (THF) replacing a base in between fluorophore and quencher; and finally a C3 spacer block that prevents amplification. When used on plant samples, a plant internal control was also included in the assay. Primers (CoxI-IPC-F and CoxI-IPC-R) and probe (CoxI-IPC-P) were developed by Miles et al. (2015) and their sequences are listed in Table 4.2. Primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA) and probes were synthesized from Biosearch Technologies, Inc. (Novato, CA).

RPA amplification conditions for *P. sojae* and *P. sansomeana*. Isothermal amplification was conducted using TwistAmp exo kit (TwistDx) and two platforms were used for the RPA assays incubation and detection: Twista® (TwistDx) and Smart-DART™ device (Diagenetix, Inc., Honolulu, HI). The *Phytophthora* genus-specific RPA reaction was conducted as reported by Miles et al. (2015), the reagents and sample volumes are listed in Table S.4.3. The reaction mix was then transferred into a TwistAmp exo kit reaction tube containing the lyophilized reagents, and mixed well to dissolve the lyophilized enzymes. To initiate the reaction, 2.5 µL of 280 mM magnesium acetate was placed on the cap, closed carefully and spun briefly to initiate the reaction. For the *Phytophthora* species-specific RPA assay, a 50 µL reaction was setup with the volumes listed in Table S.4.3 and following the procedure described above. Initiated reactions were incubated at 39ºC for 4 min, tubes were removed, mixed by inversion, spun briefly and placed into the detection unit for 25 min at 39ºC. Fluorescence was collected every 20 s following manufacturer recommendations.

Table 4.2. Primers and probes used for *Phytophthora sojae* and *Phytophthora sansomeana* recombinase polymerase amplification (RPA) genus and species-specific assay.

Primer/Probes	Sequence (5' - 3')	Length (bp)	GC%	Target
Phytophthora genus-specific				
TrnM-F[a]	ATGTAGTTTAATGGTAGAGCGTGGGAATC	29	41.4	tRNA-M
TrnM-R[a]	GAACCTACATCTTCAGATTATGAGCCTGATAAG	33	39.4	tRNA-M
TrnM-P (Probe) [a]	TAGAGCGTGGGAATCATAATCCTAATGTTG [FAM-dT] A [THF] G [BHQ1-dT] TCAAATCCTACCATCAT [3'-C3SPACER]	51	37.3	tRNA-M
Phytophthora species-specific				
Atp9-F[a]	CCTTCTTTACAACAAGAATTAATGAGAACCGCTAT	35	34.3	*atp*9
Psojae-*nad*9-R	TTAAATCTATCTTTGTATTCATATATCAA	29	17.2	*atp*9-*nad*9
Psan-*nad*9-R	TTAGTAGTTAGTACTAATATAACAAAAATATAATA	35	14.3	*atp*9-*nad*9
Atp9-P (probe) [a]	TTGCTTTATTYTGTTTAATGATGGCWTTY (T-FAM) T [THF] A (T-BHQ1) YTTATTTGCTTTTT [3'-C3SPACER]	47	22.3	*atp*9
Plant Internal Control				
Cox1-IPC-F[a]	CATGCGTGGACCTGGAATGACTATGCATAGA	31	48.4	*cox*I
Cox1-IPC-R[a]	GGTTGTATTAAAGTTTCGATCGGTTAATAACA	32	31.3	*cox*I
Cox1-IPC-P (probe) [a]	GGTCCGTTCTAGTGACAGCATTCCYACTTTTATTA [TAM-dT] C [THF] C [BHQ2-dT] YCCGGTACTGGC [3'-C3SPACER]	51	49	*cox*I

[a] Reported by Miles et al. (2015)

RPA assays sensitivity and specificity. The specificity was tested against the same panel of *Phytophthora* spp., *Pythium* spp. and *Phytopythium* spp. used for the qPCR assay. DNA was diluted to 1ng/µL and 1 µL from five species was pooled and tested for specificity. If cross-reactivity was observed, species were tested individually. Sensitivity for the RPA assay was determined with standard serial dilutions ranging from 10 ng to 1 fg prepared as described above. Standard curve plots were constructed based on the log transformed DNA concentration and the onset of amplification threshold (OT) for each concentration. The OT was established using a slope validation, where four time points had an overall slope higher than 30 mV/min.

Collection of field samples and assays validation. In order to validate qPCR and RPA assays, two-year field samples were collected in 2013 and 2014 in soybean fields with damping-off and root rot symptomatic plants. In 2013, 16 fields across Michigan and Ontario were sampled, 42 plant samples and 16 composite soil samples were collected (Table 4.4). Symptomatic plant samples were plated on semi-selective medium CMA-PARPB to isolate *P. sojae* or *P. sansomeana*, and plant samples were also tested with *Phytophthora* ELISA Kit (SRA 92601; Agdia, Inc., Elkhart, IN). In 2014, extensive sampling was conducted across 9 soybean producing states in the Midwest with collaborators of the OSCAP NIFA Project (Table 4.5). A total of 23 fields, consisting of one to three fields per state were sampled; paired plant and soil samples were collected at each field location, resulting in 74 plant samples and 18 composite soil samples. Samples were transported in coolers and shipped overnight to Michigan State University. No isolations were performed on the 2014 plant samples.

Table 4.3. Cross-platform validation of *Phytophthora* genus and *Phytophthora sojae* and *Phytophthora sansomeana* species-specific probes.

Probe	Platforms	Efficiency (%)	Slope	Intercept	R^2
Phytophthora genus (FAM labeled probe)	Step One Plus (ABI)	100.78	-3.30	38.16	0.99
	LightCycler 96 (Roche)	100.00	-3.31	35.70	0.92
	CFX96 (Bio-Rad)	95.60	-3.43	34.80	0.99
P. sojae (HEX labeled probe)	Step One Plus (ABI)	95.325	-3.44	41.175	0.99
	LightCycler 96 (Roche)	95.50	-3.56	39.35	1
	CFX96 (Bio-Rad)	95.99	-3.42	38.746	0.99
P. sansomeana (Quasar670 labeled probe)	Step One Plus (ABI)	NC*	NC	NC	NC
	LightCycler 96 (Roche)	100.50	-3.31	37.59	1
	CFX96 (Bio-Rad)	100.54	-3.31	37.59	0.99

* NC = Not compatible

Table 4.4. Isolation, ELISA, multiplex qPCR and recombinase polymerase amplification (RPA) evaluation for *Phytophthora* genus and *Phytophthora sojae* and *P. sansomeana* species-specific assays of plant samples collected in Michigan and Ontario in 2013.

Fields	Number samples	Isolation[a]	*Phytophthora* ELISA[b]	Positives qPCR genus[d]	Plant internal control	*Phytophthora* genus		*P. sojae*		*P. sansomeana*	
					Mean C_t[e]	Mean Ct	RPA[f]	Mean C_t	RPA	Mean C_t	RPA
MIPS2	3	+	+	(3/3)	13.79	26.34	(2/3)	28.31	(2/3)	ND[g]	(0/3)
MIPS3	3	+	+	(3/3)	13.40	24.12	(3/3)	26.41	(2/3)	ND	(0/3)
MIPS4	3	-	+	(3/3)	12.62	26.92	(3/3)	29.06	(1/3)	ND	(0/3)
MIPS5	3	+	+	(3/3)	14.77	20.62	(3/3)	23.39	(2/3)	ND	(0/3)
MIPS6	3	+	+	(3/3)	12.80	26.81	(3/3)	28.86	(2/3)	ND	(0/3)
MIPS7	3	+	NC[c]	(3/3)	14.28	17.23	(2/3)	20.14	(1/3)	ND	(0/3)
MIPS8	3	+	NC	(2/3)	14.47	27.43	(2/3)	30.49	(2/3)	ND	(0/3)
MIPS9	3	+	+	(2/3)	14.10	28.78	(2/3)	31.23	(3/3)	ND	(0/3)
MIPS11	3	-	+	(3/3)	13.04	24.55	(3/3)	27.29	(2/3)	ND	(0/3)
MIPS12	1	-	+	(1/1)	14.12	24.13	(1/1)	26.93	(1/1)	ND	(0/1)
ONPS1	3	+	+	(2/3)	15.16	23.87	(3/3)	27.55	(3/3)	ND	(0/3)
ONPS2	2	+	+	(1/2)	14.59	23.92	(2/2)	27.66	(2/2)	ND	(0/2)
ONPS3	3	+	+	(3/3)	16.94	17.89	(3/3)	21.98	(2/3)	ND	(0/3)
ONPS4	2	+	+	(2/2)	17.33	28.35	(2/2)	29.43	(2/2)	ND	(0/2)
ONPS5	2	-	+	(0/2)	15.20	ND	(0/2)	ND	(0/2)	ND	(0/2)
ONPS6	2	-	+	(2/2)	15.87	26.33	(2/2)	28.82	(2/2)	ND	(0/2)

[a] *P. sojae* isolation on semi-selective medium (CMA-PARPB)
[b] Enzyme Linked Immunosorbent Assay for *Phytophthora* was conducted in plant tissues collected.
[c] ELISA result not conclusive due to weak reaction.
[d] Number of positive samples detected with qPCR *Phytophthora* genus assay as defined as Bustin et al. (2009).
[e] C_t = Cycle threshold values for qPCR
[f] Number of positive samples out total samples evaluated with RPA assay
[g] ND = Non-detected

Table 4.5. Multiplex qPCR evaluation for *Phytophthora* genus and *Phytophthora sojae* and *P. sansomeana* species-specific assays of plant samples collected across nine soybean producing states in the U.S. in 2014.

State	Fields	Positives qPCR genus[a]	Plant Internal Control	*Phytophthora* genus		*P. sojae*		*P. sansomeana*	
			Mean C_t[b]	Mean C_t	RPA	Mean C_t	RPA	Mean C_t	RPA
Arkansas	ARPS2_1	(3/3)	16.18	28.17	(2/3)	28.99	(2/3)	31.26	(0/3)
	ARPS2_2	(1/3)	18.84	29.37	(1/3)	32.48	(0/3)	33.87	(0/3)
	ARPS2_3	(2/3)	14.27	28.51	(1/3)	ND	(0/3)	33.34	(0/3)
Iowa	IAPS2_1	(0/4)	19.33	ND[c]	(1/4)	ND	(0/4)	ND	(0/4)
	IAPS2_2	(0/3)	19.69	ND	(1/3)	ND	(0/3)	ND	(0/3)
Illinois	ILPS2_1	(3/3)	17.61	26.13	(3/3)	27.82	(1/3)	ND	(0/3)
	ILPS2_2	(2/3)	18.36	28.09	(1/3)	31.54	(1/3)	ND	(0/3)
	ILPS2_3	(1/3)	22.81	23.94	(1/3)	ND	(0/3)	ND	(0/3)
	ILPS2_4	(1/3)	16.26	21.88	(1/3)	ND	(0/3)	24.57	(0/3)
Indiana	INPS2_1	(4/4)	17.65	27.70	(1/4)	29.16	(1/4)	ND	(0/4)
	INPS2_2	(2/3)	16.95	28.15	(1/3)	30.82	(1/3)	29.76	(0/3)
Kansas	KSPS2_1	(4/4)	18.90	22.98	(3/4)	25.00	(2/4)	ND	(0/4)
	KSPS2_2	(1/3)	19.18	27.60	(1/3)	30.52	(0/3)	ND	(0/3)
Michigan	MIPS2_1	(2/3)	20.68	28.17	(2/3)	29.60	(1/3)	ND	(0/3)
	MIPS2_2	(3/4)	18.84	28.30	(2/4)	30.06	(0/4)	ND	(0/4)
	MIPS2_3	(3/3)	16.63	29.21	(0/3)	30.25	(0/3)	ND	(0/3)
N Dakota	NDPS2_1	(3/3)	15.71	27.44	(1/3)	28.88	(1/3)	ND	(0/3)
	NDPS2_2	(2/3)	17.72	25.80	(1/3)	25.30	(1/3)	ND	(0/3)
	NDPS2_3	(1/3)	15.69	26.53	(1/3)	25.82	(1/3)	ND	(0/3)
Nebraska	NEPS2_1	(2/3)	19.70	25.57	(1/3)	27.46	(2/3)	ND	(0/3)
	NEPS2_2	(3/3)	20.24	22.82	(3/3)	24.93	(3/3)	ND	(0/3)
S Dakota	SDPS2_1	(3/3)	17.40	24.96	(3/3)	26.78	(3/3)	ND	(0/3)
	SDPS2_2	(3/3)	17.09	22.76	(2/3)	24.70	(2/3)	ND	(0/3)

[a] Number of positive samples detected with qPCR *Phytophthora* genus assay as defined as Bustin et al. (2009).
[b] C_t = Cycle threshold values for qPCR
[c] ND = Non-detected
[d] Number of positive samples out total samples evaluated with RPA assay

DNA extraction from soybean roots. Collected soybean roots with or without visible symptoms of root rot were washed with tap water, and patted dry with paper towel. Plants from each location were divided into three biological replicates consisting of five plants. Roots of each of the three biological subsamples were cut longitudinally; one half was frozen at -20ºC for later use with the RPA assay. The second half was air-dried, and then ground using a Wiley mill (1 mm screen) and then used for DNA extraction. DNA was isolated following the standard USDA-APHIS procedure for *P. ramorum* (APHIS 2004) employing Qiagen DNeasy kit (QIAGEN, Valencia, California).

DNA extraction from soil. DNA was extracted from three soil subsamples per field location using a FastDNA SPIN kit for soil (MP Bio, Solon, OH). Briefly, 400 mg of soil were placed in a lysing matrix E tube, followed by addition of 978 µL of phosphate buffer and 122 µL of MT buffer. Tubes were homogenized in a FastPrep FP120 instrument (MP Bio) at speed 6 for 40 s. DNA extraction was performed according to manufacturer's recommendations. Three independent DNA extraction were done. The recovered DNA was tested with multiplex qPCR assay undiluted and ten-fold diluted. To determine if PCR inhibitors or soil contamination was a problem, 25 µL DNA were purified using Mag-Bind® E-Z Pure kit (Omega Bio-tek Inc., Norcross, GA). The fraction purified by magnetic beads was re-evaluated using the multiplex qPCR assay.

Crude extracts from soybean roots and RPA assay. Root samples preserved at -20ºC for the RPA assays were further subsampled into 0.5 g of root tissue. Root tissue was placed into plastic mesh sample bags (ACC 00930; Agdia) with 5 mL GEB2 extraction buffer dissolved according to the manufacturers recommendations (ACC 00130; Agdia) and macerated with a tissue homogenizer tool (ACC 00900; Agdia). Crude extracts were collected in centrifuge tubes

and frozen at -20ºC for later use. Reactions were conducted as mentioned above and described in Table S.4.3.

Confirmation of Phytophthora spp. identity. In order to confirm the identity of *Phytophthora* spp. detected by the RPA genus specific assay, but negative for the species-specific assay, amplifications were used to conduct a nested PCR as described by Miles et. al (2015). Briefly, 1 µL of 10-fold dilution of product was used as template, and amplified using primers Nad9_Fseq1 and Nad9Rseq1. Products were evaluated in agarose gel and processed for sequencing as described above.

Results

qPCR probe design for *P. sojae* and *P. sansomeana*. *atp*9-*nad*9 sequences of *P. sojae* isolates from different geographical origins were aligned in order to evaluate intraspecific variation of this species across different locations around the world (Table S.4.1). No differences for this locus were observed among 53 isolates sequenced, which indicates that the assay could be use without limitations to identify *P. sojae.* A similar approach was carried out with 22 isolates of *P. sansomeana*, even though this species has only been reported in the U.S. and in China. Since we only had access to U.S. isolates and *P. sansomeana* has been reported as infecting different hosts within the U.S., isolates from different host plants were sequenced and aligned. In this case, we observed SNPs that separated most *P. sansomeana* isolates from Douglas fir and other hosts from those obtained from soybean and corn (Fig. S.4.1). These SNPs were taken into account when designing the probe to allow for detection of *P. sansomeana* infecting any host.

For *P. sojae* only one TaqMan probe was evaluated since there was no variation in the isolates observed across the locus, whereas two probes that annealed at different locations of the *atp*9-*nad*9 spacer were designed and tested for *P. sansomeana* (Table 4.1 and Table S.4.4). The probe selected for *P. sansomeana* was able to amplify isolates from different hosts, ensuring amplification of all known genotypes. In addition, the *Phytophthora* genus-specific probe labeled with FAM and the *P. sojae* species-specific probe labeled with HEX were modified to include an internal quencher, in order to reduce background signal, improving sensibility and amplification signal.

Sensitivity and specificity of qPCR for species-specific. In order to determine the specificity of the species-specific assays, a panel of *Phytophthora* spp. representing 96 valid taxa and 14 provisional species across the different clades of this genus, 21 *Pythium* spp. and *Phytopythium vexans* were included to test the assay specificity (Table S.4.5). No amplification was observed from any of the non-target *Phytophthora* spp., including those in Clade 7 and Clade 8 where *P. sojae* and *P. sansomeana* are contained, respectively. There was consistent amplification of the target species for *P. sojae* and *P. sansomeana,* respectively. No cross amplification was detected between the species-specific assays with the genera *Pythium* and *Phytopythium*.

A serial dilution of genomic DNA of *P. sojae* and *P. sansomeana* was used to determine the sensitivity of the assay. A linear correlation of all of the probes tested with the concentration of DNA for the respective pathogen was observed (Fig 4.1 and Fig. S.4.2), resulting in corresponding amplification efficiencies of 101% for *P. sansomeana*, 95% for *P. sojae* and 96% for *Phytophthora* genus assay. The limit of detection (LOD) for quantification purposes, which is defined by consistent amplification of replicates at the lowest concentration of the standards

used, was established for *Phytophthora* genus (LOD C_t = 28), *P. sojae* (LOD C_t = 32) and *P. sansomeana* (LOD C_t = 31). All of these LOD thresholds were established at 100 fg of the respective species pure genomic DNA and only within range of the establish C_t accurate quantification can be made, based on Bustin et al. (2009) was determined by >95% (100% observed in this study) amplification of the lowest amount of target genomic DNA (Fig. 4.2). However, detection can occur above these C_t indicating the presence of the pathogen at low concentrations (Fig. 4.2). In addition, the presence of plant, specifically soybean did not affect assay performance (Fig. S.4.3). In addition, there was no difference between uniplex or multiplex conditions, therefore all the reactions including standards were run under multiplex conditions. For instance, the *Phytophthora* genus assay had efficiency of 96.5% in uniplex versus 95.6% in multiplex assay, maintaining the assay conditions within MIQE guidelines (Fig. 4.2). No evidence of cross-reactivity was detected.

