EFFECT OF SOYBEAN SEED TREATMENTS ON OOMYCETE EVOLUTION AND DIVERSITY FOR IMPROVED SEEDLING DISEASE MANAGEMENT

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Pathology–Doctor of Philosophy
Ecology, Evolutionary Biology and Behavior–Dual Major

2019

ABSTRACT

EFFECT OF SOYBEAN SEED TREATMENTS ON OOMYCETE EVOLUTION AND DIVERSITY FOR IMPROVED SEEDLING DISEASE MANAGEMENT

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Soybean (*Glycine max* L.) is the second most important crop in the United States. Soil conservation efforts combined with earlier planting dates has led to increased crop residue and cooler soil at planting. This exposes seeds and developing seedlings to adverse conditions for extended periods of time, which can increase disease pressure from many oomycete pathogens causing pre- or post-emergence damping-off. In North America, at least 84 oomycete species within the genera *Pythium*, *Phytophthora*, *Phytopythium*, and *Aphanomyces* are associated with soybean seedlings. The number of oomycete species makes management decisions difficult and seed applied anti-oomycete chemicals (oomicides) are the primary management tool against the majority of these species. Therefore, the overall theme of this dissertation was to determine the effect of soybean seed treatments on oomycete evolution and diversity to improve management recommendations.

First, in chapter 1, I provide a review of current literature and background information on soybean seedling disease management, fungicide (including oomicide) resistance theory, Peronosporalean taxonomy and evolution, and review current methods to study oomycete diversity. Secondly, since an essential step in monitoring for oomicide resistance is *in vitro* testing, I provide clarification of terms and models involved in the analysis of *in vitro* dose-response data for improved reproducibility (chapter 2). Next, in chapter 3, the level of interspecific variation in mefenoxam and ethaboxam sensitivity was determined using a newly developed high-throughput assay for oomycetes that utilized growth curves and Z'-factor for

quality control. This revealed that that interspecific variation in sensitivity to ethaboxam was greater than mefenoxam. Therefore, in chapter 4, the genetic and evolutionary mechanism of ethaboxam insensitivity was investigated. This revealed for the first time that inherent insensitivity to ethaboxam was linked to the convergent evolution of a specific substitution in the target gene, which resulted in lineage-specific insensitivity to ethaboxam. In chapter 5, effect of location, and seed treatments containing either mefenoxam or ethaboxam and metalaxyl on the recovery of oomycetes from soybean taproot or lateral root tissue. This study demonstrated that oomycete communities were largely structured by location and that the recovery oomycetes from soybeans was dependent on the unique combination of location, tissue, and seed treatment. Finally, in chapter 6, an oomycete metabarcoding approach (amplicon sequencing) was used to study the influence of soybean seed treatment and genotype on oomycete rhizosphere diversity from a location with or without a history of seedling disease. This indicated that oomycete community diversity was driven by location and that an imbalance of oomycete taxa rather than simply the presence-absence of certain taxa might be responsible for differences in disease pressure. Additionally, there was no substantial evidence that genotype or seed treatment influenced oomycete diversity in soybean rhizosphere samples.

Finally, in chapter 7, I discuss the overall conclusions and impacts of the studies presented herein. Overall, data from these studies provide essential new information for the management of oomycete communities with soybean seed treatments. Importantly, these studies advance our knowledge about the effect of soybean seed treatments on the evolution and diversity of oomycetes in a soybean agroecosystem.

To mom and dad.

Mom, you taught me patience, to follow my dreams, and to not grow up too quickly.

Dad, you taught me common sense, hard work, and perspective.

Most importantly, you both taught me love, humility, kindness, generosity, to never quit, to take pride in everything I did, to believe in myself, and that it was acceptable to fail gracefully.

To my wife.

You have been a constant source of love and support, and I look forward to our many future adventures together.

Thank you.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my PhD advisor Dr. Martin Chilvers for pushing me outside of my comfort zone, for forcing me to think critically, but most importantly believing in me when it seemed others did not. You fostered an environment where productivity and intellectual freedom join. Secondly, I would like to thank my committee: Dr. George Sundin, Dr. Gregory Bonito, and Dr. Jim Smith. All three committee members have challenged me to think beyond my project with course content, discussions, feedback, and advice. Without George, I probably would have never come to Michigan State University or met Marty. Greg has been an instrumental influence on the way that I think about microbial ecology. Lastly, Jim has been an exceptional teacher, mentor, and colleague. I aspire to teach like Jim someday.

I would also like to thank USDA National Institute for Food and Agriculture, North Central Soybean Research Program, United Soybean Board, Michigan Soybean Promotion Committee, Valent U.S.A., and scholarships that enabled me to conduct research and attend scientific meetings.

I would also like to thank previous and current Chilvers lab members especially Mitch Roth for being someone to continually discuss ideas (good or bad, professional or personal) and being a good friend throughout graduate school. Additionally, I would like to thank Janette Jacobs and Adam Byrne for help getting me up to speed in the lab and field.

Lastly, I would like to thank my friends and my entire family, but especially my parents, my sister, and my wife for being a constant source of love and encouragement throughout this process.

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Chapter 1

Literature Review

Chemical management of oomycete communities from a field crop's perspective

This chapter is written as a review of the current literature within chemical management of oomycetes associated with soybean. Though some parts of this review were gathered from oomycetes outside of soybean, this review will have a focus on oomycetes associated with soybean. This chapter is split into five parts that review (1) an introduction to management of oomycete seed and seedling diseases, (2) background on fungicide resistance evolution, monitoring, and management, (3) the extent of resistance in soybean associated oomycetes, (4) Peronosporalean evolution and taxonomy, and (5) using metabarcoding to study oomycetes and perspectives on the effect of seed treatments on oomycete ecology.

Introduction to management of soybean oomycete seedling diseases

Soybean (*Glycine max* L.) accounts for a large portion of U.S. agricultural production, with production values over $30 billion every year since 2008. Soybean acreage planted has increased in the past two decades with about 75 million acres planted in 2000 increasing to almost 90 million acres in 2017 and 2018 (USDA-NASS). For comparison, the land acreage planted to soybean in 2018 was almost as large as the land area of Montana, the fourth largest state in the U.S. with 94 million acres.

Soybean production primarily occurs in the Midwest, which consistently plants about 80% of the soybeans in the U.S. In the past forty years soybean production has changed due in part to growers primarily purchasing instead of saving seed in the late 1980s and the release of glyphosate resistant soybeans in 1996 (Fernandez-Cornejo 2004). This makes seed one of the most important operating costs for a soybean grower. For example, the national average operating cost in the U.S. in 2016 was $131.90 per acre and the cost of seed accounted for 44.5% of that operating cost (Meade et al. 2016). Therefore, protection of seeds and developing

seedlings from pests and pathogens is essential. However, changes in soybean production practices in the past twenty years such as conservation tillage and earlier planting dates can create unique plant disease challenges in soybean production.

Papendick and Elliott (1983) recognized the challenge for plant disease management as more growers would begin using "conservation-tillage" (i.e., reduced or no-tillage) to maintain soil quality and reduce soil erosion. Indeed, soil conservation has contributed to the adoption of minimum or no-till operations, which increases the crop debris left over from the previous season and increases water retention in the soil. This organic debris can act as a reservoir that can harbor inoculum for many soil-borne plant pathogens (Pankhurst et al. 1995). Additionally, growers are planting earlier, sometimes starting in mid to late April, when soil temperatures are still cool (i.e., less than the recommended 13ºC) to increase growing days and yield potential (Vossenkemper et al. 2015). As a result, soybean seeds and seedlings can be exposed to cool moist conditions for extended periods of time. Consequently, there has been an increase in the incidence of soybean establishment (number of germinated and emerged seedlings) issues due to seedling diseases (Koenning and Wrather 2010).

These changes to production practices along with the increased cost in seed was met with a dramatic surge in the usage of seed treatments in the past twenty-five years to protect soybean seed against pests and pathogens. Based on industry estimates, fungicide use on soybean seed increased from 8% in 1996 to over 30% in 2008, and by 2013 over 70% of soybeans contained a seed-applied fungicide, insecticide, or nematicide (Munkvold 2009; Gaspar et al. 2014). Based on simulated data, about 80% of corn and 44% of soybean hectares planted in 2011 contained a neonicotinoid insecticide (Douglas and Tooker, 2015). In Brazil, over 90% of soybeans are treated with a fungicide (Campo et al. 2009).