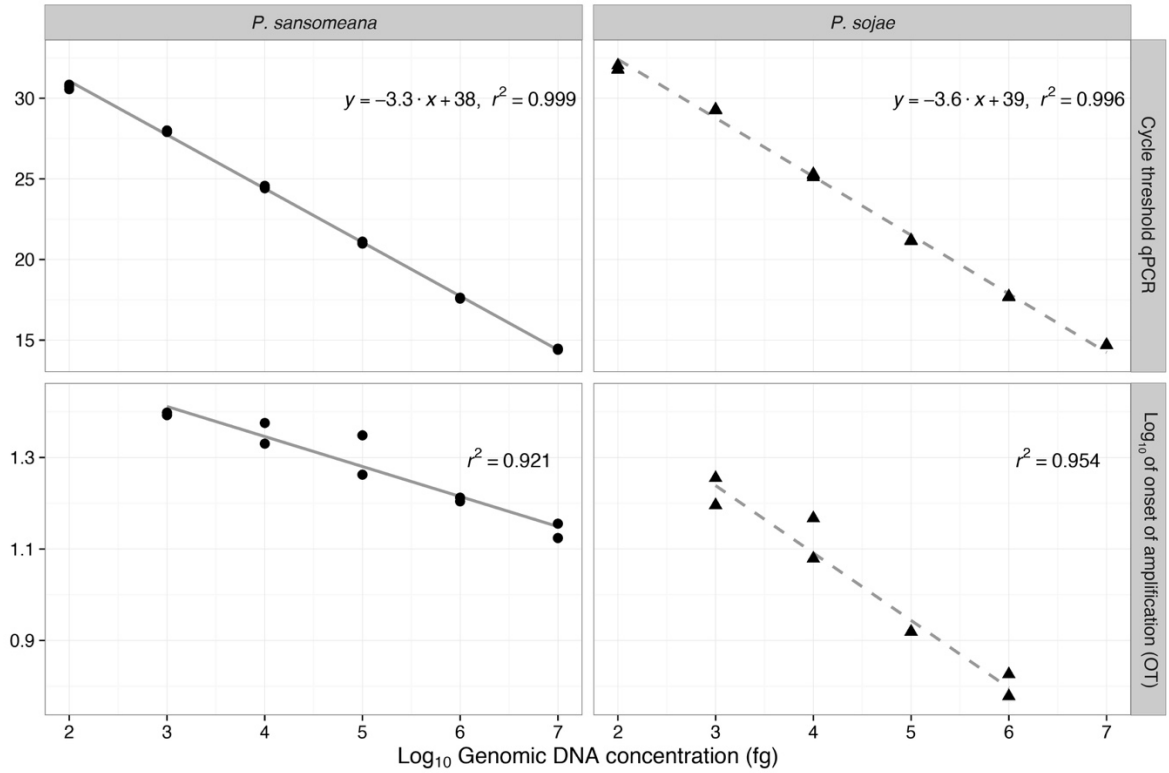

Figure 4.1. Standard curve for *Phytophthora sojae* and *P. sansomeana* genomic DNA (fg) using the qPCR assay (top panels) and recombinant polymerase amplification (RPA) *atp9-nad9* species-specific assay (bottom panels). Genomic DNA was ten-fold diluted and the sensitivity was determined to be 100 fg (2 $\log_{10}$) for qPCR and 1 pg (3 $\log_{10}$) for RPA. Three technical repeats for each DNA concentration on the qPCR and two technical repeats were used for RPA.

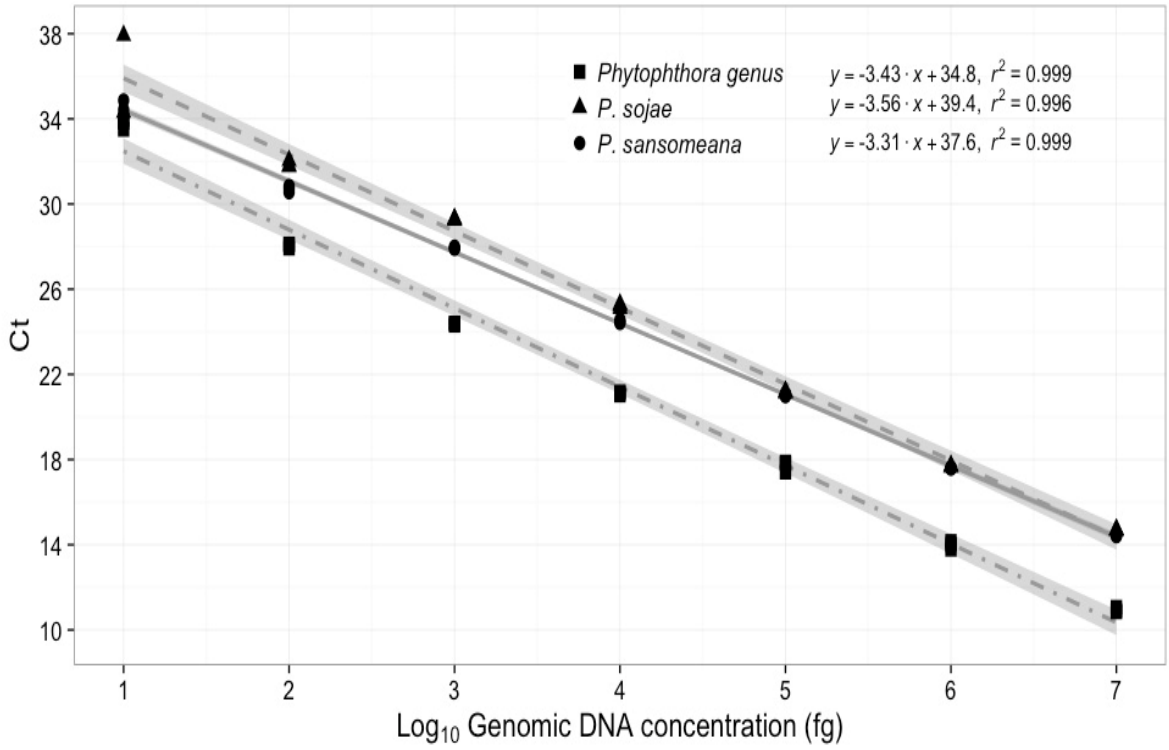

Figure 4.2. Standard curve for *Phytophthora* genus, *P. sojae* and *P. sansomeana* on genomic DNA (fg) using the multiplex qPCR assay. Genomic DNA was ten-fold diluted and the sensitivity was determined to be 100 fg (2 $\log_{10}$) for qPCR, as >95% (100% observed) of standards amplified consistently as designated by Bustin et al. (2009). Squares represent *Phytophthora* genus, triangles represent *P. sojae* and circles represent *P. sansomeana.*

Cross-platform validation. Three different platforms were evaluated using the serial standard diluted DNA for both pathogens to test for assay consistency. Assay conditions were the same across the platforms. Overall amplification efficiencies ranged from 93.1 to 100.8% for the *Phytophthora* genus assay, from 95.3 to 95.9% for *P. sojae* and 100 to 101% for *P. sansomeana* indicating minimal variation across platforms (Table 4.3). However, the *P. sansomeana* probe was not useful with the StepOnePlus system since the fluorophore Quasar670

has a spectral absorption at 650 nm and emission at 670 nm, which is not detected by this system. In all the platforms, the respective software set the C_t threshold. The StepOnePlus platform exhibited high background noise that was resolved by adjusting the baseline from cycle 3 to 14.

Field application of qPCR. The developed multiplex assay was used to diagnose soybean seedling samples collected in Michigan and Ontario in 2013 (Table 4.4), and across different soybean producing states in 2014, including Arkansas, Iowa, Illinois, Indiana, Kansas, Michigan, North Dakota, South Dakota and Nebraska (Table 4.5). In 2013, *P. sojae* was isolated from the soybean tissue collected from 7 out of 10 sampled Michigan fields; however, no isolates of *P. sansomeana* were obtained from these field locations (Table 4.4). In samples collected from Ontario, *P. sojae* was isolated from 4 out of 6 sampled locations. Subsamples from plant tissue were tested with the *Phytophthora* ELISA assay from AGDIA. The ELISA resulted in 8 positive samples for Michigan and 6 samples positive for Ontario. However, the *Phytophthora* ELISA assay did not produce conclusive results (i.e. weak reaction) for the detection of *Phytophthora* spp. in samples isolated from two fields in Michigan.

The multiplex qPCR assay resulted in amplification of the plant internal control with C_t values ranged from 12.6 to 17.3, indicating amplifiable template could be generated from these extracted DNAs, reducing the possibility of false negatives due to amplification inhibition (Table 4.4). For the *Phytophthora* genus assay, C_t values ranged from 17.2 to 28.8 with four fields where the pathogen was not cultured but there was a positive qPCR result. The *P. sojae* assay resulted in C_t values ranging from 20.1 to 31.2, where three fields in Michigan that were negative for isolation resulted in positive qPCR samples. One Ontario field (ONPS5) was positive for

ELISA but *Phytophthora* was not detected using the molecular assays even though there was a positive amplification for the plant internal control. The rest of the samples were positive for both the *Phytophthora* genus and *P. sojae* assay. With respect to the *P. sansomeana* assay, positive results were not obtained for any of the fields evaluated in 2013.

In 2014, 23 fields were sampled resulting in 73 plant samples evaluated with the multiplex qPCR assay. Amplification of the plant internal control was detected for all of the samples and the *Phytophthora* genus-specific assay resulted in 21 positive fields, and the remaining two samples did not produce amplification for *Phytophthora* genus and species-specific assays (Table 4.5). Among the 21 positive fields for the genus specific assay, 18 fields were positive using the *P. sojae* assay, where one sample had a C_t of 32, reflecting a low titer of the pathogen. Amplification using the *P. sansomeana* assay detected this pathogen in plant samples from fields ARPS2_1, ARPS2_2, ARPS2_3, ILPS2_4 and INPS2_2 (Table 4.5). Three samples were also positive for *P. sojae*, indicating the presence of both pathogens in these plants. Only one sample produced amplification for the genus specific assay, but it was negative for both species-specific assays. The DNA sequence of the species specific amplicon indicates it is an undescribed species similar to *Phytophthora megakarya.*

Soil samples were collected from the same locations as the plant samples and evaluated using the multiplex qPCR assay to determine the presence of the pathogens in the sampled fields. In 2013, three different types of DNA extraction treatments were processed from the same soil sample: undiluted, tenfold diluted, and a paramagnetic bead-purified DNA fraction from the undiluted sample (Table S.4.6). For all the reactions, an internal control was used to determine presence of PCR inhibitors that could affect amplification. Overall, amplification of the internal control was detected in all reactions, but C_t values were improved by either diluting samples or

using the paramagnetic bead purified DNA as template (Table S.4.6). With respect to detection of pathogens, the *Phytophthora* genus and species-specific assays resulted in detectable amplification, but C_t values were greater than 30, so an accurate quantification of the amount of pathogen present in the soil could not be made. Although C_t values improved with paramagnetic bead purification, those C_t remained higher than 30 (Table S.4.6). A C_t below the LOD for *P. sojae* by the genus and species specific assay was obtained only for MIPS9 soil samples following purification with paramagnetic beads. The use of diluted soil DNA as template delayed amplification and increased the C_t values in most cases, except by that from field ONPS1, which actually resulted in detectable amplification in comparison to no detection in the undiluted sample. Soil DNA samples from 2014 were further purified with paramagnetic bead purification. These samples revealed a low concentration of the pathogen with a C_t around or higher than 32 cycles (Table S.4.6 and S.4.7).

Recombinase polymerase amplification. The development of a recombinase polymerase amplification (RPA) species-specific assay for *P. sojae* and *P. sansomeana* was based on the alignment noted above for designing the qPCR species specific assay. The criteria for the selection of the primers was initially based on performance using genomic DNA, followed by a preliminary specificity test with closely related species. Since primer design for RPA assays are not as straight forward as designing PCR primers, multiple primers were tested for species specificity. In total, 10 reverse primers of different length were designed for *P. sojae* (Table S.4.8), and evaluated in combination with the general *Phytophthora* forward primer designed by Miles et al. (2015). The selected primer Psoj_n9_rev_twexo9 has a length of 29 bp and GC% content of 17.2. In the case of *P. sansomeana*, only six primers were tested, and the best performing primer was Psan_n9_rev_twexo1 with a length of 35 bp and GC% content of

14.3. Both primers were assessed against the panel of *Phytophthora* spp., *Phytopythium vexans* and *Pythium* spp. resulting in no cross amplification with any of the non-target taxa.

In order to test the sensitivity of the RPA assay, a ten-fold *P. sojae* or *P. sansomeana* genomic DNA serial dilution from 10 ng to 1 pg was tested in independent RPA reactions; DNA concentrations below this level were not detected. The log10 of the onset of amplification, namely the time at which the reaction meets the criteria for the slope validation, was plotted against the log concentration of the genomic DNA. While the *P. sojae* RPA assay amplification occurred between 6 to 18 min, depending on the concentration, with a R^2 value of 0.954 (Fig. 4.1), amplification for the *P. sansomeana* RPA assay occurred between 12 to 24 min with a R^2 value of 0.921 (Fig. 4.1). The lowest concentration for a positive detection for both assays was between 10 pg to 1 pg.

Field application of RPA genus and species-specific assays. Plant samples collected in 2013 and 2014 were divided for both qPCR and RPA testing. A total of 115 plant samples were evaluated, 42 in 2013 and 73 in 2014. From the 42 samples obtained in 2013, 36 resulted in positive amplification by the *Phytophthora* genus RPA assay, of which 29 were also positive for detection of *P. sojae* using the RPA species-specific assay (Table 4.4). From 73 samples tested in 2014, 34 were positive for detection with the *Phytophthora* genus specific assay of which 22 were positive with the *P. sojae* RPA assay (Table 4.5). In both years *P. sansomeana* was not detected in any plant samples.

Sensitivity of the RPA genus and species-specific assays was evaluated in contingency tables in comparison to qPCR genus assay using the number of true positives (positive for qPCR and RPA) divided by the sum of true positives (positive for qPCR and RPA) and false RPA negatives (positive qPCR, but negative for RPA). Out of 115 samples, the RPA genus assay

resulted in an overlap of 70 samples designated as true positives, and only 15 samples designated as false negatives (positive for qPCR but negative for RPA). Results from the RPA genus specific assay correlated with the qPCR assay 82.4% of the time. There were 4 samples designated false positives (negative with qPCR, and positive with RPA) and 26 were samples designated as true negatives. Out of the 115 samples at the species level, 51 samples were designated as true positives, 34 samples were designated as false negatives, resulting in a correlation between RPA and qPCR of 60.4% for species-specific detection. Both assays coincided in 30 samples being designated as true negatives for the species assay, indicating no issues with specificity.

Discussion

We developed a multiplex hierarchical genus and species-specific qPCR assay, utilizing the *Phytophthora* genus assay developed by Bilodeau et al. (2014), which simultaneously determines if a sample is infected by any *Phytophthora* spp. in addition to determining if the sample is infected with either *P. sojae, P. sansomeana,* or both. The assay is specific to these two species as tested against a panel of 110 different *Phytophthora* taxa (including valid and provisional species) and other oomycete species that could be associated with roots of soybean plants. In addition, the assay exhibited a high sensitivity consistently detecting as little as 100 fg of *P. sojae* or *P. sansomeana* DNA. The assay also includes a plant mitochondrial internal control to determine if the concentration of PCR inhibitors was high enough to prevent amplification. In order to allow the use of this assay on soil samples, an artificial internal control was added to the master mix to monitor the effect of PCR inhibitors on amplification efficiency as reported by Bilodeau et al. (2012). Furthermore, the qPCR assay was cross-validated on

different platforms in laboratories in Michigan and California demonstrating the transferability of the assay.

The selection of the DNA target plays an important role in assay sensitivity and specificity, and in this case, the mitochondrial region between the *atp*9-*nad*9 was identified as harboring enough variation to generate species-specific probes for different *Phytophthora* spp. (Bilodeau et al. 2014, Miles et al. 2015, Miles et al. 2016). Both TaqMan probes, primers and RPA primers have already been validated for this mitochondrial locus which will allow us to utilize this *atp*9-*nad*9 marker system on a variety of crops outside of soybeans as demonstrated by Miles et al. (2016). In comparison to other assays that target multicopy genes or genomic regions like rDNA in fungal pathogens (Bilodeau et al. 2012, Schena et al. 2013, Wang et al. 2014), the current assays targeting the mitochondrial DNA are very sensitive due to the presence of multiple mitochondria per cell, however, it is unknown if there is consistency in the numbers of mitochondria per cell during different phases of plant infection and survival in soil; this should be evaluated further prior to using this locus for pathogen quantification.

The use of the multiplex qPCR, resulted in the detection of *P. sojae* in soybean plants collected from 33 out 39 fields. While the sampling was targeted towards *Phytophthora* stem and root rot symptomatic fields, it agrees with the broad presence of *P. sojae* reported in other studies (Dorrance et al. 2016). With respect to *P. sansomeana,* fields in Arkansas, Indiana and Illinois were designated as positive which highlights the distribution of this recently described species. The tools reported in this study will be of great help to confirm the presence and impact of *P. sansomeana* in soybean and corn fields, since reports of its recovery in some states, like Wisconsin, are increasing (Phibbs et al. 2014). In 2013, isolations of *P. sojae* from symptomatic plants were performed and results corroborated the qPCR detection. There were 4 cases out of 5

where *P. sojae* was not isolated, but it was detected with the qPCR assay. These results provided support for the reliability of the assay, and reinforced the advantage of using molecular diagnostics to establish the presence of pathogen. The plant internal control was amplified from all plant tissue DNA extractions, confirming that inhibitors were not preventing amplification. However, this type of internal control does not evaluate the influence of PCR inhibitors on amplification efficiency, so it is possible that pathogen detection was impacted if pathogen target DNA was present at low amounts, which in turn can result on false negatives. Samples that were positive for qPCR genus assay, but negative for the species-specific qPCR assay were sequenced to determine the if there were issues with specificity, the resulting sequences were negative for *P. sojae* and *P. sansomeana*, confirming the specificity of the assay.

In addition to the field samples reported in the current study, the multiplex qPCR assay was also used on diagnostic samples submitted to and in collaboration with the Michigan State University Diagnostic Laboratory. Four samples were subsampled and processed into three different tissues (tap root, lateral roots and stem) and analyzed in the CFX-96 (Bio-Rad platform). From these, two samples were positive for both pathogens. The processing of these samples by tissue type (tap root, lateral roots and stem) demonstrated a trend were *P. sojae* infected primarily stem and lateral roots, while *P. sansomeana* infected tap root (data not shown). This preliminary data suggests spatial variation on the infection of these pathogens, however this requires further evaluation. The availability of these tools can be used in conjunction with microscopy to track the pathogen infection as reported in other pathosystems (Martín-Rodrigues et al. 2013).

When evaluating soil samples with the qPCR assay, it was apparent additional template cleanup was needed to eliminate the influence of PCR inhibitors on the sensitivity and accuracy

of data collection (Table S6). Amplification of the internal control was inhibited in many soil samples following DNA extraction with just the FastDNA SPIN kit, diluting samples 1:10 improved amplification for many but not all templates. Subsequent purification of DNA extracts with paramagnetic beads further improved amplification (reduced C_t closer to what was observed for amplification without added soil extracted DNA) for nearly all samples. The improvement in the amplification was also observed for the internal control, where in most cases there is a reduced cycle threshold that suggests better amplification conditions. Additional research on techniques to improve the quality of the extracted DNA will likely improve the sensitivity of the described assays. Other problems with developing molecular techniques for quantification of soilborne pathogens include ensuring the pathogen propagules have been disrupted and the field sampling strategy is adequate to ensure enough of the pathogen has been recovered to be detected and reflects pathogen distribution within the field. It is possible that oospores were recalcitrant to DNA extraction using the DNA extraction procedures used in this report and further procedures are necessary to increase DNA yield, however the extraction technique used in this experimentation was similar to what was reported for disruption of microsclerotia of *Verticillium dahliae* (Bilodeau et al. 2012).

Given the non-random distribution and low inoculum densities of many soilborne pathogens, developing a procedure to collect a representative sample from the field and economically process more than 500 mg of soil at a time should improve assay sensitivity and reliability for estimating pathogen propagule density. The use of larger amount of samples could improve the detection since *P. sojae* could be present in a low density in the soil and plant tissue, therefore the requirement of sophisticated enrichment and isolation methods to improve the recovery of this pathogen (Dorrance et al. 2008). Soil type and composition may also influence

subsequent purity of the DNA samples and may require additional screening of extraction procedures (Okubara et al. 2005, Bilodeau et al. 2012). Although C_t values from most soil samples evaluated in the study were above the limit of consistent detection (LOD) determined by DNA standard curves (thereby limiting data interpretation for estimation of propagule density), some level of amplification was observed for many samples suggesting the pathogen was present but at low inoculum density. Following the above noted considerations for assay optimization, assays should also be optimized in order to improved pathogen DNA yield, since it has been observed that increased concentration of pathogen propagules does not assure higher extraction and detection efficiency (Chilvers et al. 2007).

A rapid isothermal molecular assay was also developed based on the same mitochondrial locus, in order to discriminate between the two species focus of this study, *P. sojae* and *P. sansomeana*. Using an established *Phytophthora* genus assay developed by Miles et al. (2015), a hierarchical approach was also used to diagnose field samples, validating the use of this novel technology to establish the presence of pathogen in plant samples directly in the field. The sensitivity of the RPA assays was around 1 pg of genomic DNA from both pathogens, exhibiting high specificity, as evaluations against a panel of *Phytophthora* spp., *Pythium* spp. and *Phytopythium* resulted in no amplification of the non-target species.

The recombinase polymerase amplification (RPA) assay exhibited a high level of specificity, however the assay was not as sensitive as previously reported. While Miles et al. (2015) reported sensitivities between 200-300 fg of DNA, the experiments reported in this manuscript show sensitivity of 1-10 pg, which is likely the reason for false negatives in the RPA assay when qPCR results were positive; many of these samples had a high C_t indicating a low pathogen DNA concentration. Lot variability in fluorometric RPA exo kit amplification was

observed over the course of these experiments, and during this process we identified several factors that impacted assay performance. After discussions with the manufacturer, one is likely due to changes in formulation resulting in changes in the ratios of enzymes involved in amplification in the current kits available. To overcome the changes in chemistry, additional optimization of assay conditions to improve detection sensitivity should be conducted. This would include evaluation of using a larger amount of tissue for extraction or the adding a larger amount of sample extract to the amplification reaction. The sample handling and extraction conditions were also different in the current study; due to time limitation and the number of samples to process the tissue used for RPA amplification was placed fresh at -20° C and removed at a later time for maceration in buffer whereas in Miles et al. (2015) the tissue was macerated fresh. It is possible that during the slow freezing to -20° C and subsequent thaw step prior to maceration that some of the target DNA may have been degraded. Trials processing samples fresh or flash freezing in liquid nitrogen may provide more optimal conditions for pathogen DNA extraction. Optimizing other conditions such as magnesium concentration may also increase sensitivity since this drives the amplification reaction. For example, preliminary data showed that increasing the magnesium concentration in the *P. sojae* RPA assay had a positive impact in amplification sensitivity. Currently there are two manufacturer's of RPA kits (Twistdx Inc., and Agdia Inc.) creating products with different overall reaction volumes (50 and 25 µl, respectively). Field samples from the current study were evaluated with Agdia Inc. kits, and these produced consistent results (data not shown).

The primer development for RPA is also critical in assay optimization, although this is not well characterized, different primer lengths may help to improve the assay performance (Boyle et al. 2014). We observed that different primer lengths had different behavior on the two

species. For example, in our case *P. sojae* with the 29-mer primer assay has a faster amplification than the *P. sansomeana* assay with a 35-mer primer, but both of them were the best among the primers tested (Table S.4.8). The primers developed in this study in conjunction with primers from other studies will be important to develop criteria and guidelines for optimal RPA primer design and reaction conditions.

Both qPCR and RPA assays were validated with soybean samples collected in commercial fields in 2013 and 2014, demonstrating the applicability of the assays to detect *Phytophthora* in soybean fields. While the RPA assay demonstrated similar levels of detection at the genus level, the sensitivity at the species level was lower. Nevertheless, the rapid detection of the isothermal assay provides a tool with the potential to diagnose field samples in just 20 to 40 minutes, with the potential to be more sensitive than culture based techniques. The results obtained with field samples using the *P. sojae* RPA assay indicate the robustness of the method in comparison to the qPCR assay, since RPA uses a crude extract rather than purified DNA, being more recalcitrant to inhibitors present in the sample and the reaction happens at a low temperature (Craw and Balachandran 2012). For instance, food and tissue samples processed with isothermal techniques, such as RPA, have demonstrated the consistent amplification when compared to real-time quantitative PCR, which is affected by different inhibitors present in the samples (Kim and Lee 2016). In addition, the availability of different and inexpensive platforms, such as the portable and battery operable BioRanger (formerly Smart-DART; Diagenetix Inc.; Jenkins et al. (2011)), facilitates the rapid and robust detection of pathogens directly in the field.

The qPCR and RPA assays were presented in a diagnostician workshop as part of the North Central-APS meeting in 2015, and the assays were demonstrated and used by a variety of

diagnosticians and researchers (Wang et al. 2016). Materials from the workshop are available on github page (http://chilverslab.github.io/2015MSU_Diag_Workshop/). In addition, a webcast was recorded on the Plant Health Management Network, where both qPCR and RPA assay basics and the development of *P. sojae* and *P. sansomeana* assays were discussed (Rojas 2016). These tools will help diagnostic clinics and researchers throughout soybean growing regions to identify more quickly and accurately *P. sojae* and *P. sansomeana*. The *Phytophthora* species-specific qPCR and RPA assays presented in the current study allow the accurate, sensitive and specific detection of *P. sojae* and *P. sansomeana* within the limits of the corresponding technology. Using molecular detection tools more routinely will give more clear insights into the epidemiology of *P. sojae* and *P. sansomeana,* and it will open new avenues for the study of the infection process of these pathogens and the effect of management strategies on the abundance of these *Phytophthora* spp. In addition, coupling these tools with isolation data might help us identify new *Phytophthora* spp. that have a detrimental effect on soybean and corn or the identification of potential host specific forms of *P. sansomeana*.

APPENDIX

Table S.4.1. *Phytophthora sojae* and *Phytophthora sansomeana* isolates used in this study to evaluate *atp*9-*nad*9 locus variation.

Species	Isolate	Geographic Origin	Source
Phytophthora sojae	P0405*	Mississipi	Soybean
P. sojae	P6497*	Mississipi	Soybean
P. sojae	Br2.4	Brazil	Soybean
P. sojae	P10704*	New Zealand	Soil
P. sojae	P7061*	Ontario	Soybean
P. sojae	P3114*	Wisconsin	Soybean
P. sojae	INPS_2-4	Indiana	Soybean
P. sojae	IASO_3-41.17	Iowa	Soybean
P. sojae	C-KSSO2_3-11	Kansas	Soybean
P. sojae	C-MIPS_2-16	Michigan	Soybean
P. sojae	C-MIPS_2-17	Michigan	Soybean
P. sojae	C-MIPS_3-2	Michigan	Soybean
P. sojae	C-MIPS_3-7	Michigan	Soybean
P. sojae	C-MIPS_5-14	Michigan	Soybean
P. sojae	C-MIPS_5-17	Michigan	Soybean
P. sojae	C-MIPS_5-7	Michigan	Soybean
P. sojae	C-MIPS_7-7	Michigan	Soybean
P. sojae	C-MIPS_8-6	Michigan	Soybean
P. sojae	C-MIPS_9-20	Michigan	Soybean
P. sojae	C-MIPS_9-4	Michigan	Soybean
P. sojae	C-MIPS_9-5	Michigan	Soybean
P. sojae	MISO_4-27	Michigan	Soybean
P. sojae	P-MIPS_5-14	Michigan	Soybean
P. sojae	P-MIPS_5-7	Michigan	Soybean
P. sojae	P-MIPS_9-20	Michigan	Soybean
P. sojae	P-MIPS_9-5	Michigan	Soybean
P. sojae	V-MISO2_3-44	Michigan	Soybean
P. sojae	V-MISO2_3-63	Michigan	Soybean
P. sojae	V-MISO2_3-66	Michigan	Soybean
P. sojae	MNPS_2-1	Minnesota	Soybean
P. sojae	MNPS_2-8	Minnesota	Soybean
P. sojae	MNPS_2-9	Minnesota	Soybean

Table S.4.1 (cont'd)

P. sojae	MNSO_5-17.2r	Minnesota	Soybean
P. sojae	V-MNSO2_1-47	Minnesota	Soybean
P. sojae	C-NESO2_5-5	Nebraska	Soybean
P. sojae	NESO_2-25	Nebraska	Soybean
P. sojae	V-NESO2_2-44	Nebraska	Soybean
P. sojae	V-NESO2_2-49	Nebraska	Soybean
P. sojae	V-NESO2_6-55	Nebraska	Soybean
P. sojae	NDPS_1-1	North Dakota	Soybean
P. sojae	NDPS_1-2	North Dakota	Soybean
P. sojae	NDPS_2-3	North Dakota	Soybean
P. sojae	NDPS_4-1	North Dakota	Soybean
P. sojae	NDPS_4-3	North Dakota	Soybean
P. sojae	NDPS_7-3	North Dakota	Soybean
P. sojae	NDPS_8-1	North Dakota	Soybean
P. sojae	NDPS_9-4	North Dakota	Soybean
P. sojae	NDPS_9-5	North Dakota	Soybean
P. sojae	ONSO2_1-66	Ontario	Soybean
P. sojae	ONSO2_1-83	Ontario	Soybean
P. sojae	ONSO2_1-84	Ontario	Soybean
P. sojae	ONSO2_1-96	Ontario	Soybean
P. sojae	V-SDSO2_2-48	South Dakota	Soybean
P. sansomeana	Psan_1819B*	Indiana	Soybean
P. sansomeana	Psan_44*	New York	White Clover
P. sansomeana	Psan_72*	New York	White Cockle
P. sansomeana	Psan_22*	Oregon	Douglas-fir
P. sansomeana	Psan_77*	Oregon	Douglas-fir
P. sansomeana	P3163*	New York	White Cockle
P. sansomeana	C-IASO2_6-15	Iowa	Soybean
P. sansomeana	C-KSSO2_3-6	Kansas	Soybean
P. sansomeana	KSSO_6-1-1	Kansas	Soybean
P. sansomeana	V-KSSO2_1-7	Kansas	Soybean
P. sansomeana	V-KSSO2_3-6	Kansas	Soybean
P. sansomeana	C-MISO2_3-19	Michigan	Soybean
P. sansomeana	MICO_3-15	Michigan	Corn
P. sansomeana	MICO_3-24	Michigan	Corn

Table S.4.1 (cont'd)

P. sansomeana	MICO_3-28	Michigan	Corn
P. sansomeana	C-NESO2_5-19	Nebraska	Soybean
P. sansomeana	C-NESO2_5-6	Nebraska	Soybean
P. sansomeana	V-NESO2_5-44	Nebraska	Soybean
P. sansomeana	V-NESO2_5-45	Nebraska	Soybean
P. sansomeana	ONPS_2-5	Ontario	Soybean
P. sansomeana	ONSO2_1-114	Ontario	Soybean
P. sansomeana	ONSO2_1-65	Ontario	Soybean

* Isolates obtained from the World Phytophthora Genetic Resource collection. The remaining isolates were collected in a survey (Rojas et al. 2016)

Table S.4.2. Multiplex qPCR reactions for detection of *Phytophthora* genus and *Phytophthora sojae* and *Phytophthora sansomeana* species-specific pathogens in plant and soil samples.

Reagents	Initial concentration	Volume per reaction (µL)	
		Plant Samples	Soil Samples
Primers			
PhyG_ATP9_2FTail	10 µM	1.0	1.0
PhyG-R6_Tail	10 µM	1.0	1.0
Probes			
Phytophthora genus-specific TaqMan probe	10 µM	0.05	0.05
P. sojae species-specific TaqMan probe	10 µM	0.2	0.2
P. sansomeana species-specific TaqMan probe	10 µM	0.1	0.1
Plant Internal Control			
FMPI2b	1 µM	0.4	…
FMPI3b	1 µM	0.4	…
Plant-IC probe	1 µM	0.4	…
Internal Control (Soil)			
PPF	10 µM	…	0.2
Internal Control		…	1.0
Real Master Mix without Rox (5 Prime)	2.5X	8.0	8.0
Mg^{++}	25 mM	2.0	2.0
DNA		2.0	2.0
PCR-grade water		4.45	4.45
Total volume		20 µL	20 µL

Table S.4.3. *Phytophthora* genus and *Phytophthora sojae* and *Phytophthora sansomeana* species-specific recombinase polymerase amplification (RPA) reaction setup for detection in plant samples.

Reagents	Initial concentration	Volume per reaction (µL)		
		RPA *Phytophthora* genus	RPA *P. sojae* species-specific	RPA *P. sansomeana* species-specific
Genus-specific				
TrnM-F	1 µM	1	…	…
TrnM-R	10 µM	2.9	…	…
TrnM-P	10 µM	0.6	…	…
*S*pecies-specific				
ATP9-F	1 µM	…	0.5	1
Psojae-*nad*9-R	10 µM	…	4.15	…
Psan-*nad*9-R	10 µM	…	…	4.1
ATP9-P	10 µM	…	0.9	0.9
Plant Internal Control				
Cox1-IPC-F	10 µM	1.25	…	…
Cox1-IPC-R	10 µM	1.25	…	…
Cox1-IPC-P	10 µM	0.6	…	…
Buffer		29.5	29.5	29.5
Water		8.9	10.95	10.5
Crude Plant Extract/DNA		1.5	1.5	1.5
Total Volume[a]		50 µL	50 µL	50 µL

[a] Total volume after the addition of the magnesium acetate, which takes place before starting the reaction.

Table S.4.4. Species-specific hydrolysis probes tested for development of *P. sojae* and *P. sansomeana* qPCR diagnostic assay.

qPCR Probes tested	Sequence(5' - 3')
P. sojae	[Hex] TTGATATATGAATACAAAGAT AGATTTAAGTAAAT [BQH-1]
P. sojae ZEN	[HEX] TTGATATAT [ZEN] GAATACAAAG ATAGATTTAAGTAAAT [IABkFQ]
P. sansomeana	[Quasar670] TATTAGTACTAA**Y**TACTAATA TGCATTATTTTTAG [BQH-2]
P. sansomeana	[Quasar670] TACTAATATGCATTA TTTTTAGAAAAAATATAT [BQH2]

Table S.4.5. Panel of *Phytophthora* species utilized in conducting species-specific tests on species specific TaqMan and RPA reactions for *P. sojae* and *P sansomeana*.