The fungicidal component of seed treatments protects seeds and developing seedlings from soil-borne fungal and fungal-like (oomycete) plant pathogens. Note that for simplicity the term fungicide is used to refer to anti-oomycete chemicals (i.e., oomicides) (Govers 2009). Fungal organisms causing seed or seedling diseases primarily includes *Rhizoctonia solani* and *Fusarium* spp. However, for this dissertation I will be concerned with only oomycete organisms. Oomycetes are a group of fungal-like organisms that favored by moist conditions often experienced early in the growing season. Oomycetes require water for infection since they contain motile propagules called zoospores that swim via flagella chemotactically toward seed and root exudates where they encyst (lose the flagella) form a germ tube and infect the host plant. Infection can occur rapidly. For example, *Pythium ultimum* sporangia can germinate in the spermosphere (environment directly surrounding a germinating seed) within one to four hours following exposure to germinating seeds (Nelson 2004). Seeds may rot before emergence (pre-emergence damping-off) while infection of root and stem tissue (post-emergence damping-off) may kill the seedling or reduce root health and plant vigor. Because oomycete infection does not always result in plant death the yield loss associated with oomycete infection can be difficult to quantify (Martin 2009; LéVesque 2011).

Upon infection, oomycetes grow within the plant, and sporangia can develop causing secondary infection from direct germination or the formation of zoospores. Alternatively, oospores can be formed. Oospores (sexual propagules) and chlamydospores can persist in the soil or on crop residue for many years. Sporangia typically develop from oospores and release zoospores (asexual propagules), but direct germination of oospores and sporangia is observed in some *Pythium* spp. (Martin and Loper 1999). Oospores are formed through fertilization of an

oogonium with an antheridium. Meiosis, karyogamy, and formation of a mature oospore completes an oomycete life cycle (Fig. 1.1).

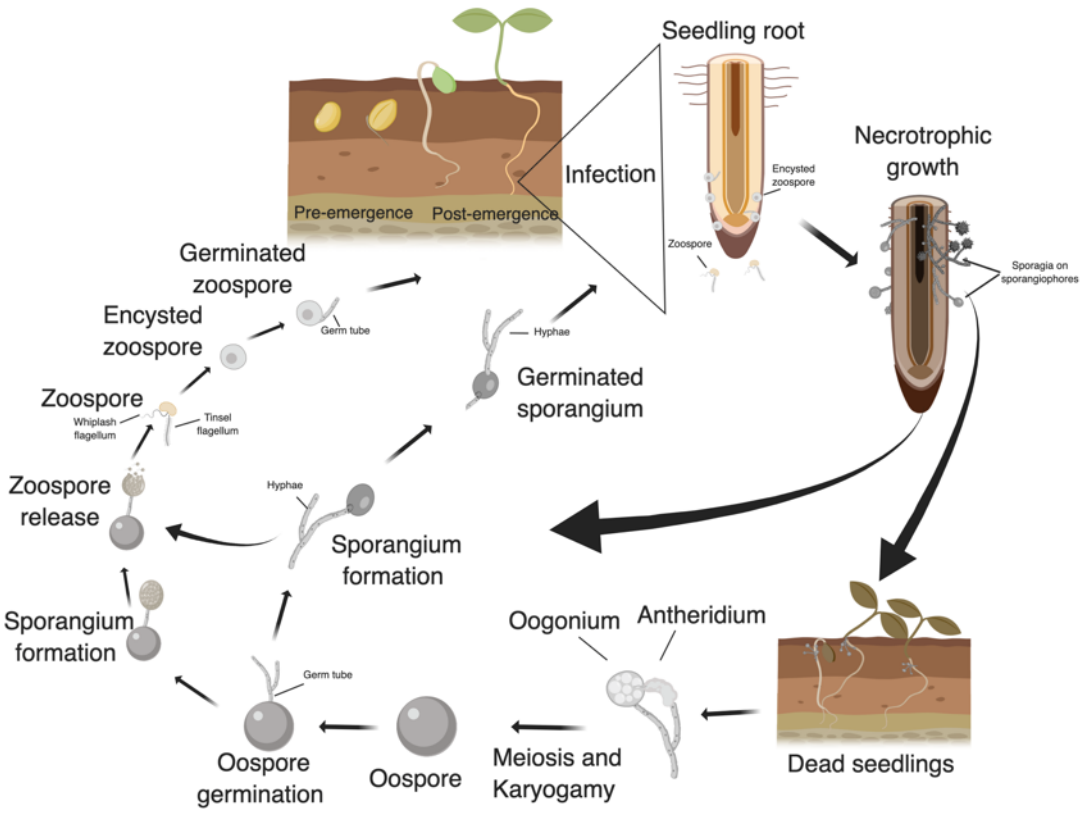

Figure 1.1. A general life cycle for soybean associated oomycetes.

Members of the four oomycete genera *Pythium*, *Phytophthora*, *Phytopythium*, and *Aphanomyces* are associated with seedling root and stem rots of many plant species including soybean (Broders et al. 2007; Broders et al. 2009; Rojas et al. 2017a, b; Zitnick-Anderson and Nelson 2015). Of the 84-oomycete species currently known to be associated with soybeans in North America, 43 were found to be pathogenic on soybean seeds or seedlings (Rojas et al. 2017a). The number of potential pathogenic species presents an enormous challenge for plant pathologists since species vary in pathogenicity and virulence, and community composition may

be influenced by location, temperature, and edaphic factors (Broders et al. 2009; Rojas et al. 2017b). Furthermore, the etiology of seedling root and stem rots may be confounded since multiple species can infect soybean. Some species, like *Pythium ultimum* and *Py. aphanidermatum* have a broad host range and can cause disease on many plant species. *Phytophthora sojae* has a narrow host range, only infecting soybean and lupins and *Py. arrhenomanes* prefering monocot grasses (Martin and Loper 1999). Other soybean associated *Pythium* spp. can be beneficial. For example, *Py. oligandrum*, *Py. acanthicum* and *Py. pleroticum* (all members of *Pythium* clade D) have been studied for their biological control properties as mycoparasites (i.e., fungal or oomycete pathogens) (Ali-Shtayeh and Saleh 1999; Martin and Loper 1999) and have been isolated from soybeans. Disease caused by seedling associated oomycetes may go unreported or may be underreported due to inconsistent infection within a field, non-lethal symptoms, plant stunting, or infection not resulting in noticeable yield loss due to compensation of neighboring plants. Thus, the oomycete community present in a field must be considered for effective disease management, not just a single species.

Soybean breeding efforts against oomycete pathogens have been primarily focused on *P. sojae* due to the gene-for-gene interactions with *P. sojae* effectors (*Rps* genes) and soybean R-gene (resistance genes) products (Dorrance and Grunwald 2009; Whitham et al. 2016). Additionally, R genes can be overcome and shifts in the composition of *Rps* genes has been observed over time (Dorrance et al. 2016). Additionally, partial resistance to *P. sojae* does not develop until two to four weeks after planting (Dorrance and McClure 2001; Dorrance et al. 2009). In contrast with *P. sojae*, fewer studies have been conducted for breeding against *Pythium* (Rosso et al. 2008; Rupe et al. 2011; Kirkpatrick et al. 2006; Lin et al. 2018; Stasko et al. 2016; Ellis et al. 2013). Consequently, germplasm used in soybean production does not have or is

unknown to have resistance to *Pythium* spp. making chemical intervention a necessary part of an integrated management strategy. Therefore, treatments containing fungicides applied as seed treatments remain the primary management tool for oomycetes in soybean production.

Several fungicides have been applied as seed treatments for control against oomycetes. The broad-spectrum fungicide, captan, has been used for protection of against a variety of soil-borne pathogens including oomycetes. In commercial seed treatments captan has been substituted for the more specific azoxystrobin and trifloxystrobin for control against fungi and oomycetes. Metalaxyl or its active stereoisomer metalaxyl-M (i.e., mefenoxam) is a commonly applied seed treatment fungicide specific to oomycetes (i.e., oomicide). In terms of total sales, the phenylamide chemical class, which includes mefenoxam, is estimated to be about four times that of other oomycete specific fungicides at about $405 million (Gisi and Sierotizki 2015). Ethaboxam is a newer oomycete specific chemical released in 2014 that was the first chemical after metalaxyl and mefenoxam to be used in soybean seed treatments specifically for oomycete control. Consequently, ethaboxam use has gained popularity as a component in soybean and corn seed treatments. For example, in 2017, an estimated 40% of (36 million acres) corn seed in the U.S. was treated with ethaboxam (Dair McDuffee, *personal communication*).

With the increasing usage in seed treatments in the past two decades it is essential for growers to have an idea of how inputs such as seed treatments are going to help improve their livelihood by providing effective disease control. However, recently there is concern of resistance evolution and loss of product efficacy especially surrounding the commonly used mefenoxam, which has lost the ability to control oomycetes in some production systems (Gisi and Sierotzki 2015; Taylor et al. 2002). Additionally, there is information lacking on the efficacy of mefenoxam and ethaboxam against the diversity of oomycete species associated with

soybeans. Therefore, the following two sections will review fungicide resistance evolution, methods for monitoring resistance, resistance management, and review the current status of fungicide resistance in soybean associated oomycetes.

Fungicide resistance evolution, monitoring, and resistance

The first reported usage of a chemical to control a plant disease is difficult to determine. Leukel (1936) argued that the practice of treating cereal seed dates back to almost the 1600s. However, the first intentional use of a chemical for plant diseases was likely from Forsyth (1802), the gardener to King George III of England, who detailed how to combine lime and sulfur to create a mix suitable for control of "mildew" on fruit trees. However, the commercial development and use of chemicals for reduction in plant disease largely began at the turn of the 20th century, utilizing inorganic compounds such as sulfur, copper, mercury, antimony and arsenic (Eckert 1988). These compounds were successful in controlling plant pathogens, especially sulfur containing compounds such as lime sulfur for control of powdery mildews, apple scab (*Venturia inaequalis*) and peach leaf curl (*Taphrina deformans*) as well as copper containing compounds like the Bordeaux mixture for control of downy mildew (*Palsmopara viticola*) on grapes (Millardet 1885).

Despite the effectiveness of these compounds, the targets were not specific and posed health risks to field workers, had non-target environmental hazards, and phytotoxicity risks to crops (Eckert 1988). Following the development of antibiotics for human therapy, synthetically derived selective anti-fungal compounds such as benomyl, dimethirimol, and carboxin started flooding the market in the 1960's. Another flood of newer chemistries in the 1970's and 1980's introduced compounds such as the phenylamides, the organophosphates, the dicarboximides, and the sterol inhibitors. These chemistries were more specific toward a target organism and were not

as dangerous to humans or plants. However, this specificity came at a cost. The incidence of resistant fungal genera increased from less than five in 1960 to over 60 by 1988 (Eckert 1988). With the increase in fungicide resistance incidence there has been a large body of literature, including the formation of the Fungicide Resistance Action Committee (FRAC) dedicated to monitoring and management of fungicide resistance.

To discuss fungicide resistance, the term 'fungicide resistance' must be defined. Typically, in plant pathology literature by 'fungicide resistance' is used to describe 'practical resistance' situations in which fungicides no longer work, or are efficacious, in field settings because the pathogen population has evolved resistance to the fungicide (Brent and Hollomon 2007). 'Field resistance' describes situations where resistant isolates are detected from a field and regardless of the number or level of insensitivity may not necessarily pose management problems. 'Laboratory resistance' describes situations when resistance is artificially induced in a laboratory by various genetic manipulation experiments (Brent and Hollomon 2007).

An important distinction to fungicide resistance literature is that it usually deals with what is termed 'acquired resistance' as opposed to 'inherent resistance' (Brent and Hollomon 2007; Angelini et al. 2011). 'Inherent resistance' describes the pathogen species that the fungicide is not active against, where 'acquired resistance' describes the proportion of a population that has been selected for resistance from the application of a fungicide (Brent and Hollomon 2007). A good example of 'inherent resistance' is the resistance to strobilurin fungicides in the pinecone cap fungus *Strobilurus tenacellus*, from which the strobilurin fungicide class acquired its name (Anke et al. 1977). For oomycetes, Blum et al. (2012) used a phylogenetic approach to the evolutionary and molecular mechanism of 'inherent resistance' to the oomycete specific cellulose synthesis inhibiting carboxylic acid amide (CAA) chemical mandipropamid and

discovered that the derived character state (sensitivity) evolved in the Peronosporales order due to differences in the target gene (Ces3A) sequence. Practically, 'inherent resistance' is seldom investigated or of interest. However, many oomycete species are associated with soybeans and thus both types of resistance are important and necessary to characterize.

If fungicide resistance does evolve there are two ways fungicide resistance manifests in a population, termed 'quantitative resistance' or 'qualitative resistance'. Quantitative resistance, also known as 'multi-step resistance' or 'continuous resistance' is when highly insensitive and mildly insensitive individuals are present in a population and the repeated application of a fungicide selects in a step-wise manner towards an insensitive population. Quantitative resistance, also known as 'discrete resistance', is when highly insensitive and sensitive individuals exist in a population and selection from a fungicide results in insensitive individuals existing in the population.

Once fungicide resistance has is present in a population it is essential to have an effective fungicide resistance management scheme. The central component of fungicide resistance management is about limiting pathogen exposure to fungicides. This includes 'pathogen factors' and 'fungicide factors'. 'Pathogen factors' include life history traits that influence pathogen exposure such as life cycle (monocyclic or polycyclic), dispersal, and fitness of resistant individuals. Similarly, 'fungicide factors' include factors that influence the exposure of a fungicide and the target organism such as target (broad spectrum vs. site specific) and use patterns. Manipulation of these factors that limit exposure to the fungicide can be a strategy for managing fungicide resistance.

Many foliar diseases, like *Venturia inaequalis* (causal agent of apple scab) and *Cercospora sojina* (causal agent of frog-eye leaf spot of soybean), have polycyclic disease cycles. This

means they can infect multiple times in one growing season, and if fungicides are applied multiple times to control the pathogen, this increases the exposure to fungicides. Multiple applications increase the probability that resistant mutants in the population will be exposed and survive while sensitive isolates will die (i.e., selection). Additionally, since oomycetes are primarily diploid rather than haploid or dikaryotic like most fungi, mutations resulting in resistance occurring on one allele may be masked by the other dominant wild-type allele (Angelini et al. 2011). Other pathogen factors that influence the dissemination of disease can be important as well. For example, *Phytophthora infestans* produce a large number of deciduous sporangia on leaf surfaces, which can be disseminated by wind to neighboring farms. In this case, there is a higher probability that resistant isolates will be spread quickly. In contrast to many foliar pathogens, often soil borne pathogens, like oomycetes causing root rot of soybean, have limited dispersal or dissemination, and thus fungicide resistant isolates or alleles are slow to accumulate in a population.

Fungicide mode of action and how that fungicide is used play an essential role in managing fungicide resistance. For example, QoI (Quinone outside Inhibitors), otherwise known as "strobilurins" have had a history of developing fungicide resistance quickly, largely due to a single target mutation in the quinol oxidation site of the *cytb* gene that confer discrete resistance to all QoI fungicides (Sierotzki 2011). Though in-furrow application of fungicides has been recommended for soybeans, the most popular application of a fungicide for oomycete pathogens are seed treatments. For seed treatments the considerations of management of fungicide resistance change slightly because the risk of developing fungicide resistance is considered lower due to the interactions of pathogen factors and fungicide use patterns. For example, the lack of rapid dispersal of soil-borne organisms along with seed treatments being localized to a seed and

not broadly sprayed on foliage or on the soil is thought to decrease exposure and slow the evolution of fungicide resistance (Brent and Hollomon 2007; Gisi and Sierotzki 2015; Urech 1988).

However, there is debate. Some fungicides applied as seed treatments are targeted toward foliar pathogens. Brent et al. (1989) indicated that the selection pressure from seed treatments against powdery mildew on barley (*Blumeria graminis* f. sp. *hordei*) were like foliar applied fungicides. Kitchen et al. (2016) simulated epidemics of *Zymoseptoria tritici* on winter wheat with foliar applied succinate dehydrogenase inhibitor (SDHI) or as a seed treatment. Kitchen et al. (2016) concluded that seed treatments posed a similar selection pressure to those applied as foliar sprays and should be included in a resistance management regime that limits the applications per growing season of a fungicide. However, in both of these studies it was unclear if the seed treatment alone or a prior exposure to foliar applied fungicides initially selected for practical resistance. Therefore, little experimental evidence about the risk of fungicide resistance evolution posed by seed treatments alone is known.

Additionally, few studies have focused on fungicide resistance or more broadly, antibiotic resistance, at a community level rather than a population level. This shift in thinking is taking hold in medical literature (Andersen et al. 2019). Beardmore et al. (2018) found that by altering fungicide and glucose concentrations, populations of *Candida albicans* (sensitive to fluconazole) and *Candida glabrata* (resistant to fluconazole) hit "tipping points" such that one species would dominate given certain environmental conditions. This observation has the important consequence that resistant species can accumulate without selection from a fungicide, but also points out that novel methods of resistance management exist through manipulation of the environment and thus community dynamics. de Vos et al. (2017) demonstrated that

community dynamics can be important for the survival of individual pathogen populations in the presence and absence of antibiotic treatments. Since many oomycete species are associated with soybean it is necessary to study fungicide resistance considering many potential pathogen populations.

At a molecular level, there are four common molecular mechanisms of resistance in oomycete and fungal plant pathogens (Fig. 1.2). First, and most frequently observed (Ma and Michailides 2005), is a non-synonymous mutation in the target protein gene, which alters the protein structure such that the fungicide can no longer bind to the target protein. Second is a target protein overproduction where there is no mutation in the target protein, but the expression of the target gene enables the target protein to simply 'out-compete' the fungicide. Third is over expression of efflux proteins, like ABC transporters, where the fungicide is pumped out of the cell so it cannot reach the intended target. Fourth is fungicide metabolism where the fungi will express an enzyme capable of breaking down the fungicide such that it is no longer active. Regardless of the type of fungicide resistance and the molecular mechanism involved another central component of fungicide resistance management is accurate and robust methods to monitor fungicide resistance.