Species DNA		Isolate Number[a]	Isolation source	Origin
Phytophthora	*alni subsp alni*	P16203	*Alnus glutinosa*	Netherlands
Phytophthora	*alni subsp multiformis*	P16202	*baiting wetland ecosystem*	
Phytophthora	*alni subsp uniformis*	P16206	*Alnus sp.*	Sweden
Phytophthora	*alticola*	P16053	*Eucalyptus sp*	South Africa
Phytophthora	*andina*	P13365	*Solanum brevifolium*	Ecuador
Phytophthora	*asparagi*	P10690	*Asparagus officinalis*	New Zealand
Phytophthora	*austrocedrae*	P15132	*Austrocedrus chilensis*	Argentina
Phytophthora	*bahamensis*	P3930		Bahamas
Phytophthora	*bisheria*	P10117	*Fragaria sp.*	USA
Phytophthora	*boehmeriae*	P6950	*Boehmeriae nivea*	Taiwan
Phytophthora	*botryosa*	P3425	*Hevea brasiliensis*	Malaysia
Phytophthora	*brassicae*	P10414	*Brassica oleracea*	Netherlands
Phytophthora	*cactorum*	P0714	*Syringa vulgaris*	Netherlands
Phytophthora	*cajani*	P3105	*Cajanus cajani*	India
Phytophthora	*cambivora*	P0592	*Abies procera*	USA
Phytophthora	*canalensis*	P10456	Canal water	USA
Phytophthora	*capensis*	P1819	*Curtisia dentata*	South Africa
Phytophthora	*capsici*	P3605	*Capsicum annuum*	USA
Phytophthora	*capsici like Brazil*	P0630	*Theobroma cacao*	Brazil
Phytophthora	*captiosa*	P10719	*Eucalyptus saligna*	New Zealand
Phytophthora	*cinnamomi*	P2110	*Cinnamomum burmannii*	Indonesia
Phytophthora	*citricola*	P0716	*Citrus sinensis*	Taiwan
Phytophthora	*citricola clade E*	P1321	*Rubus* sp.	USA
Phytophthora	*citricola like*	P0911	*Persea americana*	USA
Phytophthora	*citricola, not*	P6880	*Fragaria sp.*	Bulgaria
Phytophthora	*citrophthora*	P6310	*Theobroma cacao*	Indonesia
Phytophthora	*citrophthora like1a*	P0318	*Citrus sp.*	Australia
Phytophthora	*citrophthora like1b*	P10341	*Syringa sp.*	UK
Phytophthora	*citrophthora like2*	P1200	*Theobroma cacao*	Brazil
Phytophthora	*clandestina*	P3942	*Trifolium subterraneum*	Australia
Phytophthora	*colocasiae*	P6317	*Colocasia esculenta*	Indonesia
Phytophthora	*cryptogea*	P1088	*Callistephus chinensis*	USA
Phytophthora	*cryptogea like*	P3103	*Solanum marginatum*	Ecuador
Phytophthora	*cryptogea like2*	P10811	*Zantedeschia aethiopica*	Japan
Phytophthora	*cuyabensis*	P8213	Tropical lowland rainforest	Ecuador
Phytophthora	*drechsleri*	P10331	*Gerbera jamesonii*	USA
Phytophthora	*erwinii*	P3132	*Banksia integrifolia*	Australia
Phytophthora	*erythroseptica*	P1699	*Solanum tuberosum*	USA
Phytophthora	*europaea*	P10324	*Quercus rhizosphere*	France
Phytophthora	*europaea*	P10324	*Quercus rhizosphere*	France
Phytophthora	*fallax*	P10725	*Eucalyptus fastigata*	New Zealand

Table S.4.5 (cont'd)

Phytophthora	*foliorum*	P10969	*Rhododendron sp.*	USA
Phytophthora	*fragariae*	P3821	*Fragaria x ananassa*	UK
Phytophthora	*fragariae*	P3821	*Fragaria x ananassa*	UK
Phytophthora	*frigida*	P16059	*Eucalyptus sp.*	South Africa
Phytophthora	*gallica*	P16826	-	-
Phytophthora	*gemini*	P15880	*Zostera marina*	Netherlands
Phytophthora	*glovera*			
Phytophthora	*gonapodyides*	P6135	*Ilex paraguariensis*	UK
Phytophthora	*hedraiandra*	P11056	*Rhododendron sp.*	USA
Phytophthora	*heveae*	P3428	*Hevea brasiliensis*	Malaysia
Phytophthora	*heveae like1*	P1000	*Persea americana*	Guatemala
Phytophthora	*heveae like2*	P0578	*Theobroma cacao*	Malaysia
Phytophthora	*hibernalis*	P3822	*Citrus sinensis*	Australia
Phytophthora	*humicola*	P3826	soil, citrus grove	Taiwan
Phytophthora	*hungarica*	P10281	soil containing Alder roots	Hungary
Phytophthora	*hydropathica*	P16857	-	-
Phytophthora	*idaei*	P6767	*Rubus idaeus*	UK
Phytophthora	*ilicis*	P3939	*Ilex sp*	Canada
Phytophthora	*infestans*		-	-
Phytophthora	*insolita*	P6195	soil in citrus orchard	Taiwan
Phytophthora	*inundata*	P8478	*Aesculus hippocastanum*	UK
Phytophthora	*ipomoeae*	P10225	*Ipomoea longipedunculata*	Mexico
Phytophthora	*iranica*	P3882	*Solanum melongena*	Iran
Phytophthora	*irrigata*	P16861	-	-
Phytophthora	*katsurae*	P10187	*Castanea crenata*	Japan
Phytophthora	*katsurae like1*	P1372	*Cocos nucifera*	USA
Phytophthora	*katsurae like2*	P15169	*Agathis australis*	New Zealand
Phytophthora	*kelmania*	P10613	*Abes fraseri*	USA
Phytophthora	*kernoviae*	P10681	*Annona cherimola*	New Zealand
Phytophthora	*lacrimae*	P15880	*Zostera marina*	Netherlands
Phytophthora	*lagoariana*	P8217		Ecuador
Phytophthora	*lateralis*	P3888	*Chamaecyparis lawsoniana*	USA
Phytophthora	*lateralis*	P3888	*Chamaecyparis lawsoniana*	USA
Phytophthora	*lateralis*	P3888	*Chamaecyparis lawsoniana*	USA
Phytophthora	*macrochlamydospora*	P10267	*Glycine max*	Australia
Phytophthora	*meadii*	P6128	*Elettaria cardamomum*	India
Phytophthora	*meadii like1*	P6262	*Hevea brasiliensis*	India
Phytophthora	*medicaginis*	P10683	*Medicago sativa*	USA
Phytophthora	*megakarya*	P8516	*Theobroma cacao*	Sao Tome
Phytophthora	*megasperma*	P1679	*Malus sylvestris*	USA
Phytophthora	*megasperma*	P3136	*Brassica napus var. napus*	Australia
Phytophthora	*melonis*	P10994	*Trichosanthes dioica*	India
Phytophthora	*mengei*	P1273	*Persea americana*	USA
Phytophthora	*mexicana*	P0646	*Solanum lycopersicum*	Mexico
Phytophthora	*mirabilis*	P3005	*Mirabilis jalapa*	Mexico
Phytophthora	*multivesiculata*	P10410	*Cymbidium*	Netherlands

Table S.4.5 (cont'd)

Phytophthora	*multivora*	P1821	*Ocotea bullata*	South Africa
Phytophthora	*multivora*	P7902	*Pinus radiata*	USA
Phytophthora	*napoensis*	P8221	lowland tropical rainforest	Ecuador
Phytophthora	*nemorosa*	P10288	*Lithocarpus densiflorus*	USA
Phytophthora	*nicotianae*			
Phytophthora	*niederhauserii*	P10617	*Thuja occidentalis*	USA
Phytophthora	*novaeguine*	P3389	*Auracaria*	New Guinea
Phytophthora	*ohioensis*	P16050	oak forest soil	USA
Phytophthora	*palmivora*	P0255	*Theobroma cacao*	Costa Rica
Phytophthora	*parsiana*	P15164	*Ficus carica*	Iran
Phytophthora	*parvispora*	P8495	*Beaucamea sp.*	Germany
Phytophthora	*personii*	P11555	*Nicotiana tabacum*	USA
Phytophthora	*Pgchlamydo*	P10669	*Idesia polycarpa*	New Zealand
Phytophthora	*phaseoli*	P10145	*Phaseolus lunatus*	USA
Phytophthora	*pini*	P0767	*Syringa sp.*	Canada
Phytophthora	*pini*	P10204	*Rhododendron sp.*	USA
Phytophthora	*pinifolia*	P16100	*Pinus radiata*	Chile
Phytophthora	*pistaciae*	P6197	*Pistacia vera*	Iran
Phytophthora	*pistaciae*	P6197	*Pistacia vera*	Iran
Phytophthora	*plurivora*	P10679	*Juglans regia L.*	New Zealand
Phytophthora	*polonica*	P15005	*Alnus glutinosa rhizosphere*	Poland
Phytophthora	*porri*	P7518	*Allium porrum*	Netherlands
Phytophthora	*porri like1*	P10728	*Daucus carota*	France
Phytophthora	*porri like2*	P6207	*Allium cepa*	Switzerland
Phytophthora	*primulae*	P10333	*Primula acaulis*	Germany
Phytophthora	*pseudosyringae*	P10437	*Quercus robur*	Germany
Phytophthora	*pseudotsugae*	P10339	*Psendotsuga menziesii*	USA
Phytophthora	*psychrophila*	P10433	*Quercus robur*	Germany
Phytophthora	*quercetorum*	P15555	*Quercus rubra rhizosphere*	USA
Phytophthora	*quercina*	P10334	*Quercus robur*	Germany
Phytophthora	*quininea*	P3247	*Cinchona officinalis*	Peru
Phytophthora	*ramorum*	P10301	*Rhododendron sp.*	Netherlands
Phytophthora	*richardiae*	P6875	*Zantedeschia aethiopica*	USA
Phytophthora	*richardiae*	P7788	*Daucus carota*	UK
Phytophthora	*richardiae*	P7788	*Daucus carota*	UK
Phytophthora	*robiniae*	P16350	-	-
Phytophthora	*rosacearum*	P3315	*Prunus sp.*	USA
Phytophthora	*rubi*	P3289	*Rubus sp.*	USA
Phytophthora	*salixsoil*	P10337	*Salix matsudana*	UK
Phytophthora	*sansomea*	P3163	*Silene latifolia subsp. alba*	USA
Phytophthora	*sinensis*	P1475		
Phytophthora	*siskiyouensis*	P15122	seasonal tributary	USA
Phytophthora	*sojae*			
Phytophthora	*sulawesiensis*	P6306	*Syzygium aromaticum*	Indonesia
Phytophthora	*syringae*	P10330	*Rhododendron*	Germany

Table S.4.5 (cont'd)

Phytophthora	*tentaculata*	P8497	*Chrysanthemum leucanthemum*	Germany
Phytophthora	*thermophilum*	P10457	canal water	USA
Phytophthora	*trifolii*	P7010	*Trifolium sp.*	USA
Phytophthora	*tropicalis*	P10329	*Macadamia integrifolia*	USA
Phytophthora	*uliginosa*	P10413	rhizosphere of Quercus robur	Poland
Phytophthora	*vignae*	P3019	*Vigna unguiculata*	Australia
Pythium	*ultimum*	P2006	peas, cucumber	Wisconsin
Pythium	*undulatum*	P10342		
Phytopythium	*vexans*	P8419	*Solanum tuberosum*	Canada
Phytopythium	*vexans*	P3980		

[a] Isolate number in the *Phytophthora* World Collection.

Table S.4.6. Multiplex qPCR evaluation for *Phytophthora* genus and *Phytophthora sojae* and *Phytophthora sansomeana* species-specific of soil samples collected in Michigan and Ontario in 2013.

State/ Province	Fields	Dilution[a]	*Phytophthora* genus		*P. sojae*		*P. sansomeana*		Internal control	
			Mean Ct[b]	SD[c]	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD
Michigan	MIPS2	1	ND[d]	-	ND	-	ND	-	37.17	5.27
		1:10	ND	-	ND	-	ND	-	32.32	1.08
		MB	34.60	1.83	45.40	3.40	ND	-	33.37	0.52
	MIPS3	1	32.14	2.36	32.17	0.81	ND	-	31.47	2.60
		1:10	32.73	1.10	33.83	1.14	ND	-	32.84	2.38
		MB	31.01	1.14	33.22	1.40	ND	-	34.55	3.16
	MIPS4	1	ND	-	ND	-	ND	-	30.64	4.20
		1:10	ND	-	ND	-	ND	-	31.95	1.14
		MB	ND	-	ND	-	ND	-	40.63	3.56
	MIPS5	1	33.40	0.55	ND	-	ND	-	37.62	6.59
		1:10	ND	-	ND	-	ND	-	32.57	0.71
		MB	33.23	1.93	34.54	1.71	ND	-	33.69	0.88
	MIPS6	1	ND	-	ND	-	ND	-	38.91	0.10
		1:10	33.32	1.20	ND	-	ND	-	36.60	1.30
		MB	ND	-	ND	-	ND	-	38.07	3.42
	MIPS7	1	35.29	1.30	ND	-	ND	-	37.89	1.80
		1:10	ND	-	ND	-	ND	-	36.83	0.83
		MB	40.78	7.20	38.56	2.10	ND	-	32.81	0.68
	MIPS8	1	ND	-	ND	-	ND	-	37.07	0.10
		1:10	ND	-	ND	-	ND	-	36.60	0.75
		MB	35.48	3.58	ND	-	ND	-	32.88	0.53
	MIPS9	1	38.54	4.81	ND	-	37.53	2.30	37.08	1.77
		1:10	ND	-	ND	-	ND	-	36.87	2.07
		MB	27.95	4.16	29.02	4.14	ND	-	33.14	1.12
Ontario	ONPS1	1	ND	-	ND	-	ND	-	39.44	0.11
		1:10	33.69	2.10	ND	-	ND	-	38.13	3.08
		MB	36.11	1.16	ND	-	ND	-	31.67	0.86
	ONPS2	1	34.91	0.95	35.66	0.89	ND	-	38.84	5.93
		1:10	34.37	0.16	35.44	0.05	ND	-	34.61	1.37
		MB	33.42	0.72	35.44	0.86	ND	-	31.60	0.90
	ONPS3	1	ND	-	ND	-	ND	-	36.15	0.58
		1:10	ND	-	ND	-	ND	-	34.53	1.37
		MB	34.23	1.90	ND	-	35.21	0.39	31.81	0.52
	ONPS4	1	34.43	1.30	ND	-	ND	-	33.11	9.52
		1:10	35.46	0.97	ND	-	ND	-	35.56	1.55
		MB	33.96	1.79	ND	-	ND	-	31.69	0.98
	ONPS5	1	32.86	2.30	ND	-	ND	-	38.69	1.34
		1:10	33.22	0.90	ND	-	ND	-	34.88	0.80

Table S.4.6 (cont'd)

	MB	32.67	1.21	ND	-	ND	-	31.50	0.96
ONPS6	1	34.33	2.20	ND	-	ND	-	37.08	4.20
	1:10	ND	-	ND	-	ND	-	37.64	5.37
	MB	37.37	1.23	ND	-	ND	-	31.46	1.38

[a] Dilution level of samples: 1 - undiluted; 1:10 – tenfold diluted; MB – Magnetic bead fraction purification

[b] Ct = Mean cycle threshold values.

[c] SD = Standard deviation

[d] ND = Non-detected

Table S.4.7. Multiplex qPCR evaluation for *Phytophthora* genus and *Phytophthora sojae* and *Phytophthora sansomeana* species-specific of soil samples collected across nine soybean producing states in the U.S. in 2014. All soil samples were extracted and followed with magnetic bead purification.

State	Fields	***Phytophthora* genus**		***P. sojae***		***P. sansomeana***		**Internal control**	
		Mean Ct[a]	SD[b]	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD
Arkansas	ARPS2_1	31.49	1.11	40.10	1.52	34.17	0.62	32.74	0.82
	ARPS2_2	33.15	1.74	44.48	2.30	ND	-	33.22	0.90
Iowa	IAPS2_1	31.67	1.13	33.72	1.85	ND	-	33.82	0.83
Illinois	ILPS2_1	25.65	3.44	27.19	3.44	ND	-	31.41	3.69
	ILPS2_2	32.90	0.60	48.44	2.50	ND	-	32.73	1.30
Indiana	INPS2_1	34.19	1.71	ND	-	ND	-	32.42	0.73
	INPS2_2	ND[c]	-	ND	-	ND	-	33.10	0.91
Kansas	KSPS2_1	32.03	2.47	32.61	1.52	ND	-	32.62	0.99
Michigan	MIPS2_1	32.32	1.60	34.09	1.28	34.56	0.04	31.20	1.98
	MIPS2_2	32.39	0.97	35.82	0.60	ND	-	31.94	2.56
	MIPS2_3	ND	-	ND	-	ND	-	29.30	2.01
N Dakota	NDPS2_1	ND	-	ND	-	ND	-	32.33	1.32
	NDPS2_2	34.50	1.71	ND	-	35.97	0.58	33.11	1.10
	NDPS2_3	32.38	1.54	37.97	2.93	36.55	1.80	32.89	1.47
Nebraska	NEPS2_1	42.16	8.29	37.24	2.70	ND	-	33.69	0.99
	NEPS2_2	35.31	2.63	35.78	1.20	ND	-	33.58	0.98
S Dakota	SDPS2_1	34.77	1.38	36.12	1.46	ND	-	32.91	0.77
	SDPS2_2	30.73	0.28	32.00	0.21	ND	-	33.26	1.04

[a] Ct = Mean cycle threshold values.
[b] SD = Standard deviation
[c] ND = Non-detected

Table S.4.8. Reverse species-specific primers tested for development of *P. sojae* and *P. sansomeana* recombinase polymerase amplification (RPA) diagnostic assay.