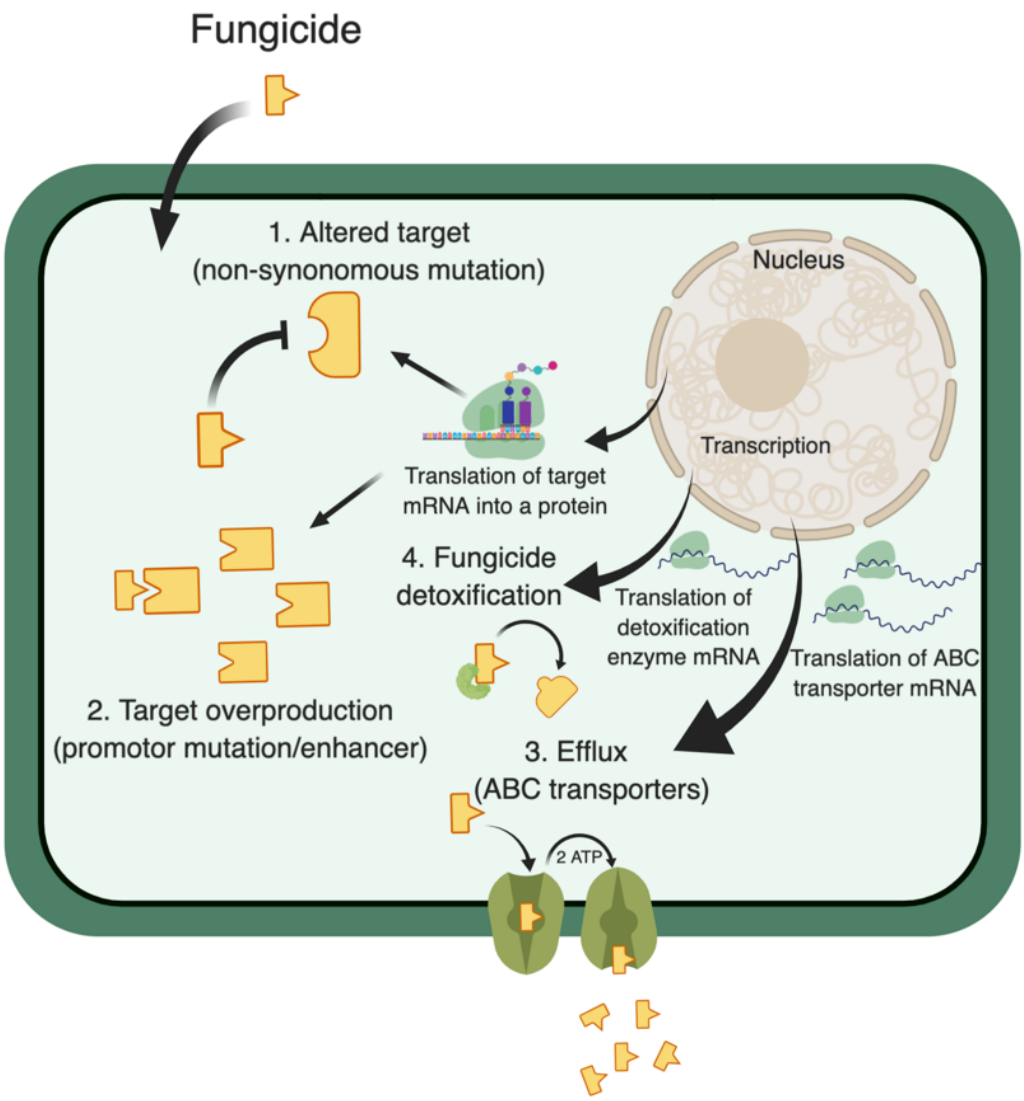

Figure 1.2. Schematic of the common molecular mechanisms of fungicide resistance in fungi and oomycetes. First, the target protein may be mutated such that the fungicide no longer can bind to the protein. Second, the target protein is not mutated but is overexpressed resulting in more target protein than fungicide and the target protein can still carry out its function. Third, the fungicide is pumped out of the cell by an efflux protein. Fourth, is a fungicide metabolism mechanism where a fungal enzyme breaks down or changes the fungicide structure so that it can no longer bind to the intended target protein.

Effective fungicide resistance management requires monitoring to assess the proportion of insensitive isolates present in a pathogen population or community. Monitoring strategies include using molecular methods. However, in order to utilize and develop molecular methods of monitoring, the molecular mechanism must be known. Additionally, molecular monitoring methods may only be applicable for a single pathogen species since polymerase chain reaction (PCR) primers and reaction conditions may be only applicable to the pathogen species the assay was designed for. More traditionally, the sensitivity level is measured *in vitro*. The sensitivity level is measured for each individual isolate by estimating the effective concentration to reduce mycelial growth by half (EC_{50}). The EC_{50} is the standard value used in plant pathology for resistance monitoring. If the EC_{50} increases over time the fungicide will no longer be a viable management tool. The 'gold standard' *in vitro* method used to test oomycete sensitivity is by measuring colony growth on fungicide-amended agar medium (Georgopoulos 1982; King-Watson 1988). In this method, several concentrations of fungicides (the dose) are amended into agar medium, and colony diameter (the response) is monitored.

Dose-response data are often analyzed by regressing the response (colony diameter) against the log-transformed concentrations. The EC_{50} is then estimated from the inflection point of sigmoidal curves. This modeled EC_{50} is known as the relative EC_{50} (Sebaugh 2011) whereas the FRAC-defined EC_{50} is the concentration at which 50% relative growth occurs (the absolute EC_{50}). To date, there has not been an extensive exploration of how the use of absolute vs. relative EC_{50} change interpretation of pathogen populations in the context of plant pathology. Furthermore, there are many statistical models to describe dose-response data. The most common are the three- and four-parameter log-logistic models (Ritz et al. 2015). Other models describe hormetic growth which model stimulation at sub-lethal concentrations of a fungicide

(Brain and Cousens 1989; Cedergreen et al. 2005; Knezevic et al. 2007). Hormetic effects can be important for oomycete or fungal pathogens because sublethal concentrations of mefenoxam can enhance *Pythium aphanidermatum* virulence on geraniums (Garzón et al. 2011; Garzón et al. 2013). The influence of these models on the interpretation of pathogen populations has not been explored in a plant pathology context.

Nonetheless, using the 'gold standard' amended agar medium assay to screening hundreds of isolates can be slow, labor intensive, and expensive. Thus, the development of alternative methods with higher throughput are desired (Brent and Hollomon 1998). Alternatively, testing the sensitivity of single-celled organisms to antimicrobial compounds in microtiter plates is common and efforts to adopt these methods have been applied to filamentous fungi and oomycetes (Vega et al. 2012; Cox et al. 2009; Rampersad 2010; Kuhajek et al. 2003; Olsen et al. 2013; Frac et al. 2016). However, these methods are not applicable to the diverse set of oomycete species that is relevant to soybean production, because methods developed for oomycetes mainly relied on zoospores as an inoculum source and not all oomycetes form zoospores as readily as *Phytophthora*, or different conditions may be required to produce zoospores for all oomycete species associated with soybean. In contrast, mycelium is easily produced.

A mycelium-based fungicide sensitivity method (Olson et al. 2013; Lookabaugh et al. 2015; Huzar-Novakowiski and Dorrance 2018) has been developed and is executed by visually rating the growth of oomycetes on fungicide-amended agar or broth medium in 48 well microtiter plates. The reliability and repeatability of visual assessment of mycelial growth could be improved by collecting quantitative optical density data. Hunter et al. (2018) measured the growth of *Phytophthora* spp. by placing an inoculated agar plug into a 24 well plate, allowing the

isolate to grow for a specified amount of time, then read optical density at 620 nm. Similarly, a method could be developed for use on all oomycetes associated with soybean. To date no method has been developed that is quicker than the 'gold standard', has been demonstrated to be applicable to many culturable oomycetes, and is assessed using optical density. This is a severe limitation for assessing fungicide sensitivity of soybean associated oomycetes since only a limited number of isolates per species can be tested at once. Additionally, there is a lack of quality control statistics in high-throughput fungicide sensitivity assays. In high-throughput drug screening assays, the reliability of a high-throughput drug-screening assay is usually assessed using quality control statistics like Z'-factor (Zhang et al. 1999). No similar efforts for quality control of high-throughput assays in plant pathology have been made.