***P. sojae* primers tested**	**Sequence (5' - 3')**	**Length**
Psoj_n9_rev_twexo1	TTAAATCTATCTTTGTATTCATATATCAATATAAA	35
Psoj_n9_rev_twexo2	ACTTAAATCTATCTTTGTATTCATATATCAAT	32
Psoj_n9_rev_twexo3	TCTATCTTTGTATTCATATATCAATATAAA	30
Psoj_n9_rev_twexo4	CTTTGTATTCATATATCAATATAAA	25
Psoj_n9_rev_twexo5	AAATCTATCTTTGTATTCATATATCAAT	28
Psoj_n9_rev_twexo6	TCTATCTTTGTATTCATATATCAAT	25
Psoj_n9_rev_twexo7	CTTAAATCTATCTTTGTATTCATATATCAAT	31
Psoj_n9_rev_twexo8	ATATTTACTTAAATCTATCTTTGTATTCATATAT	34
Psoj_n9_rev_twexo9	TTAAATCTATCTTTGTATTCATATATCAA	29
Psoj_n9_rev_twexo10	CTTAAATCTATCTTTGTATTCATATATCAATAT	33
***P. sansomeana* primers tested**		
Psan_n9_rev_twexo1	TTAGTAGTTAGTACTAATATAACAAAAATATAATA	35
Psan_n9_rev_twexo2	AGTTAGTACTAATATAACAAAAATATAATA	30
Psan_n9_rev_twexo3	GTACTAATATAACAAAAATATAATA	25
Psan_n9_rev_twexo4	TAATGCATATTAGTARTTAGTACTAATATAAC	32
Psan_n9_rev_twexo5	GCATATTAGTARTTAGTACTAATATAAC	28
Psan_n9_rev_twexo6	TATTAGTARTTAGTACTAATATAAC	25

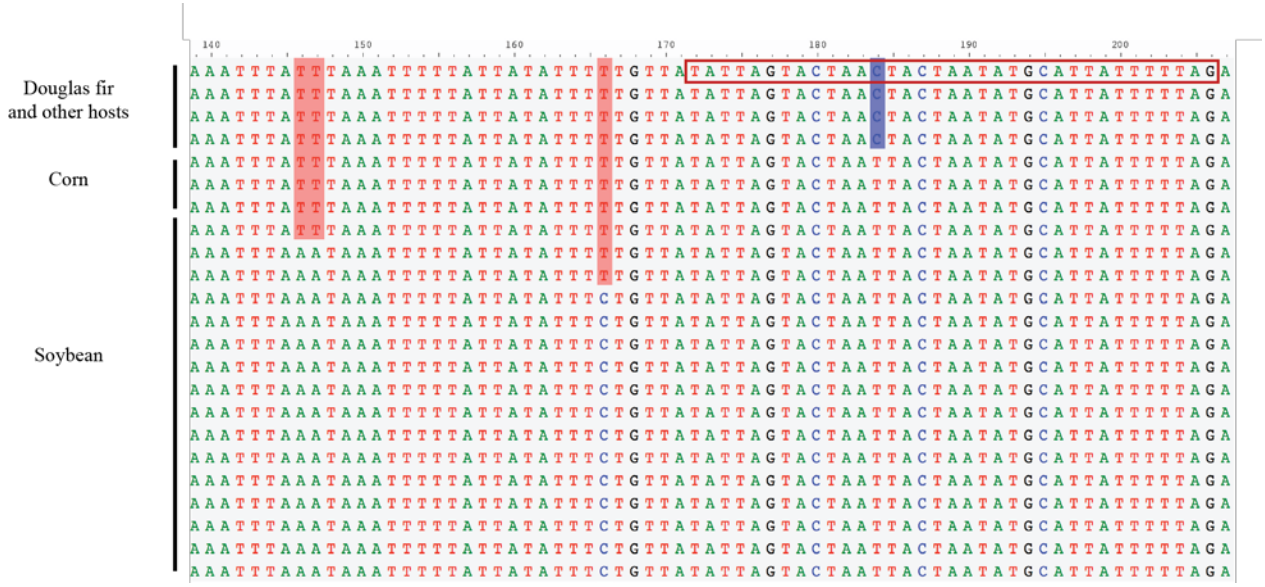

Figure S.4.1. *Phytophthora sansomeana atp*9-*nad*9 alignment sorted by hosts. *P. sansomeana* probe sequence is highlighted in the red box.

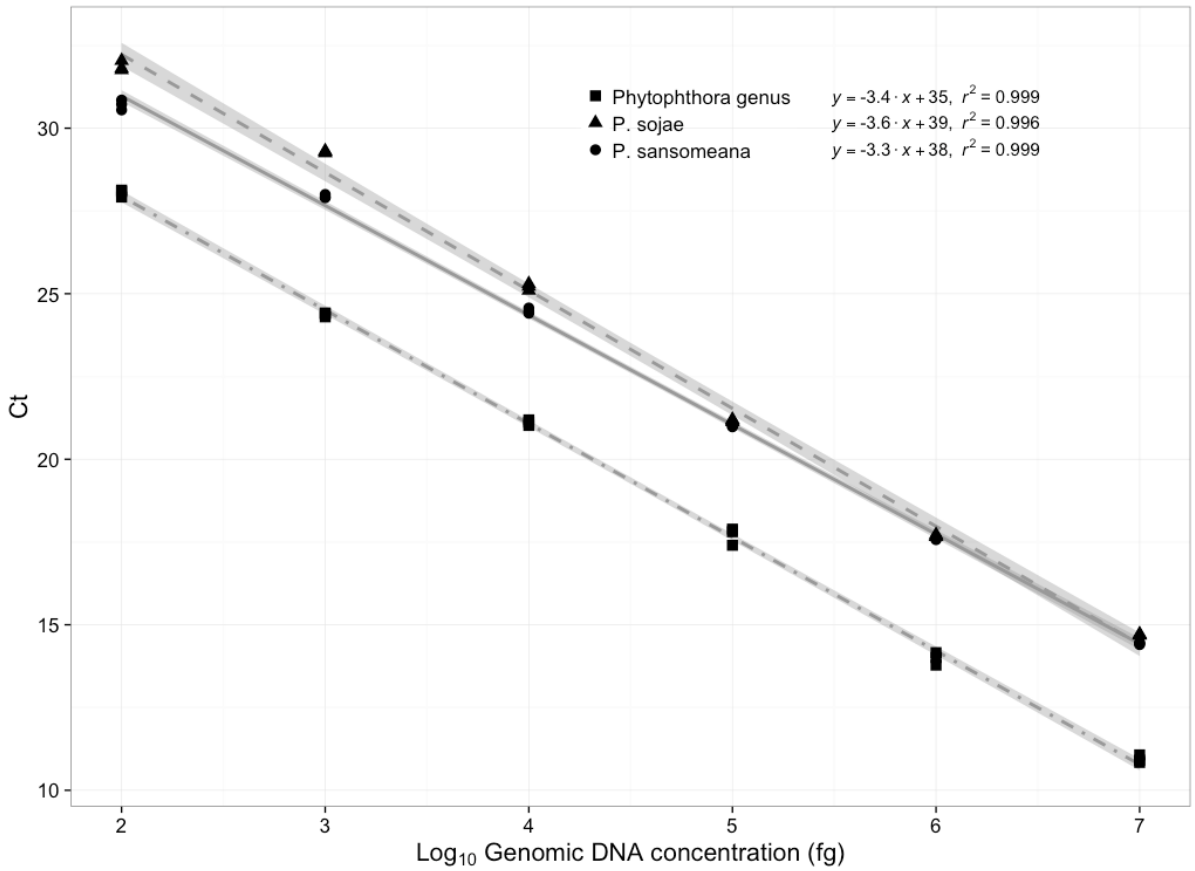

Figure S.4.2. Standard curve for *Phytophthora sojae* and *Phytophthora sansomeana* genomic DNA (fg) using the genus-specific and species-specific qPCR assay. Genomic DNA was ten-fold diluted and the sensitivity was determined to be 100 fg. Three technical repeats for each DNA concentration and confidence intervals are in light gray.

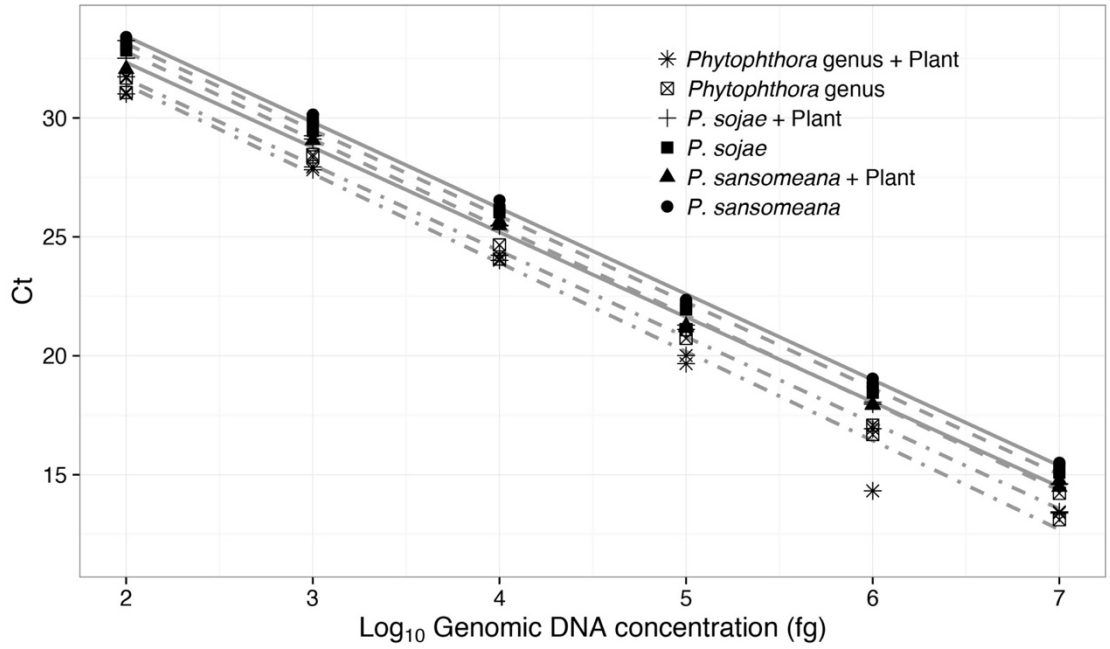

Figure S.4.3. Detection *Phytophthora sojae* and *P. sansomeana* genomic DNA (fg) and genomic DNA spiked with 20 ng of plant DNA using the TaqMan *atp9-nad9* genus-specific and species-specific assay. Genomic DNA was ten-fold diluted and the sensitivity was determined to be 100 fg. Three technical repeats for each DNA concentration.

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Chapter 5

A continental view of soil oomycete community structure associated with soybean fields

ABSTRACT

Plant pathogens have a detrimental effect on agricultural systems, nonetheless these organisms are also subject to ecological processes which affects the structure of their communities. The identification of these ecological processes could improve our understanding of the distribution of these organisms and our ability to manage them. The present study focuses on the distribution and community composition of oomycetes and fungi associated with soybean fields across the continental U.S. Composite soil samples were collected by the OSCAP Extension Network from 125 fields with a history of soybean establishment issues across the primary soybean production area in the U.S. DNA was extracted from the soil samples and amplicons were sequenced for two barcodes: internal transcribed spacer 1 (ITS1) for fungi and oomycetes and cytochrome oxidase subunit I (*cox*I) for oomycetes. In addition, environmental and edaphic data was collected from databases using geographic information systems. On average, 4000 sequence reads were recovered per sample after quality trimming and molecular operational taxonomic units picking. The composition of oomycete communities was similar across locations, however, this similarity increased as distance between sampled sites decreased. An ecological perspective on the distribution of oomycete communities could provide information on processes that drive community assembly. Thus, giving a new perspective on plant diseases and their management through the tailoring of specific management measures based on pathogen and community distribution.

Introduction

Soybean has been designated as a key crop for global food security and oilseed production (Singh et al. 2007). Production worldwide is around 223 million tons, which ranks soybean as the fourth most important crop in the world and second in the U.S. in terms of land area planted (FAOSTAT 2010, http://faostat.fao.org/default.aspx). In the last ten years, the U.S. soybean planting area has grown from 26 to 34 million hectares (American Soybean Association, http://soystats.com/). The production costs for soybean are around $421 dollars per hectare, where seed alone represents 35% of operating costs. If seed treatments are included these operating costs increase to 52%, which emphasizes the importance of management and understanding of diseases at the seedling stage. Crop germination and stand are key factors for a successful cropping season for growers. During seed establishment, seedlings are subject to attack by a number of soilborne pathogens, resulting in lack of germination, damping off or reduced plant vigor. Poor plant stands due to disease result in replanting and increased costs to growers. The impact of these soilborne diseases is not only limited to the beginning of the season, as root infections can occur at later stages, often reducing yield without significant above ground symptoms. In 2005, loss to soybean seedling diseases in the US was estimated at 1 million tons. From 2006 to 2014, soybean yield losses due to seedling diseases have increased from 1 to 1.8 million tons, ranking second only to soybean cyst nematode (Wrather and Koenning 2009, Koenning and Wrather 2010).

Soilborne seedling and root rot diseases are typically attributed to fungi and fungi-like organisms (oomycetes). It is estimated that the oomycetes, particularly the genera *Pythium* and *Phytophthora* have increased in severity over the last ten years (Broders et al. 2007). With respect to fungi, *Fusarium* and *Rhizoctonia* are often associated with soybean seedling diseases

causing damage to emerging plants (Rizvi and Yang 1996). Multiple species of *Fusarium* have been associated with disease in soybeans, with varied aggressiveness (Arias et al. 2013), likewise *Rhizoctonia solani* has also been demonstrated to cause significant damage to soybean seedlings (Dorrance et al. 2003, Bahramisharif et al. 2014). The root and rhizosphere of plants are habitat for multiple organisms that interact with each other in order to produce different outcomes, such as disease or healthy plants, and the understanding of those interactions will help to predict those outcomes (Aguilar-Trigueros et al. 2014).

However, it is not only due to the interaction of microbes, but also the cultural practices that could affect these ecosystems (Pérez-Jaramillo et al. 2015). There are different management practices that are used to reduce the prevalence of pathogens, like crop rotation, which is a common practice used by growers, although there are benefits for disease and insect control with this rotation, it has been observed that this scheme has been not effective in reducing soilborne pathogens, as many species are capable of causing disease on other crops part of the rotation (Zhang and Yang 2000, Broders et al. 2007). The increased incidence of seedling disease is also related to cultural practices that have been adopted by growers such as no-till or minimum tillage practices and earlier planting dates. These practices and the environment interact and influence the microbial communities present in the soil, including the microbial seed bank, which refers to dormant spores present in the soil. Different cues could stimulate certain plant pathogenic species inducing the germination of these dormant spores, causing population shifts in the species present in the soil (Arcate et al. 2006).

Recently, different studies were carried using culture-based approach to understand the diversity of oomycetes associated with soybean and corn seedling diseases within and across states (Zhang et al. 1998, Broders et al. 2007, Murillo-Williams and Pedersen 2008, Zitnick-

Anderson and Nelson 2015, Rojas et al. 2016). In previous study across soybean fields in North America, a total of 84 species of oomycetes were identified and 43 of those species were designated as pathogenic based on seedling cup assay. The sampling recover ca. 3500 oomycete isolates that represented fields from the soybean belt in the U.S. and Ontario, Canada. The diversity found revealed similar community structure in adjacent states and trend of higher diversity toward high latitudes. However, soilborne diseases provide a more complex system where the soil heterogeneity, the root system and the microbial species interact. With the advent of high throughput sequencing technologies, systems like the soil and rhizosphere have been studied to catalogue and understand the role of bacterial, fungal and oomycete species (Impullitti and Malvick 2013, Mendes et al. 2014, Sugiyama et al. 2014, Bai et al. 2015, Coffua et al. 2016). The use amplicon sequencing provides a new tool to study the co-existence of non-pathogenic and pathogenic species and their interaction with the environment and host.

In this study, we used amplicon community analysis to study the diversity of fungal and oomycete pathogens associated with soybean fields using soil samples to catalogue the organisms associated with disease and yield loss and to characterize the diversity of these organisms in the U.S. soybean production in 2011 and 2012. The goals of the present study were: (i) characterize the fungal and oomycete seed bank present in soybean fields across the U.S.; (ii) describe large-scale patterns of diversity of fungi and oomycetes in soil samples from soybean fields and (iii) evaluate the correlation of different environmental and edaphic factors with the oomycete and fungal community diversity and structure. We hypothesize that microbial communities present in the soil is influenced by many factors associated with a particular niche, such as plant host and environmental conditions. Under disease conducive conditions (e.g., high soil moisture, soil compaction, and susceptible hosts) pathogen populations will increase

resulting in community shifts. Multiple fungal/oomycete pathogen species will be stimulated causing the interaction among them and with the plant, developing the formation of disease complexes producing damping off and root rots. The information will provide an important framework in the understanding of disease complexes and the conditions that promote disease development.

Materials and Methods

Sample collection and DNA extraction. Fields with a history of seedling disease and plant stand issues were identified in 12 U.S. states comprising the majority of soybean producing states. Bulked soil samples were collected from 62 and 60 fields in 2011 and 2012, respectively (Fig. 5.1). Three to twenty fields were sampled across the two years per participating state by collaborators. A standard sampling procedure was followed collecting 15 to 20 soil cores to a depth of 15 cm in a W-shaped transect across each field. Soil was mixed together to form a composite soil sample and shipped to Michigan State University for processing. Soil subsamples were taken and stored at -80ºC until they were 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, USA) with a modified protocol (Bilodeau and Robideau 2014). Briefly, 200 µL 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 µL of sodium phosphate buffer from the original kit and 122 µL of the MT buffer were added and the protocol was followed as recommended by the manufacturer. DNA was stored at -20ºC until used for amplification.

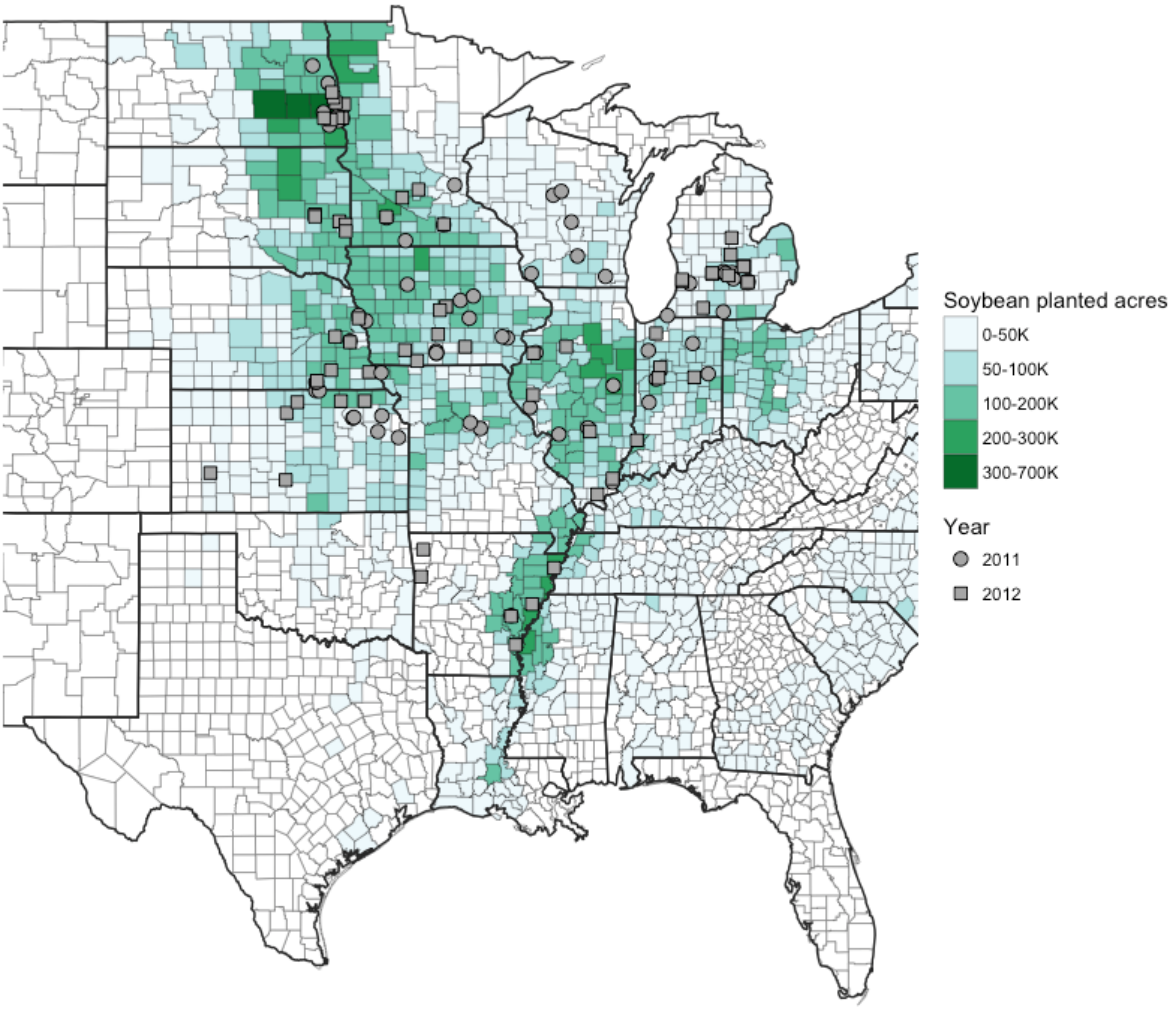

Figure 5.1. Locations of soybean fields sampled in 2011 and 2012 for amplicon community analysis. Soybean planted area designated by color intensity at county level.

Table 5.1. Primers used in this study for the amplification of oomycetes and fungi from soil samples.

Primers	Sequence	Length	Region
CS1-ITS6f[a]	ACACTGACGACATGGTTCTACA+ TAGAAGGTGAAGTCGTAACAAGG	45	Oomycete ITS1
CS2-ITS7r[a]	TACGGTAGCAGAGACTTGGTCTC+ AAGCGTTCTTCATCGATGTGC	44	Oomycete ITS1
CS1-199f_COI	ACACTGACGACATGGTTCTACA+ CCTWGGTGGTTTTGGTAAYTGG	44	Oomycete *cox*I
CS2-659r_COI	TACGGTAGCAGAGACTTGGTCT+ ACGGATCWCCTCCWCCWGAWGG	44	Oomycete *cox*I
CS1- ITS1FI2[b]	ACACTGACGACATGGTTCTACA+ GAACCWGCGGARGGATCA	40	Fungal ITS1
CS2- ITS2 [b]	TACGGTAGCAGAGACTTGGTCT+ GCTGCGTTCTTCATCGATGC	42	Fungal ITS1

[a] Modified from Cooke, D. E. et al. *Fungal Genet. Biol.* **30,** 17–32 (2000).
[b] Schmidt, P.A. *et al. Soil Biology and Biochemistry* **65,** 128–132 (2013).

Oomycete loci amplification and sequencing. The ITS1 of the rDNA and the *cox*I of the mtDNA were amplified using a two-step PCR method. Prior to the first amplification, DNA was diluted ten-fold with molecular grade water. Amplification mix consisted of 1x of Q5 reaction buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 1U of Q5 hot-start high fidelity polymerase (NEB, Ipswich, MA) and 1 µL of DNA in a total volume of 25 µL. The thermal cycling program consisted of 94ºC for 30 s, 25-30 cycles of 94ºC for 15 s, 59ºC (oomycete ITS 1) or 50ºC (oomycete *cox*I) for 30 s and 72ºC for 40 s, and final extension at 72ºC for 10 min. Primers used in this study are listed in Table 5.1, the primers were modified by adding a 2 bp pad-link and the Fluidigm CS adapters at the 5' end. Amplicons were purified by adding 5µL of a mixture of 3U of exonuclease I and 0.5U of FastAP thermosensitive alkaline phosphatase (Thermo Scientific, Waltham, MA), followed by 45 min at 37ºC, and enzymes were inactivated by incubation at 85ºC for 10 min. A non-template control and mock community containing 15 oomycete species (*Phytophthora cactorum, Phytophthora citricola, Phytophthora nicotiana,*

Phytophthora sansomeana, Phytophthora sojae, Phytopythium litorale, Pythium aff. *dissotocum, Pythium* aff. *torulosum, Pythium attrantheridium, Pythium heterothallicum, Pythium irregulare , Pythium lutarium , Pythium oopapillum, Pythium ultimum* var. *sporangiiferum, Pythium ultimum* var. *ultimum*). All species were mixed together at final concentration of 0.5 ng/µL, and the mock community was diluted 1:10 before being used for PCR.

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), using the Fluidigm CS adapters included in original primers (Table 5.1) to add the Illumina adapters and barcodes allowing the multiplexing of samples. The Research Technology Support Facility Genomics Core performed limited secondary amplification using dual barcoded Fluidigm primers. After secondary PCR, the products were normalized using Invitrogen SequalPrep DNA Normalization plates and normalized DNA products were pooled. 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 done 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.

Fungal ITS1 amplification and sequencing. An aliquot of the total DNA was submitted to the Research Technology Support Facility Genomics Core at Michigan State University for ITS1 amplicon sequencing. The ITS1 region of rDNA was amplified using a two-step PCR method, using the primers described by Schmidt et al. (2013) and listed in table 5.1. The primers were modified adding 2 bp pad link and the Fluidigm CS adapters at the 5' end to

carry out the second PCR. Secondary PCR added indexed, Illumina compatible sequences with primers which targeted the Fluidigm CS oligos. After secondary PCR, the products were normalized using Invitrogen SequalPrep DNA Normalization plates and the normalized DNA products pooled. After QC and quantitation of the pooled DNA they were loaded on an Illumina MiSeq v2 flow cell and sequenced in a PE250 format using a v2 500 cycle reagent kit. For the sequencing, index primers complementary to the Fluidigm CS oligos were added the reagent cartridge. Base calling was done 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.

Pre-processing and OTU assignment. Fastq files were preprocessed based on quality using trimmomatic (Bolger et al. 2014), eliminating sequences with length less than 150 bp, removing Illumina adapters if present. After quality processing, reads were assembled using pandaseq (Masella et al. 2012), using the simple Bayesian algorithm and setting parameters to remove primers before assembling and threshold quality of at least 0.8 for alignment of the overlapping region. Oomycete and fungal ITS1, assembled sequences were pre-processed using ITSx to extract the ITS1 region removing the adjacent 18S and 5.8S regions (Bengtsson - Palme et al. 2013). The pre-processed and assembled sequences were analyzed within Qiime 1.9.0 (Caporaso et al. 2010) using USEARCH 6.1 (Edgar 2010). The molecular operational taxonomic units (OTUs) were pick using a de novo approach clustering at 97%, filtering low abundance clusters (less than 4 members) and removing chimeras with a reference database. Taxonomy was assigned using the assing_taxonomy.py from Qiime using blast with default parameters, and reference sequences obtained from the BOLD systems (http://v4.boldsystems.org/), taxonomy reference files were constructed manually. An OTU

table was constructed adding metadata collected from all field locations to create a biom file for downstream analyses.

Environmental and edaphic metadata. Edaphic and environmental parameters of the sampled fields were acquired based on geographic information system (GIS) coordinates. Fields without this information were not included in this analyses. Soil chemical and physical properties were obtained from the National Resources Conservation Service (NRCS) soil database (http://www.nrcs.usda.gov/). Ambient temperature (maximum, minimum, and mean) and precipitation (maximum, minimum, and mean) for time ranges, including year and planting season (April, May and June), were obtained from the PRISM (Parameter-elevation Regressions on Independent Slopes Model) Climate Group (http://www.prism.oregonstate.edu/). Other parameters such as topology and images for land use, were queried from United States Geological Survey (USGS) (http://www.usgs.gov/) and National Agricultural Statistics Service (USDA NASS) (http://www.nass.usda.gov/research/Cropland/SARS1a.htm), respectively.

Community and diversity analysis. The biom files were imported in R version 3.2 (R core team 2015, Vienna, Austria) using the package 'phyloseq' (McMurdie and Holmes 2013). Estimates for α and β diversity were calculated using the 'vegan' package in R (Oksanen et al. 2013). All parameters were calculated by field, and the data was summarized by state. Fields among states were evaluated for correlation with latitude and longitude using alpha diversity measures and Spearman correlation. In order to evaluate the community structure, OTU tables were constructed and normalized as relative abundance to establish among-group diversity using Bray-Curtis distances to compare communities pairwise. The resulting dissimilarity matrices

were used to assess clustering of the communities by state, and to evaluate communities by field using principal coordinate analysis (PCoA). The environmental and edaphic metadata obtained was analyzed in conjunction with community structure and diversity data in R using the packages 'vegan' and 'MASS'. The different environmental and edaphic factors were evaluated for their associations using ordination plots, plotting the environmental factors as vectors using the 'envfit' function from vegan.

Results

Oomycete coxI and ITS sequencing and community composition. A partial fragment of cytochrome oxidase subunit 1 (*cox*I) was used as target to characterize the oomycete community present in soil samples collected from soybean fields. On average, 31,134 raw reads and after quality filtering 12,870 reads were obtained per sample. The reads were assembled resulted in 1,549,829 sequences with a mean length of 416.52 ±16 bp. The OTU picking and assignment resulted in a mean OTU number of 2043.1, the number of OTUs per state is summarized in figure 5.2a. In terms of community structure, summarizing by clade, the *cox*I marker resulted in *Pythium* clade F, clade I and clade B as the three top dominant (Figure 5.3a). These were present across the different states, but their abundances shifted across years. The three clades F, B and I accounted for 50% of the abundance present in most samples. Other groups different than *Pythium* were *Phytopythium* and *Phytophthora* clade 8, both with less than 10% abundance on average.

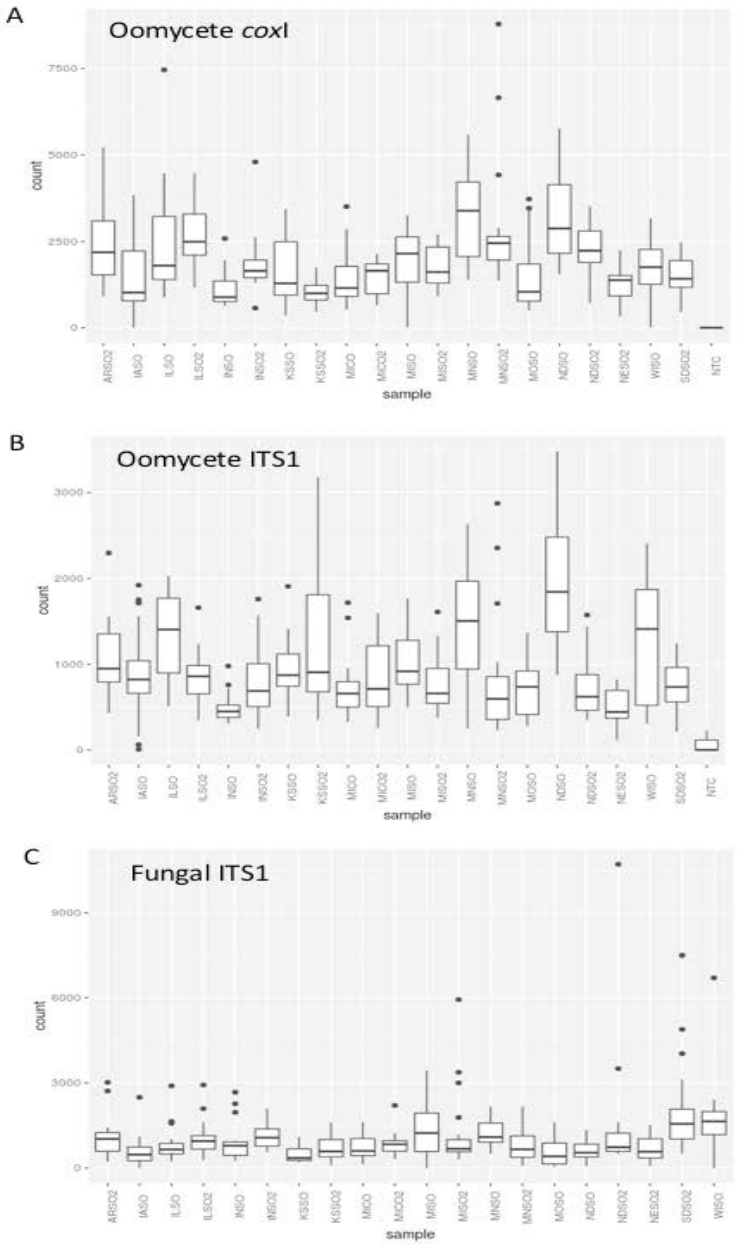

Figure 5.2. Boxplot of OTU number by state after processing samples for (a) oomycete *cox*I, (b) oomycete ITS1 and (c) Fungal ITS1. Median, quartiles and lowest and higher values are represented. Black dots represent outliers.

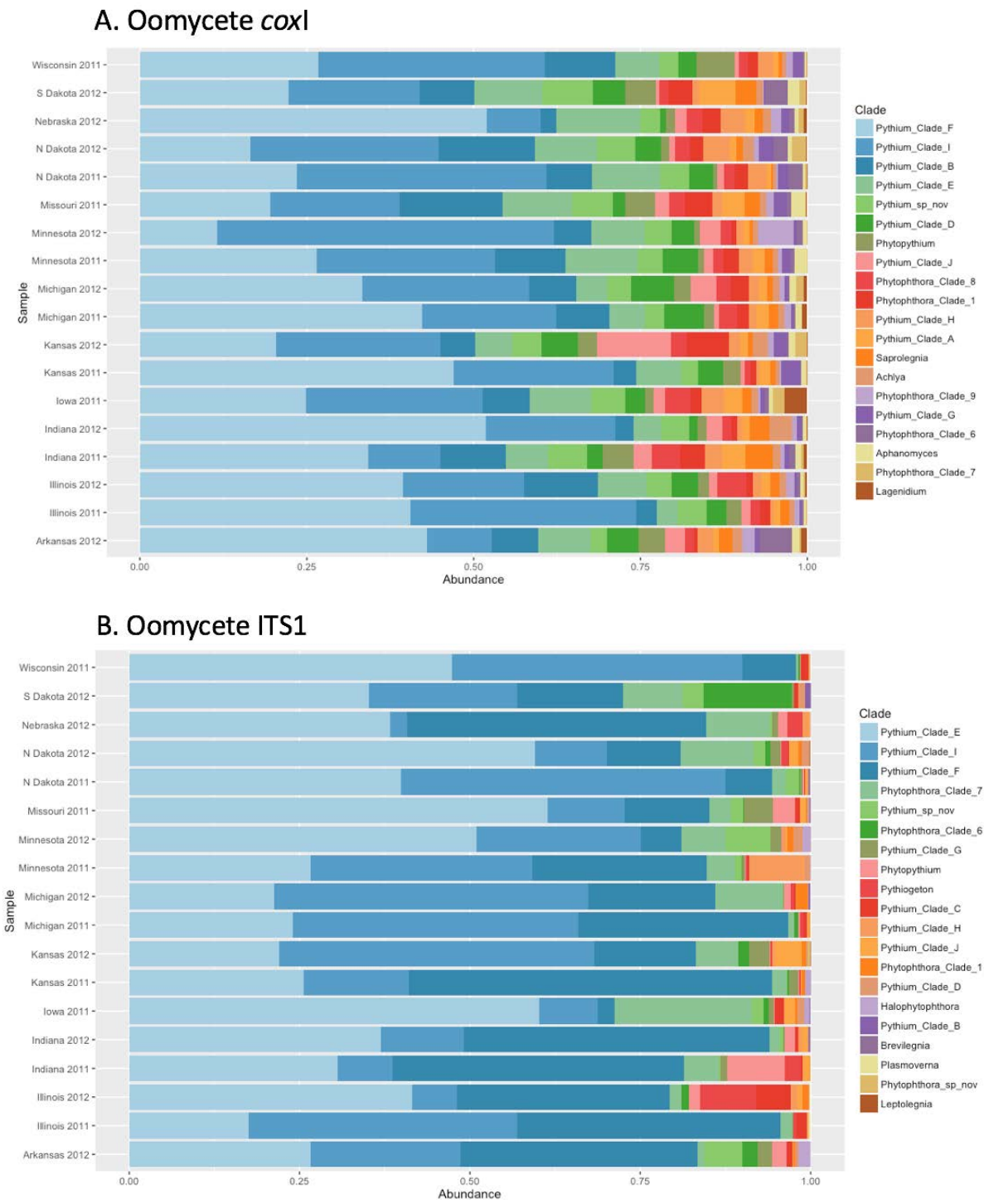

Figure 5.3. Relative abundance of the 20 most abundant OTUs for (a) oomycete *cox*I and (b) oomycete ITS1.