The extent of fungicide resistance in soybean associated oomycetes

Phenylamides (PAFs) are historically a commonly used fungicide for control of oomycetes. Phenylamides were first introduced in the late 1970s and included the compounds metalaxyl, furalaxyl, benalaxyl, ofurace, cyprofuram, and oxadixyl (Eckert 1988). The most commonly used and most studied of these chemistries is metalaxyl. Metalaxyl is a racemic mixture of both S- and R-metalaxyl. The R form is the active form and is named metalaxyl-M or mefenoxam. For clarity, the names mefenoxam or metalaxyl are used in this review and are the names used in commercial products. The first recorded report for metalaxyl insensitivity was seen in 1979 in *Pseudoperonospora cubensis* on cucumber (Reuveni et al. 1980). Daggett et al. (1993) and Bosshard and Schuepp (1983) revealed in 1977 and 1978 resistant isolates of *Phytophthora infestans* and *Plasmopara viticola*, which were found in Germany and Switzerland, respectively from populations that had never been exposed to PAFs (Gisi et al. 2000). Similar reports, of loss of efficacy to metalaxyl, were seen for *Phytophthora infestans* on potato in 1979 (Davidse et al.

1981). The first report of metalaxyl resistance in *Pythium* spp. on turfgrass was in 1983, three years after its registration for turfgrass (Sanders 1984).

The primary mode of action of all the phenylamides is ribosomal ribonucleic acid (rRNA) synthesis inhibition by inhibiting polymerase complex 1 (Davidse 1995). The segregation patterns of F1 progeny for phenylamide insensitive *P. infestans* isolates suggested a semi-dominant inheritance pattern (Knapova et al. 2002). However, it is unclear if this segregation pattern is conserved between species due to unexpected segregation patterns observed in *P. viticola* (Gisi and Sierotzki 2015; Randall et al. 2014). Recently, it was found that T1145A mutation in RPA190 (RNA polymerase 1) sequence had an 86% association with mefoxanam insensitivity in *P. infestans*. Randall et al. (2014) transferred an insensitive allele containing T1145A mutation to a sensitive isolate, which resulted in an insensitive transgenic mutant. This allele was later shown to be absent in clonal lineages resistant to metalaxyl indicating that a T1145A mutation may be a causal mutation, but other mechanisms are present (Matson et al. 2015). Today the precise mechanism of mefenoxam resistance remains unclear.

Practical resistance to metalaxyl or mefenoxam in *Phytophthora infestans*, causing late blight on potato and tomato, *Phytophthora erythroseptica*, causing pink rot, and *Pythium ultimum*, causing tuber leak of potato are consistent problems in the U.S. (Gisi and Sierotzki 2015; Taylor et al. 2002). *Pythium* spp. resistance in commercial greenhouse production (Moorman and Kim 2004) and turfgrass (Allen et al. 2004) can also be a problem. In both production systems either soil drenches or multiple foliar sprays are common, which increases exposure and the selection for less sensitive isolates (Brent and Holloman, 1998).

In contrast, and consistent with fungicide resistance theory discussed in the previous section, there are few incidences of metalaxyl or mefenoxam practical resistance reported for

oomycetes and seed treatments. Dorrance et al. (2004) reported that although some isolates were capable of growing on metalaxyl amended medium at 100 μg ml^{-1} all isolates tested had only grown 1% to 30% relative to the non-amended control (EC_{50} < 100 μg ml^{-1}). Malvick and Grunden (2004) demonstrated that all 63 isolates of *P. sojae* from Illinois were sensitive to metalaxyl since the growth of all isolates on medium amended with 1.0 μg ml^{-1} metalaxyl was less than 50 % of the control (i.e., EC_{50} < 1.0). Broders et al. (2007) reported on mefenoxam sensitivities among 12 different *Pythium* spp., and two 'species groups' now recognized as the genus *Phytopythium,* isolated from corn and soybean fields in Ohio. Similar to Dorrance et al. (2004), Broders et al. (2007) demonstrated that *Pythium* spp. isolates were capable of growing on mefenoxam amended medium, but that 96% of the isolates tested (n = 56) had an EC_{50} below 5 μg ml^{-1} with the other 4% having an EC_{50} less than 100 μg ml^{-1}. Nelson et al. (2008) reported that of the 157 *P.*sojae isolates tested, only seven had limited growth (i.e., less than 10 mm colony diameter) on 5 μg ml^{-1} metalaxyl amended medium compared to the colony diameter on non-amended medium, which grew to the edge of the plate. Matthiesen et al. (2016) indicated that the mean EC_{50} to metalaxyl of the four species tested were all below 10 μg ml^{-1} and notably, that sensitivity to metalaxyl changed with temperature for some species. For example, the mean EC_{50} for six *Pythium sylvaticum* isolates was 0.03 μg ml^{-1} at 13ºC and increased to 8.78 μg ml^{-1} at 23ºC. Radmer et al. (2017) reported that mefenoxam EC_{50} values of the 22 isolates recovered from soybean seedlings ranged from 0.020 to 4.002 but that 86% of these isolates had an EC_{50} below 1 μg ml^{-1}.

Since many oomycete species can be associated with plants, interspecific differences in metalaxyl/mefenoxam sensitivity have been investigated (Broders et al. 2007; Brantner and Windels 1998; Dorrance et al. 2004; Matthiesen et al. 2016; Radmer et al. 2017; Weiland et al.

2014; Kato et al. 1990). Broders et al. (2007) reported a range of sensitivities and found that species with proliferating and filamentous sporangia were significantly less sensitive than those with globose sporangia. These findings are unique because sporangial morphology is conserved amongst *Pythium* clades, suggesting that there might be a phylogenetic (i.e., potentially inherent) relationship with mefenoxam sensitivity (LéVesque and De Cock 2004) meaning that inherent differences rather than acquired insensitivity may result in differences in metalaxyl efficacy. On a broader evolutionary scale, Kato et al. (1990) discovered that the inhibition of growth on 600 μg ml^{-1} metalaxyl from ten species within the Saprolegnialian lineage (sister lineage to the Peronosporalean lineage) was between 36 to 93% compared to the *Pythium* and *Phytophthora* species tested which ranged from 94 to 100% inhibition. In fact, metalaxyl is commonly used within isolation medium for the Saprolegnialian lineage oomycetes *Aphanomyces cochlioides* (Chikuo and Sugimoto 1985; Windels et al. 2000) and *Aphanomyces euteiches* (Zitnick-Anderson and Pasche 2016). The level of interspecific variation in sensitivity to mefenoxam among the 84 species associated with soybean (Rojas et al. 2017a) is currently unknown. Currently, there is a lack of information connecting the level of *in vitro* sensitivity to lack of control in the field. Therefore, it is difficult to determine a discriminatory sensitivity at which control failure can be expected. Nelson et al. (2008) suggested that limited amount of growth on metalaxyl amended medium was unlikely to result in a control failure in the field. However, Dorrance et al. (2004) suggested that the small amount of growth noticed might lead to *Pythium* root rot in the field. There are, of course, examples of practical resistance issues and usage of seed treatments coinciding. For example, Falloon et al. (2000) reported resistance in *Peronospora viciae*, an obligate seed transmitted oomycete of peas. Chen and Van Vleet (2016) indicated that seed treatments containing mefenoxam or metalaxyl were ineffective in controlling

Pythium ultimum isolates in pacific northwest chickpea (*Cicer arietinum*) production. The *in vitro* sensitivity was not reported in this study (Chen and Van Vleet 2016). White et al. (2019) indicated that isolates resistant to mefenoxam were isolated from chickpea fields in Idaho. However, the history of these chickpea fields was not discussed. It is possible that fields previously in potato production resulted in the selection for resistant isolates since in-furrow treatments with mefenoxam are often used in potato production (Porter et al. 2009).

Similarly, Dorrance et al. (2004) indicated that in-furrow applications of mefenoxam had been previously applied in some Ohio counties for *P. sojae* control in the 1980s. Therefore, the true extent of practical resistance evolution due to the sole use of seed treatments is currently uncertain (Gisi and Sierotzki 2015). If practical resistance issues are present, the incidence may be low and isolated to fields with previous exposure to a different fungicide use pattern. Nonetheless, it is still important to document if shifts in sensitivity have occurred, so that appropriate use and recommendations of mefenoxam are made.

Whether or not isolates with practical resistance to metalaxyl or mefenoxam truly exist in soybean fields due to the sole usage of seed treatments, their incidence appears to be scarce. Furthermore, efforts to develop and deploy newer chemistries to manage plant pathogens have been made. Ethaboxam is an example of a novel fungicide that has proven effective in controlling the growth of phenylamide insensitive isolates in Korea and in the Pacific Northwest (Kim et al. 1999; Kim et al. 2004; White et al. 2019). In various *in vitro* and field tests, ethaboxam was shown to reduce mycelial growth of *P. infestans*, *P. capsaci*, *P. aphanidermatum*, *P. graminicola*, *P. ultimum* and prevent infection in field conditions of metalaxyl sensitive and insensitive isolates of *P. infestans* and *P. capsaci* (Kim et al. 2004). Ethaboxam was registered in the U.S. in 2014 as a seed treatment for corn and soybeans to

control *Pythium* and *Phytophthora* species. Preliminary field trials indicated that seeds treated with ethaboxam at the rates 7.5 to 15 g a.i. 100 kg $^{-1}$ seed had significantly higher stand counts and yield compared to plots with nontreated seeds (Dorrance et al. 2012). In the same study, the efficacy of ethaboxam when compared to mefenoxam was variable for *P. sojae*, *P. sansomeana*, and eight other *Pythium* species. In a similar study, Dorrance (2012) reported that ethaboxam did not affect *Py. aphanidermatum* growth at 100 µg ml^{-1}. Reports of reduced sensitivity to ethaboxam have been reported from isolates collected before the registration of ethaboxam (Matthiesen et al. 2016; Radmer et al. 2017). This indicates that some species may be inherently resistant to ethaboxam. However, the extent of interspecific variation among all oomycetes associated with soybean and the molecular mechanism of ethaboxam resistance is unknown. The mode of action is microtubule formation disruption (Uchida et al. 2005). Ethaboxam is believed to bind to the 'colchicine' site of β-tubulin due to its structural similarity to zoxamide and zarilamide (Young and Slawecki 2001; Young 2015). Young and Slawecki (2001) showed that zoxamide and zarilamide compete with each other for binding on *Phytophthora capcisi* β-tubulin, demonstrating a common binding site. Gene replacement experiments with *Phytophthora sojae* confirmed that a non-synonymous mutation, which changed the 239th amino acid from cysteine to serine (C239S), conferred resistance to zoxamide (Cai et al. 2016).

Therefore, if the inherent sensitivities of species are established, the evolutionary history of fungicide sensitivity can be investigated, the mechanism of resistance can be determined, and the breadth of activity for ethaboxam can be determined. Additionally, establishing the baseline sensitivity will allow for detection of shifts in sensitivity in future years.

Oomycete evolution and Peronosporalean taxonomy

Often, closely related species have similar biology, niches, and ecological functions. Therefore, a better understanding how traits evolved in plant pathogens is vital for management especially among those associated with soybeans. For example, the misidentification of *Phytophthora infestans* a fungus (Schiermeier 2001) instead of an oomycete has potentially led research in the wrong direction since many management strategies for fungi, like applying a fungal-specific chemical (i.e., a fungicide instead of an oomicide), would not work against *Phytophthora infestans* (Govers 2009). In soybean associated oomycetes understanding interspecific variation in sensitivity along with discovering the genetic and evolutionary mechanisms requires an understanding of oomycete evolution. Therefore, the following section covers the current understanding of the evolution of oomycetes with an emphasis on those associated with soybeans. Consequently, this section will outline the evolution of oomycetes but will mainly focus on the evolution and taxonomy of oomycetes within the genera *Pythium*, *Phytopythium*, and *Phytophthora.*

Oomycetes were initially classified within the Kingdom fungi because of similar morphology. However, it has long been recognized that oomycetes do not quite "fit in" with the rest of the fungal kingdom. Alexopoulos (1962) in the second edition of his landmark mycology book, "Introductory Mycology", grouped the oomycetes into the now obsolete Phycomycetes along with other non-septate lower fungi. Shaw (1983) described oomycetes as a "fungal geneticist's nightmare". Today it is indisputable due to molecular evidence that all species in the phylum Oomycota are more closely related to Bacillariophyta (diatoms) and the Phaeophyceae (brown algae) than they are to fungi (Beakes et al. 2012; Raffaele and Kamoun, 2012). Both fungi and oomycetes have filamentous growth in a mycelium and are osmotrophs. However,

there are some distinct differences between members of the oomycota and true fungi. Oomycota have cellulosic cell walls, tubular cristae, and are diploid. Additionally, oomycete zoospores have two anisokont (unequal length), and heterokont (different morphology) flagella (one anterior directed tinsel flagellum and one posterior directed whiplash flagellum) on haploid zoospores. Fungi have chitinous cell walls, are haploid or dikaryotic, have one flagellum (if present), and have flattened cristae.

Oomycota is a phylum of osmotrophic eukaryotic organisms with well over 1,000 species (Beakes and Sekimoto 2009). Oomycetes belong to the Stramenopile portion of the Stramenopile-Alveolates-Rhizaria (SAR) supergroup (Burki et al. 2008). Many of these organisms contain plastids and are photosynthetic, but the Oomycota are not photosynthetic. There are two hypotheses of plastid evolution of Stramenopiles; the chromoalveolate hypothesis and the horizontally inherited plastid hypothesis. The chromoalveolate hypothesis posits that plastids were inherited vertically from an ancestral species and subsequently lost from all Stramenopile lineages except for ochrophytes (Beakes et al. 2012; Lamour et al. 2007). Tyler et al. (2006) discovered photosynthesis-related genes in the *Phytophthora sojae* genome that were similar to those found in diatoms. However, no vestigial plastids organelles are found in oomycetes (Beakes et al. 2012). The competing horizontally inherited plastid hypothesis suggests that ochophytes horizontally inherited plastids through tertiary endosymbiosis, thus concluding oomycetes never had photosynthetic ancestors (Stiller et al. 2014).

The oldest convincing fossil evidence of an oomycete was found in the Paleozoic era (early Devonian) 400-million-year-old Rhynie chert (Taylor et al. 2006). Matari and Blair (2014) estimated via molecular clock dating that oomycetes diverged approximately 400-430 million-years-ago, and the divergence of the two major oomycete lineages (the Saprolegnialian and

Peronosporalean lineages) occurred approximately 200 million years ago at the beginning of the Triassic period. Interestingly, the late Silurian early Devonian (400-480 million-years-ago) marks the beginning of the colonization of land plants (Kenrick and Crane 1997). It has been suggested that oomycetes colonized land from the sea parasitizing nematodes or coastal seaweeds (Beakes et al. 2012).

Therefore, parasitism is thought to be an ancient character state of oomycetes, and they are "hard-wired" for a parasitic lifestyle (Beakes et al., 2012). Some basal oomycete lineages in the genus *Eurychasma* infect brown algae in marine environments (Grenville-Briggs 2011). Carella et al. (2017) demonstrated that *Phytophthora palmivora* could colonize, infect and complete its life cycle on the early diverging land plant *Marchantia polymorpha*. Some species in the Saprolegniales order are fish and crustacean pathogens. Lagenidiales contain a species, *Legenidium giganteum*, which is a pathogen of mosquito larvae. *Lagenisma coscinodisci* (Lagenidiales) is a diatom marine pathogen (Schnepf et al. 1978). Members of the Peronosporales contain the important plant pathogenic genera *Phytophthora*, *Phytopythium*, *Peronospora*, and *Pythium*. However, Peronosporales contain perhaps the most well-known terrestrial plant pathogens and have caused significant plant diseases that have shaped human demography and landscapes. For example, it is no mistake that the Greek translation of the word *Phytophthora* means "plant-destroyer". *Phytophthora infestans* is regarded as one of the main factors in the Irish potato famine of the 1800s and the mass migration out of Ireland (Judelson and Blanco 2005). Kovacs et al. (2011) estimated that over the past decade *Phytophthora ramorum*, the causal agent of sudden oak death, caused the removal or treatment of over ten-thousand trees and caused over 100 million dollars in property losses for single-family homes. Consequently, the molecular basis of virulence for the genus *Phytophthora* has been studied

extensively and has led to insights into the evolution of eukaryotic plant pathogens (Dong et al. 2015; Tyler et al. 2006), which is beyond the scope of this particular review.

For soybean, the most significant *Phytophthora* species for soybeans is *Phytophthora sojae*. However, Rojas et al. (2017a) isolated nine *Phytophthora* spp. from soybean roots. Two species (*Phytophthora sansomeana* and *Phytophthora drechsleri*) were pathogenic on soybean seeds whereas four species (*Phytophthora sansomeana*, *Phytophthora drechsleri*, *Phytophthora sojae*, and *Phytophthora rosacearum*) were pathogen towards soybean roots (Rojas et al. 2017a). *Phytophthora sansomeana* was not formally described until 2009 when it was differentiated from the *Phytophthora megasperma* species complex (Hansen et al. 2009), which complicates issues associated with soybean disease etiology. *Phytophthora sansomeana* has a wide host range (Malvick and Grunden 2004; Hansen et al. 2009; Zelaya-Molina et al. 2010), whereas *Phytophthora sojae* infects soybean (Erwin and Riberio 1996).

Currently, there are 244 species names under the genus *Phytophthora* in Mycobank (http://www.mycobank.org: accessed April 8th, 2019) and the Species Fungorum database currently contains 237 species (http://www.catalogueoflife.org: accessed April 8th, 2019). Most recently, there has been an increase in the rate of *Phytophthora* species descriptions with over 100 new species descriptions in the past decade (Fig. 1.3). The genus *Phytophthora* has been organized into ten phylogenic clades (Blair et al. 2008; Martin et al. 2014) and the clades do not consistently correlate with morphological features like sporangium morphology (Hyde et al. 2014). This can make species-level identification difficult based on morphology alone. An essential step in molecular identification of *Phytophthora* species has been the construction of curated databases with multiple loci like PhytophthoraDB (http://www.phytophthoradb.org/) (Park et al. 2013).