The ITS1 of the rDNA was also used as a target to characterize oomycete communities from soybean fields, on average prior to quality filtering the mean number of reads was 31,226 reads and after quality filtering it came down to an average of 4,040 reads. After assembling the ITS1 with pandaseq, there was a total 525,933 sequences with average length of 319.18 ± 35 bp. The obtained ITS1 sequences were processed with ITSx to remove non-oomycete ITS sequences, and also to remove partial sequences of 18S and 5.8S to improve clustering. This resulted in 359,007 sequences passing the ITSx criteria with an average length of 263.13 ± 39 bp. The OTU processing and assignment resulted in mean OTU number of 970.29 per sample, the distribution and number of OTU per state are represented in figure 5.2b. The community structure evaluated using the ITS1 marker revealed that *Pythium* was dominant genus, similar to the results obtained by *cox*I (Figure 5.3). The three dominant clades in this case were *Pythium* clade E, I and F, but in this case these three clades accounted for 75% of community composition. Other groups different from *Pythium* that were represented with marker were *Phytophthora* clade 7, which was the four most abundant group. Followed by *Phytophthora* clade 6 and *Phytopythium* (Figure 5.3b).

Evaluating the top 20 taxa across the ITS and *cox*I markers, there are differences in abundances of certain clades. For instance, *Pythium* clade E, F and I are amplified and recovered after analysis by both markers (Figure 5.4). However, their abundances are different, just *Pythium* clade E OTU identified are triplicated in *cox*I marker in comparison to the ITS marker. While, *Pythium* clade F is recovered using the ITS marker, but its abundance with the *cox*I marker is negligible. Other groups, such as *Phytophthora* and *Phytopythium* were also recovered by both markers, nonetheless their abundances were lower than 10,000 OTUs (Figure 5.4).

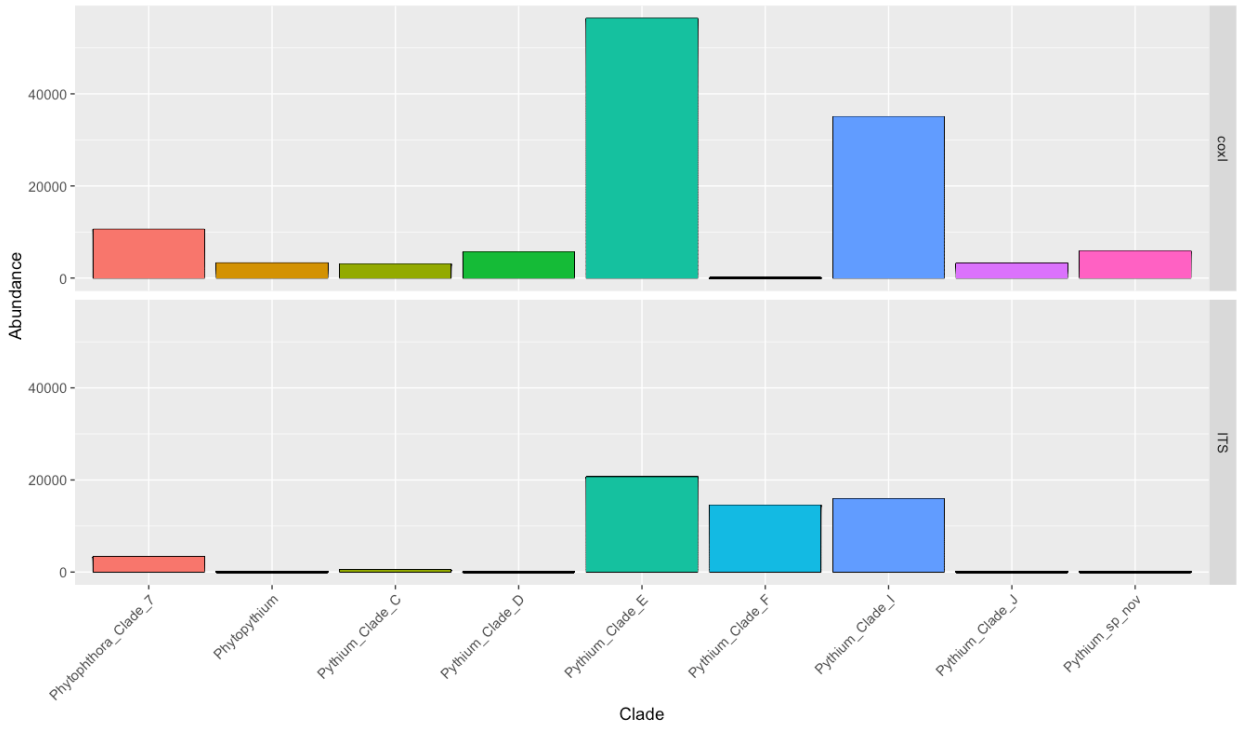

Figure 5.4. Operational taxonomic unit (OTU) abundance of the top 20 taxa summarized by clade compared across the two oomycete markers ITS1 and *cox*I.

Fungal ITS sequencing and community composition. The fungal community was evaluated by sequencing the ITS1 of the rDNA. The number of reads obtained on average per sample were around 42,857 reads, which was later reduced to 3,165 after quality filtering. Sequences were assembled with different parameters from the oomycete ITS1 since it is a shorter fragment, allowing a 60 bp overlap and an maximum length of 480 bp. After assembly, 367,966 sequences with an average length of 323.26 ± 33 bp were obtained and filtered through the ITSx software. The number of sequences passing the criteria were 357,846 with average length of 293.99 ± 33 bp. The same script produces a file containing only the ITS1 spacer. The resulting file was cluster, de-replicated and references sequences were assigned to a taxonomy using blast. The average number of OTUs per sample was 1016.4, a break down by state is presented in figure 5.2c

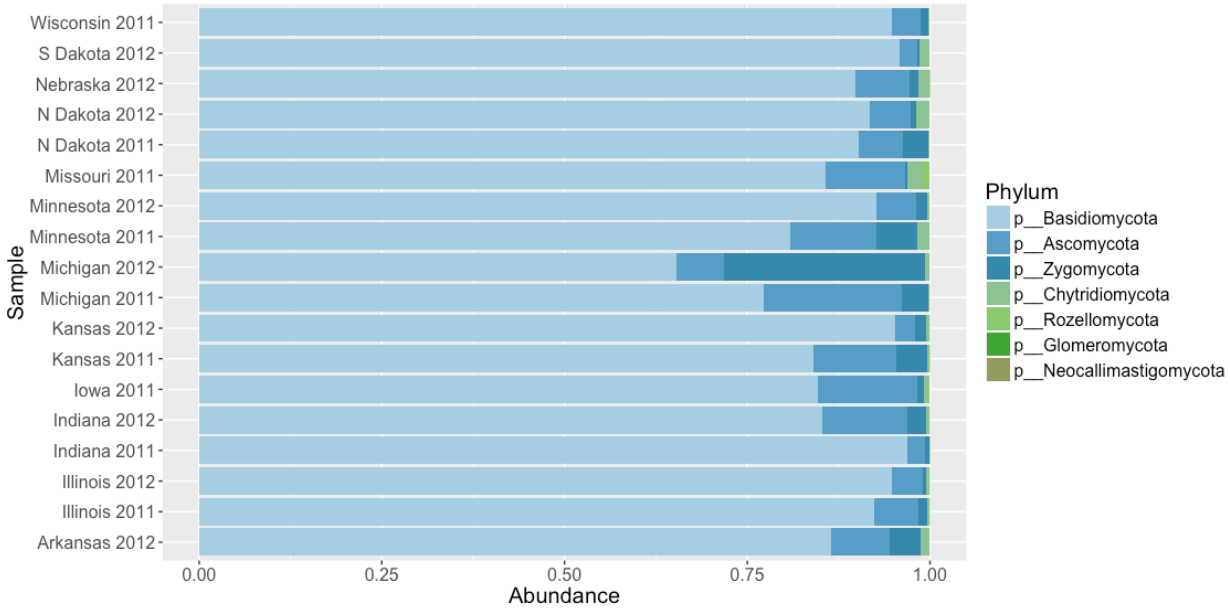

Figure 5.5. Relative abundance of OTUs summarized by fungal phyla from soybean soil samples across the U.S.

The community structure by fungal phyla found using the ITS1 showed that the phylum Basidiomycota is the most common across all the samples evaluated (Figure 5.5), accounting for more than 75% of the abundance of the community. The second most abundant phylum was the Ascomycota with abundances between 5% to 20% and third most abundant phyla was the Zygomycota that overall was below 10% on average, but only in Michigan in 2012, its abundance increased above 10%. Other phyla represented include Chytridomycota and Glomeromycota. Focusing on the three top phyla, the abundance of the top 50 taxa was evaluated summarizing by family, 13 families were represented in the Ascomycota (Figure 5.6). The most abundant family was Pezizaceae, follow unidentified and novel species, including *Trichotecium* sp, *Myrothecium* sp. and *Scolecobasidium* sp. Hypocreaceae was also present but it was a relatively low abundance in comparison to the major families. With respect to the Basidiomycota, 14 families were identified in top 50 taxa. Unidentified basidiomycota was the

most abundant group, followed by the two most abundant recognized families that were Bolbitiaceae and Agaricaceae (Figure 5.6b). Finally, the phylum Zygomycota was represented by 8 families, being the family Mucoraceae the most abundant by far in comparison to the rest of the families (Figure 5.6c). Other families represented were Kickxellaceae and Mortierellaceae with abundance around the 1000 OTUs.

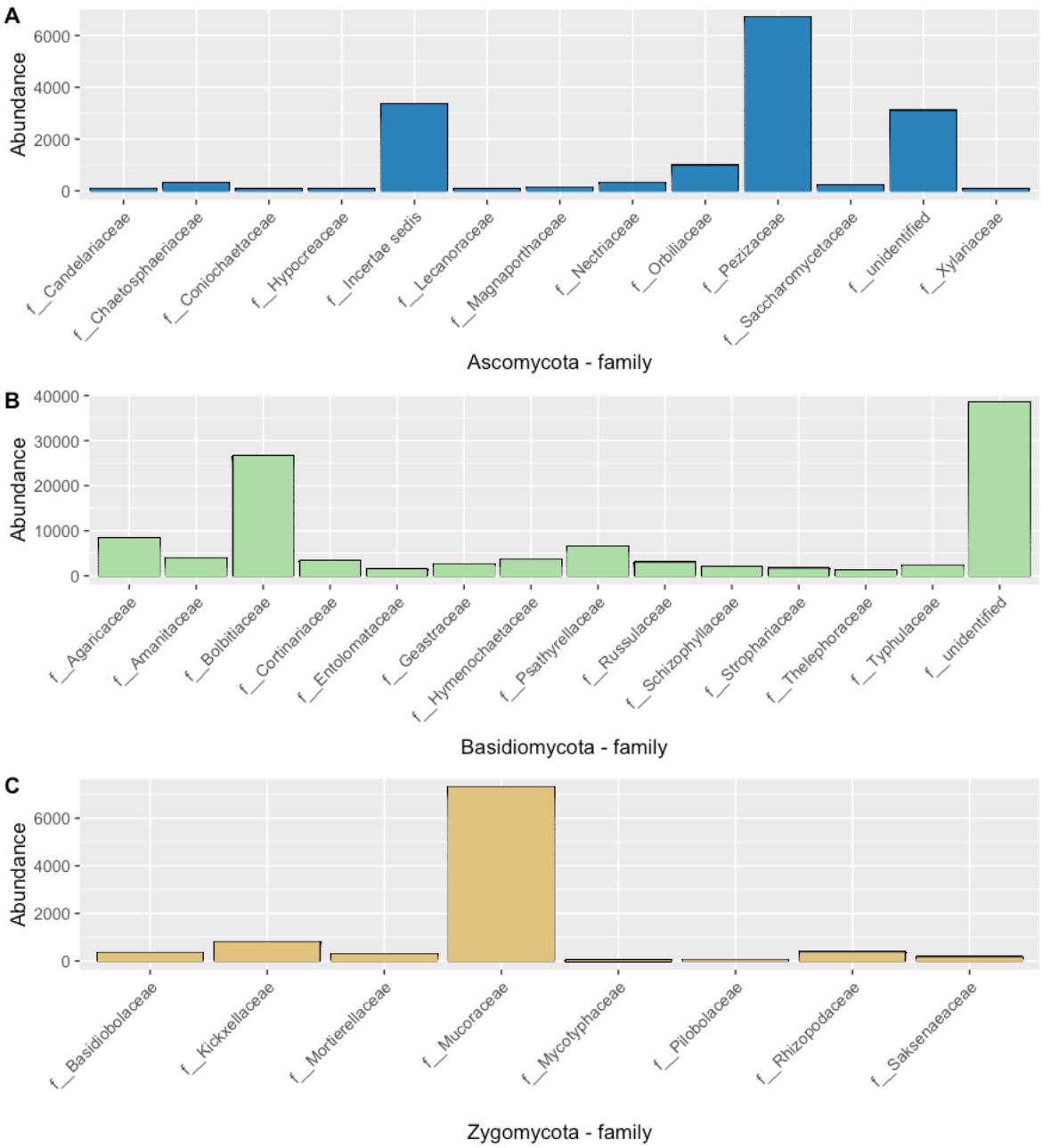

Figure 5.6. Operational taxonomic unit (OTU) abundance of the top 50 taxa summarized by fungal family of the top three phyla: (a) Ascomycota, (b) Basidiomycota and (c) Zygomycota.

Community α diversity across soybean fields in the U.S. The within-group diversity of the oomycete communities across the different states and years had similar magnitudes, the number of OTUs ranged from 241 to 425 (Table 5.2). The Shannon diversity index indicated the

diversity levels ranged from 3.80 to 4.82 and the Simpson index also indicated a similar trend with minimal variability of diversity at the state level. The evenness of the community, that measures how is the different OTUs are represented within the samples, indicated that most communities have a high degree of evenness being 1.0 the maximum (Table 5.2). This indicates that samples have OTUs represented evenly and there was not strong dominance of single OTUs at the state level.

A similar trend was observed for fungal communities, where the number of observed OTUs ranged from 148 to 295 (Table 5.3). The Shannon diversity index in this case ranged from 3.87 to 5.12, which was more variable than the diversity observed with the oomycetes. However, evenness for the fungal communities was at similar levels with respect to the oomycete communities. Interestingly, Indiana was the state with the highest diversity for both fungal and oomycete communities, but the lowest diversity for fungal communities was Missouri and for oomycete communities was Minnesota (Table 5.2 and 5.3).

Table 5.2. Oomycete community diversity and evenness by state and year. Data represents state-year average and standard deviation of soybean fields.

State/Year	Fields sampled	Observed OTUs[a]		Shannon-Wiener index		Simpson index		Evenness[d]
		Mean[b]	SD[c]	Mean	SD	Mean	SD	
Arkansas 2012	6	297.67	47.18	4.15	0.20	0.93	0.04	0.73
Illinois 2011	6	436.83	39.65	4.56	0.32	0.94	0.03	0.75
Illinois 2012	6	424.83	39.92	4.64	0.23	0.96	0.02	0.77
Indiana 2011	5	390.60	58.44	4.82	0.23	0.97	0.01	0.81
Indiana 2012	6	377.50	47.25	4.36	0.38	0.93	0.05	0.74
Iowa 2011	9	395.22	96.21	4.58	0.29	0.95	0.02	0.77
Kansas 2011	7	290.29	114.03	3.95	0.44	0.92	0.03	0.70
Kansas 2012	6	244.17	42.56	4.05	0.28	0.93	0.05	0.74
Michigan 2011	12	353.92	57.74	4.44	0.23	0.95	0.02	0.76
Michigan 2012	12	425.25	45.86	4.80	0.25	0.97	0.01	0.79
Minnesota 2011	6	306.00	32.53	4.13	0.21	0.93	0.03	0.72
Minnesota 2012	6	294.83	59.60	3.80	0.41	0.88	0.05	0.67
Missouri 2011	4	276.00	53.30	4.36	0.25	0.96	0.02	0.78
N Dakota 2011	6	388.00	46.25	4.38	0.15	0.94	0.02	0.74
N Dakota 2012	6	286.33	60.18	4.13	0.27	0.94	0.02	0.73
Nebraska 2012	6	241.50	84.23	3.89	0.40	0.92	0.05	0.72
S Dakota 2012	6	242.50	97.08	4.15	0.38	0.96	0.01	0.76
Wisconsin 2011	6	329.83	82.25	4.34	0.25	0.95	0.02	0.75

[a] OTU = Operational Taxonomic Unit defined at the 97% threshold.
[b] Mean across fields sampled for the corresponding state and year.
[c] Standard deviation for fields sampled for the corresponding state and year.
[d] Pielou's evenness: Shannon-Wiener diversity index divided by the natural logarithm of total species in a sample.

Table 5.3. Fungal community diversity and evenness by state and year. Data represents state-year average and standard deviation of soybean fields.

State/Year	Fields sampled	Observed OTUs[a]		Shannon-Wiener index		Simpson index		Evenness[d]
		Mean[b]	SD[c]	Mean	SD	Mean	SD	
Arkansas 2012	6	213.67	45.64	4.59	0.38	0.97	0.02	0.86
Illinois 2011	6	229.67	48.38	4.4	0.86	0.92	0.11	0.81
Illinois 2012	6	248.67	52.07	4.26	0.79	0.91	0.1	0.77
Indiana 2011	5	212.8	63.95	4.41	1.21	0.93	0.11	0.82
Indiana 2012	6	295.83	27.95	5.12	0.3	0.98	0.02	0.9
Iowa 2011	7	198.57	61.24	4.4	0.67	0.96	0.03	0.84
Kansas 2011	7	148.57	78.82	3.99	0.9	0.94	0.05	0.81
Kansas 2012	6	213.5	58.94	4.34	0.98	0.92	0.1	0.81
Michigan 2011	12	231.75	56.62	4.43	0.77	0.93	0.1	0.82
Michigan 2012	12	270.17	85.44	4.58	1.35	0.9	0.19	0.81
Minnesota 2011	6	225	33.94	4.55	0.5	0.96	0.04	0.84
Minnesota 2012	6	228	26.71	4.65	0.66	0.95	0.06	0.86
Missouri 2011	3	155.67	70.68	3.87	0.97	0.92	0.06	0.77
N Dakota 2011	6	234.17	56.76	4.77	0.37	0.98	0.02	0.88
N Dakota 2012	6	199	79.65	4.32	0.96	0.95	0.07	0.82
Nebraska 2012	6	180.17	83.82	4.06	0.92	0.92	0.1	0.8
S Dakota 2012	6	187.5	68.87	4.27	1.05	0.93	0.13	0.82
Wisconsin 2011	5	247.4	64.55	4.52	1.12	0.93	0.12	0.82

[a] OTU = Operational Taxonomic Unit defined at the 97% threshold.
[b] Mean across fields sampled for the corresponding state and year.
[c] Standard deviation for fields sampled for the corresponding state and year.
[d] Pielou's evenness: Shannon-Wiener diversity index divided by the natural logarithm of total species in a sample.