The genus *Pythium* is a diverse, mostly known for parasitizing plants, but also green algae, red algae, fungi, nematodes, insects, crustaceans, and mammals (Hyde et al. 2014). *Pythium ultimum* is recognized as one of the top 10 most crucial plant pathogenic oomycete species for molecular biology (Kamoun et al. 2015). The oomycete genus *Pythium* contains hemibiotrophic, necrotrophic, and saprotrophic individuals many of which are broad host range plant pathogens. The most widely studied is the aggressive pathogen *Pythium ultimum*, and the close relatives *Pythium ultimum* var. *ultimum* and *Pythium ultimum* var. *sporangiiferum*. However, other species are believed to contribute, in some fashion, to disease development while still others like *Pythium oligandrum* have been shown to prevent disease (Martin 1987).

The number of *Pythium* species is debatable. Mycobank currently has 326 species under the genus Pythium (http://www.mycobank.org: accessed April 8th, 2019), and the Species Fungorum database (http://www.catalogueoflife.org: accessed April 8th, 2019) has 275 including infraspecific taxa. There was a sharp increase in the number of described species from 1931-1933, mostly due to the work of Drechler (1930), who described almost twenty species over this time (Fig. 1.3). Since then, new species have been added almost every year with the most additions occurring in the late 1990s and early 2000s. The Species Fungorum database currently has 138 *Pythium* species with accepted names (i.e., not synonymous). The actual number of *Pythium* species is likely closer to 150 based on synonymy. *Pythium* has been previously placed within the order *Pythiales* when phylogenetic evidence suggested that the two orders, *Pythiales* and *Peronosporales*, should be split (Dick 2001). Further reclassification placed the genus *Pythium* back into the *Peronosporales*, along with the genera *Phytophthora* and *Phytopythium* (de Cock et al. 2015) citing a lack of molecular support (Beakes et al. 2014; Hyde et al., 2014). LéVesque and De Cock (2004) split *Pythium* into 11 monophyletic clades (clades A-K) based on

ITS rDNA sequences, which also followed zoosporangia morphology. Clades A-D have filamentous zoosporangia, clades E-J having globose zoosporangia and clade K having zoosporangium resembling *Phytophthora*. However, the placement of these groups is not concrete and has recently come under question.

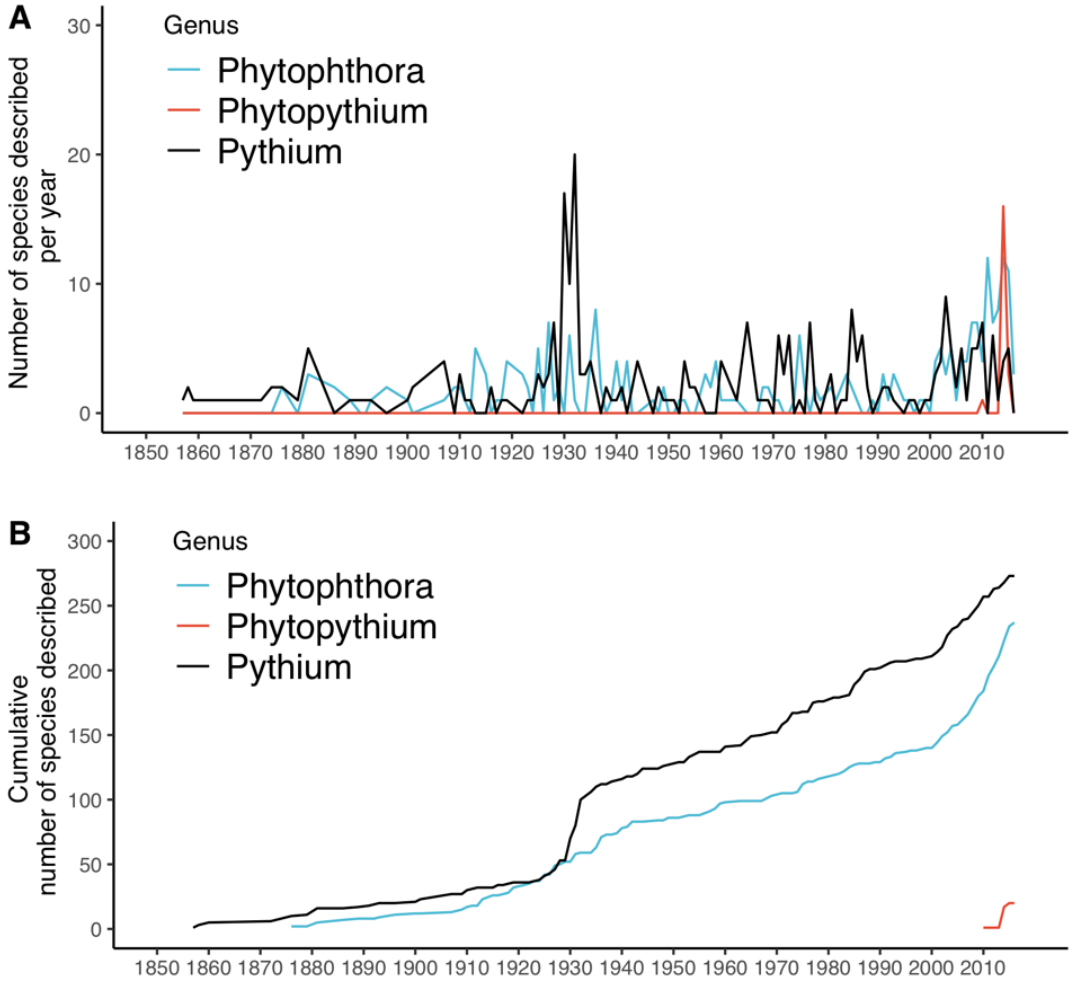

Figure 1.3. *Pythium*, *Phytopythium*, and *Phytophthora* species in the Species Fungorum from 1850-2018 accessed April 8, 2019. (A) Number of species described per year. (B) Cumulative number of species described.

Uzuhashi et al. (2010) suggested separating the genus *Pythium* into four new genera; *Ovatisporangium* (*Pythium* clade K), *Globisporangium* (*Pythium* clades E, F, G, and I), *Elongisporangium* (*Pythium* clade H), and *Pilasporangium* (represented by *Pythium apinafurcum*). *Pythium* clades A-D would remain as the genus *Pythium sensu stricto* (Uzuhashi et al. 2010). The lack of immediate adoption of these genera was mainly due to the lack of supporting phylogenetic evidence and the polyphyletic nature of the phylogeny when including genera *Pythiogeton* and *Lagena* (Hyde et al., 2014). Therefore, the current genus names remained intact. *Pythium* clade K was transferred to the new genus *Phytopythium* instead of the proposed *Ovatisporangium*, which is the only name change to have been accepted thus far (de Cock et al. 2015). Currently, some researchers are using the *Globosporangium* genus name, and some are not. For example, the National Center for Biotechnology Information (NCBI) recognizes *Pythium ultimum* as *Globisporangium ultimum*.

Phylogenies built with the markers ITS, COXI, COXII, and β-tubulin do not resolve the phylogeny of *Pythium* (Robideau et al. 2011; Hyde et al., 2014). Currently, there are eleven publicly available *Pythium sensu lato* in NCBI. Macarthy et al. (2017) reconstructed a genome-scale species phylogeny of oomycetes and found that *Pythium sensu lato* resolved into two monophyletic clades corresponding to two of the new proposed genera *Globisporangium* and *Pythium sensu stricto* (Uzuhashi et al. 2010). Even though supported by phylogenetic analysis if the newly genera names are to be used, they should be done in a way not to confuse individuals used to the genus name *Pythium.* Therefore, herein, the genus name *Pythium* refers to *Pythium sensu lato*.

Studying the diversity of oomycetes has undergone a dramatic change with curated oomycete ITS databases (Lévesque and de Cock 2004; Robideau et al. 2011; Park et al. 2013)

and improved strategies to preferentially amplify oomycetes ITS sequences from environmental samples (Sapkota and Nicolaisen 2015; Riit et al. 2016; Taheri et al. 2017). There is an increasing interest and ability to characterize oomycete communities using metabarcoding (Rojas et al. 2019; Agler et al. 2016; Coince et al. 2013; Vannini et al. 2013; Sapkota and Nicolaisen 2015; Singer et al. 2016; Bakker et al. 2017; Riit et al. 2016; Durán et al. 2018; Coffua et al. 2016). The next section reviews literature focused on metabarcoding studies of oomycete communities.

Studies on oomycete communities using metabarcoding and perspectives on the effect of seed treatments

The plant microbiome is defined as all organisms contained within the soil or associated with the plant (van der Heijden and Hartmann 2016). Plant pathology has entered a new era where the use of techniques like high-throughput amplicon sequencing (metabarcoding) from environmental samples or diseased plants is becoming commonplace as a tool for understanding the etiology of plant diseases caused by many causal agents, and conditions that promote or suppress disease development.