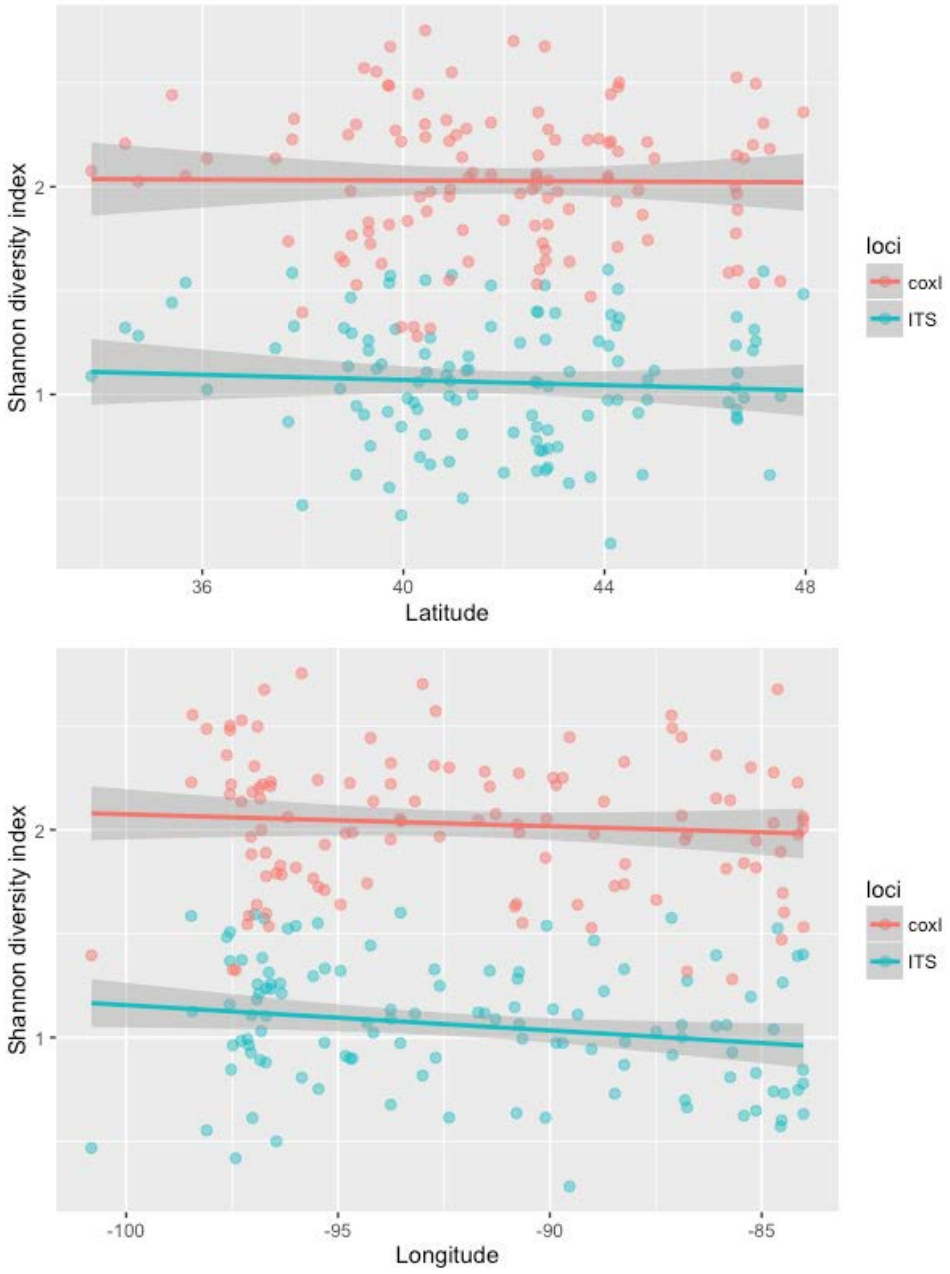

Figure 5.7. Diversity of oomycete communities in bulked soil samples expressed as Shannon diversity index across (a) latitude and (b) longitude of samples collected from soybean fields in the U.S. Both coxI and ITS are presented.

Using diversity expressed as Shannon diversity on oomycete communities obtained from both ITS and *cox*I markers was plotted against latitude and longitude (Figure 5.7). Since diversity observed across different states was of similar magnitudes, there was not a significant

correlation with respect to latitude. On the other hand, longitude is negatively correlated with diversity, which means that at lower longitudes there is a reduce diversity, however, this effect was significant only for the communities obtained with ITS marker, but not for those obtained with *cox*I marker.

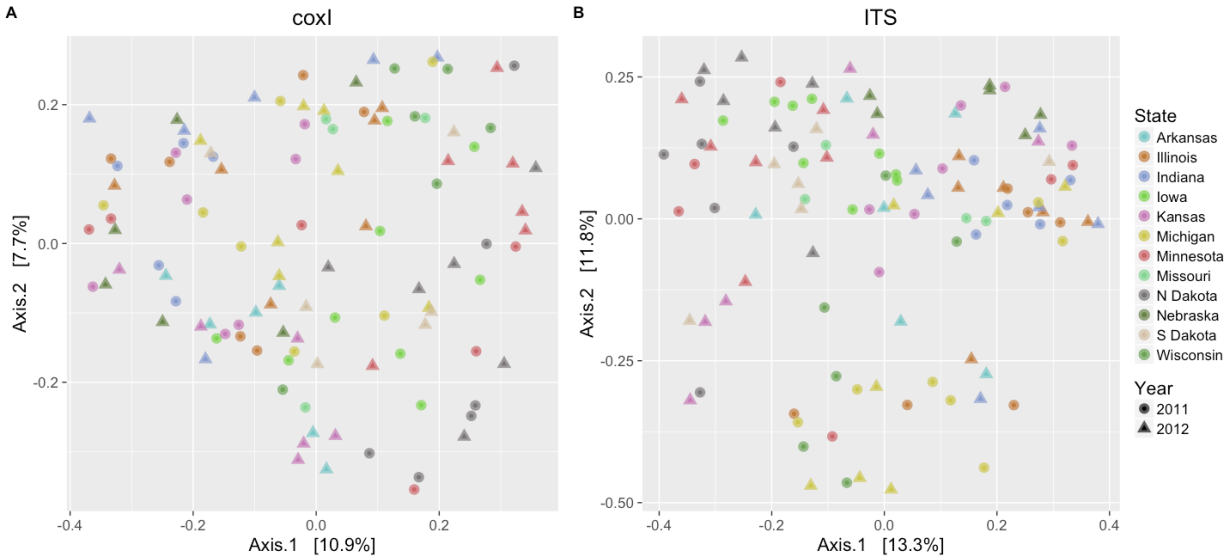

Figure 5.8. Principal coordinate analysis (PCoA) of oomycete communities collected from soils collected from soybean fields in the US using (a) *cox*I marker and (b) ITS1 marker. Bray-Curtis distance was computed from taxon counts.

Community β diversity across soybean fields in the U.S. The among-group diversity was evaluated using Bray-Curtis distances and principal coordinate analysis. The axes for *cox*I explained 10.9% and 7.7% of the variability, respectively (Figure 5.8). While ITS showed a little more variability, it was still low, 13.3% and 11.8%. There is not clear clustering of the communities on either marker. Therefore, environmental data was fitted using 'envfit' function

of the vegan package, and it demonstrated that latitude, longitude and temperature correlate with the horizontal axis (Figure 5.9), which means that there is an effect of both parameters in the community composition. With respect to the vertical axis, longitude and precipitation are also correlated with principal coordinate axes.

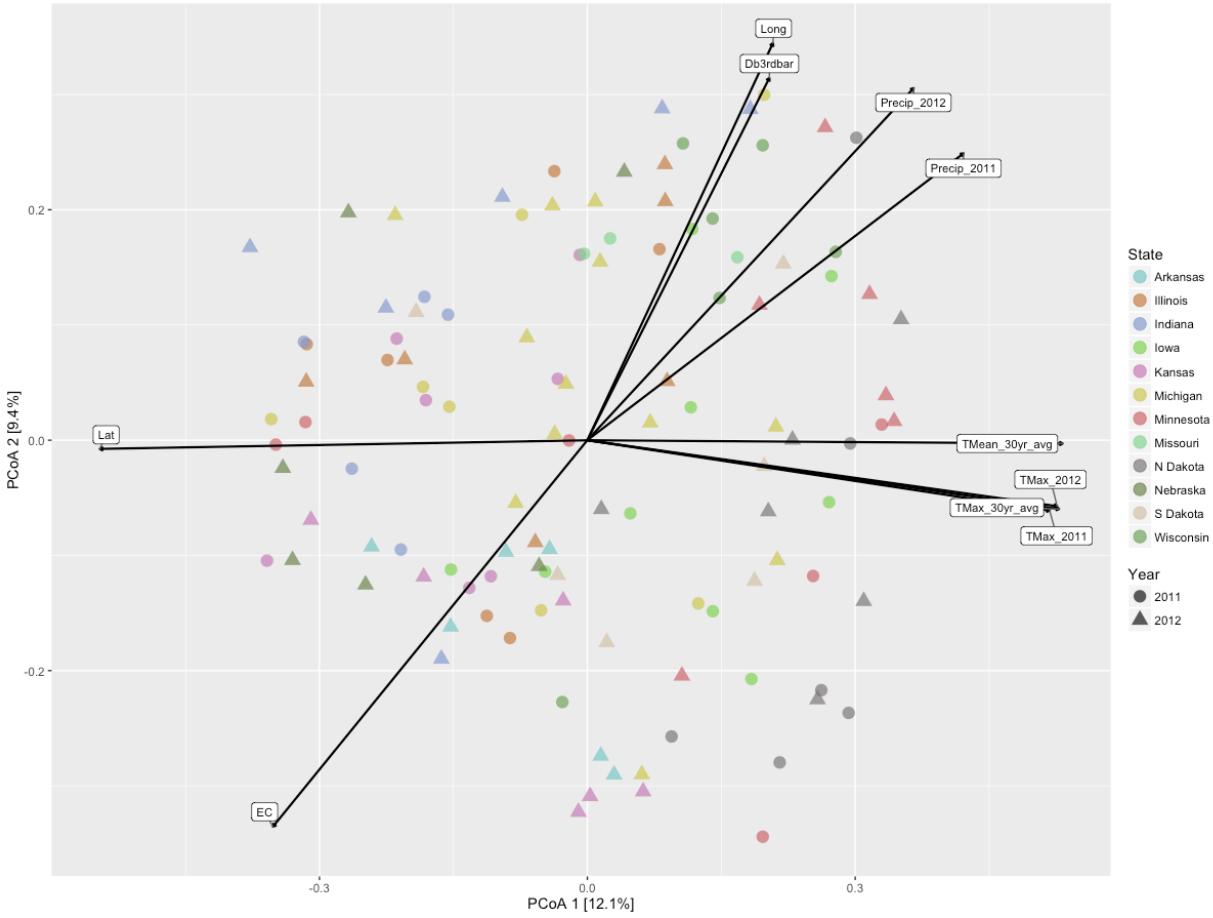

Figure 5.9. Principal coordinate analysis (PCoA) of oomycete communities obtained from bulked soils collected from soybean fields in the US. Distances were computed using Bray-Curtis distance from taxon counts. Environmental factors are plotted as vectors based on correlations with community distance.

Discussion

The present study is one of the first approaches to study oomycete community ecology in agricultural ecosystems using illumina amplicon sequencing. The communities represent the soybean belt, where most of the soybeans are produced in the U.S. In previous study, we found that oomycete communities in from symptomatic soybean seedlings are dominated by *Pythium* species and very few members of other oomycete species were found. The present approach allowed the study in depth of these communities and revealed that these communities are still dominated by *Pythium* species, but the diversity is in terms of different genera is greater than the one observed through culturing. *Phytophthora* clades were represented across the different sample in greater number than observed before (Rojas et al. 2016), suggesting those species are present in most fields, but special conditions are required to induce plant infection or to achieve isolation. This is the case of *Phytophthora sojae*, which was present in the communities from different states, but previously in culture-based approach was present in limited numbers. A recent study revealed that using a bait approach and saturating the soil water capacity for extended periods of time results in the isolation of *Ph. sojae* from most soil samples.

The current approach using amplicon-based community analysis provides a powerful method to characterized oomycete communities from different ecosystems. In the current study, two different markers were used in the same set of samples, and it was conspicuous that there are biases on the amplification of different species. In fact both markers favor *Pythium* species, but the *cox*I marker is able to capture a bigger diversity than the ITS marker. One of the issues with the ITS marker is the length variability across different species (Thines et al. 2005, Robideau et al. 2011), this could favor species that have a shorter ITS because of the sequencing technology. In addition, the presence of hybrid species in the samples could also raise issues when processing

the reads on two different steps, the red assembly and chimera filtering (Schloss et al. 2011). Nonetheless, the use of comprehensive databases with curated sequences could help to improve some of these issues. The biases of primers is common issue not only oomycetes but also in fungi and bacteria (Kennedy et al. 2014, Tedersoo et al. 2015). However, most of the ecological signals were similar for both markers despite their different OTU composition, but it will be required to go further on the analysis of the potential biases of these markers using artificial communities.

With respect to the fungal community results, most of the community was dominated by the phylum Basidiomycota and this could be also due to primer biases, since it has been reported that the primers used in the current study tend to amplify members of this phylum primarily than species from the rest of the fungal phyla. Nonetheless, it has been reported that Basidiomycota are quite abundant on agricultural soils (Kjøller and Rosendahl 2014, Detheridge et al. 2016). It was reported that the user of cover crops increases the diversity of the dungal community and increases the number of members of the Basiodiomycota. The diversity of Ascomycota observed did not revealed strong presence of plant pathogenic fungi, however, families that contain some of the major plant pathogens of soybean were present. This is the case of the hypocreales, which contains the *Fusarium* spp. (Arias et al. 2013, Wang et al. 2014). In the Zygomycota, Mucoraceae and Mortierallaceae were present and those are commonly found associated with soybean plants when using a culture-based approach (Rojas et al. 2016). The Mortierallaceae is an special case since this group zygomycetes is widely found and it establishes interactions with plants enhancing plant growth (Detheridge et al. 2016).

Similar results were obtained in terms of community structure to our previous approach using a culture-based method, however the use of amplicon based technology reduces the cost of

the study and increases the sampling power to evaluate other systems. This amplicon based method can be used as proxy to detect the dominant species present in agricultural fields and using the information device strategies to culture specific organisms of interest, as it has been done with other systems (Bonito et al. 2016). The present study also provides a powerful tool that can be used to study more specific questions, such as the effect of different hosts or fungicides in the oomycete community associated with fields crops or other plants. By using the information on diversity and amplicon-based approaches, we hope that ecology approaches could be implemented to understand the epidemiology of multiple species associated with a host, like it is often the case on root rot diseases and soilborne pathogens

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Chapter 6

Conclusions and Impacts

Conclusion and Impacts

Soybean is the second most important crop after corn in the United States, and there are 76 million acres planted with soybean, but often growers have to deal with seedling diseases that caused damping off and plant stand issues. These issues often result in losses or replanting, which is already a large percentage of the cost input from growers. For instance, just seed and chemicals are 40% percent of the costs, that on average results in $87 dollars per acre.

The seedling diseases are caused by microbes that live in the soil, these are mainly fungi and fungi-like organisms known as oomycetes. All of them survive in the soil and have the potential to infect the soybean plants at the root level. In the specific case of the oomycetes, there are two main genera *Pythium* and *Phytophthora,* but we do not know the range of different species that could cause disease on the soybean seedlings across different states. Therefore, my research focused identifying and characterizing oomycete species collected from multiple fields across 11 states that comprise most of the soybean production in the US. We identified 84 different species, of these 17 species caused seed rot and 43 caused seedling root rot. We wanted to go further due to large area sampled and we used an ecological approach to understand distribution and abundance of the oomycete species found. The goal was to incorporate environmental data, such as temperature and precipitation, with soil parameters, such as clay content, water content, organic matter, in order to determine factors that could explain the distribution of the oomycete species.

By using this ecological approach, it was possible to identify that nearby states have similar species present on the fields, and that latitude, which is associated with temperature and precipitation are also correlated with the abundance of the species. This provides a major result, since it suggests that management decisions can be taken at the state level. In addition, we

found the recognized pathogens like *P. sojae* that are very aggressive were not abundant at seedling stage, but still present at low frequencies. However, *Pythium* is the dominant genus causing disease, and other species are also present and virulent like other *Phytophthora* and *Phytopythium*.

Based on the results, *P. sojae* and *P. sansomeana* were identified as aggressive species and diagnostic assays were developed to detect these pathogens as early as possible to provide management recommendations. The diagnostic assays were developed using a hierarchical approach, which means it establishes if the infection is cause by *Phytophthora,* followed by a detection of the two species. Two approaches were developed a qPCR and isothermal amplification. The isothermal amplification can be done in the field and a result can be obtained in 25 minutes.

Nonetheless it is known that multiple species can interact with root tissue at the same time, therefore a different approach was developed to detect multiple species in one sample. This is based on the extraction of DNA from plant or soil samples. This DNA is use as a template to amplify specific markers for oomycetes and characterize a complete community from a sample. This technology allows us to do characterization of communities in culture independent manner, providing enough power to detect multiple species with enough depth to detect differences. This approach is important to study the effect of different cultivars, seed treatments, or other management practices. The goal is to understand better the role of different factors on the abundance and distribution of different species, linking this information with knowledge that has been produced on differential virulence at different plant stages to device new strategies to control pathogens. All of this with ultimate goal in mind of enhancing plant health and increasing crop yield.