With the decreasing cost of sequencing technologies, it is now possible to look at complex interactions of microbial communities, their hosts, and with anthropogenic perturbations. Microbial communities assemble and disassemble, and exploring the factors, like soybean seed treatments, that influence the assembly of pathogen species on soybean seeds or seedlings can greatly enhance disease management. Many factors can influence community assembly like biotic or abiotic filters (filter models), inter- and intraspecific competition, niche exploitation, fitness (coexistence models) and stochastic assembly processes (neutral theory) like dispersal limitations, births, and deaths (Chesson 2000; Hubbel 2006). By figuring out which factors are

most important for assembly of oomycetes on soybean seedlings, perhaps the alteration of these factors can be used to enhance disease management.

Despite the importance of oomycetes in plant disease and natural ecosystems, the number of studies including the diversity of oomycetes pales in comparison to those focused on bacteria or fungi. A search for the term “oomycete community” in the scientific literature search engine Scopus (https://www.scopus.com: accessed April 22, 2019), revealed 101 peer-reviewed documents. For comparison, the same search with “bacterial community” or “fungal community” resulted in 64,000 and 12,433 peer-reviewed articles, respectively. However, interest in sequencing oomycete communities has grown in the last twenty years leading to an exponential increase in the cumulative number of citations (Fig. 1.4A). Most manuscripts from the “oomycete community” search were published in journals at the interface of plant pathology, ecology, and microbiology (Fig. 1.4B). Studies have used soil baiting, isolation from plant roots, or metabarcoding usually to describe pathogenic species (Broders et al. 2007; Rojas et al. 2017a; Suffert and Guibert 2007; Zitnick-Anderson and Nelson 2015). However, fewer studies have focused solely on the factors that drive oomycete diversity. Rojas et al. (2017b) examined the ecology of oomycete species associated with soybeans in regions of the U.S. Isolates were identified via Sanger sequencing of ITS1 and ITS2 along with the 5.8S region. Oomycete communities were dominated by *Pythium* spp., but change in isolation medium recovered more *Phytophthora.* Oomycete communities were driven by location, precipitation, temperature, clay content and electric conductivity (Rojas et al. 2017b). Similarly, Broders et al. (2009) used direct colony polymerase chain reaction (PCR) of the ITS1 region followed by single-strand conformational polymorphism (SSCP) analysis on over 7,000 isolates from Ohio and found that location, pH, calcium, magnesium, and field capacity (i.e., water holding capacity) were the main

factors that drove oomycete diversity. These studies demonstrated that location plays a significant role in oomycete diversity, indicating that species compositions are associated with specific fields. Unique communities in soybean fields have enormous implications for chemical management since intraspecific variation to fungicides is a primary concern.

Despite the values of these studies (Broders et al. 2009; Rojas et al. 2017a, b) and the extensive culture collections produced, the time and cost could have been reduced if a culture-independent approach like targeted metagenomics, or high-throughput amplicon sequencing had been used. However, at the time methods to study oomycete communities were lacking. For example, only four oomycete species were found using a pyrosequencing approach in forest soils (Coince et al. 2013). Sapkota and Nicolaisen (2015) argued that methods were not suitable for quality studies of oomycete communities due to inefficiencies and biases in PCR protocols. Both fungi and oomycetes can be amplified using primers ITS6 and 7, which targets the internal transcribed spacer (ITS) 1 region of the rDNA, however by increasing the annealing temperature Vannini et al. (2013) and Sapkota and Nicolaisen (2015) were able to recover 79 and 95% oomycete reads, respectively, with the majority of those reads with similarity to *Pythium* species. Oomycete communities were examined in peat bogs and other oligotrophic environments of Switzerland and France using high-throughput sequencing of the v9 region of the SSU rDNA (Singer et al. 2016). The objective of this study was to explore underrepresented environments for the discovery of basal oomycete lineages. *Pythium* reads were obtained, mostly from terrestrial environments, whereas other lineages, like *Saprolegnia*, *Haptoglossa*, *Albugo*, and *Peronospora* were more abundant in peat bogs, likely parasitizing the vascular plants and crustaceans present in these environments.

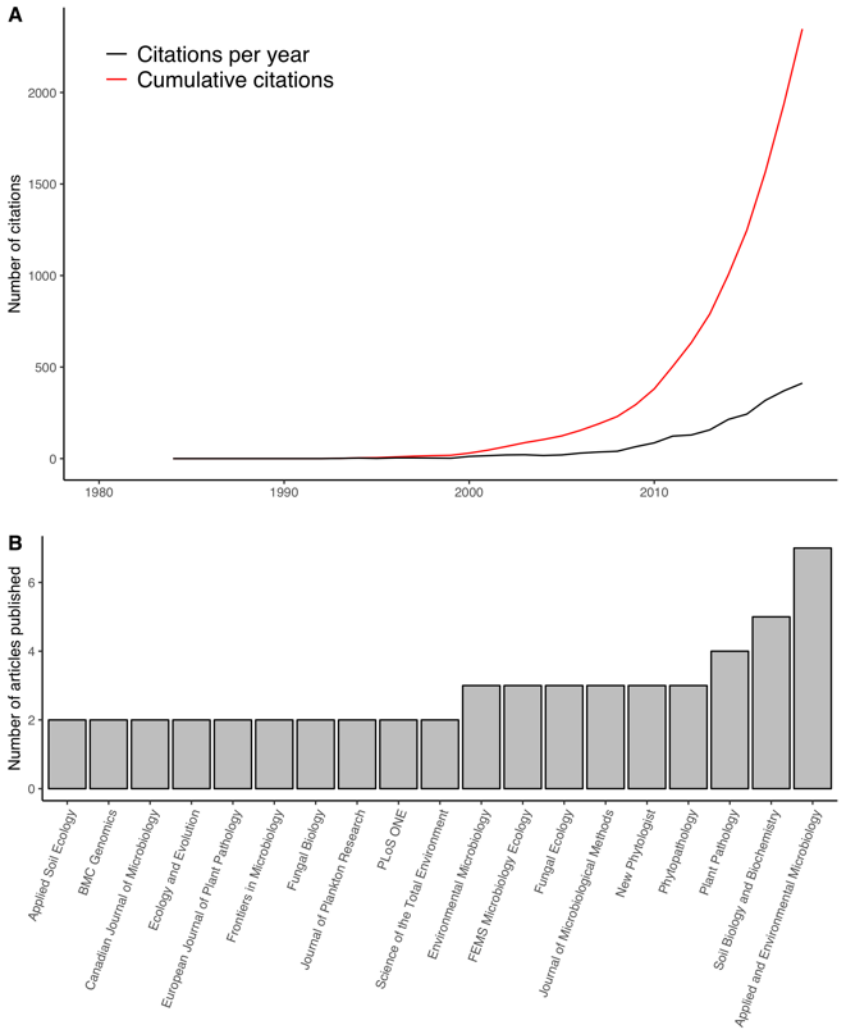

Figure 1.4. Search results of articles from peer reviewed journals from Scopus with topic = "oomycete AND community" accessed April 22, 2019. (A) The cumulative number of citations and number of citations per year from search results. (B) Peer-reviewed journals with at least five articles published matching the search topic results.

"Hub" species are species that significantly influence the structure of a microbial community. Agler et al. (2016) demonstrated that *Albugo* spp. were "hub" species in the *Arabidopsis thaliana* phyllosphere. The presence of *Albugo* spp. infection on *A. thaliana* leaves changed the colonization patterns of leaf-associated bacterial communities such that alpha diversity (within sample diversity) decreased and beta diversity (between sample diversity) became more homogenous (Agler et al. 2016). The profound effect on microbial community structure points to the importance of oomycetes in microbial community assembly and has implications for host-pathogen or microbe-microbe interactions.

For soybeans-oomycete interactions, there is evidence to suggest that *Pythium* are weak soil competitors (Martin and Loper 1999; Nelson 2004). Therefore, fast colonization of seed and root tissue may allow *Pythium* to colonize seedlings before other microbes resulting in competitive exclusion. Sporangia of *P. ultimum* can germinate within three to four hours after exposure to seed and complete seed colonization occurring within 12 to 24 hours after planting (Nelson 2004). One could hypothesize that seed treatment chemicals may slow the growth rate of infecting *Pythium* spp. long enough for other microbes to colonize and outcompete *Pythium* spp. Similarly, seed treatment chemicals like insecticides may influence the physiology of the plant. For example, some neonicotinoid insecticides resemble molecules involved in systemic acquired resistance (SAR) and can induce defense responses (Ford et al. 2010). The use of these insecticides may heighten plant defenses and exclude pathogens from associating with the developing roots. Interestingly, Rossman et al. (2018) found that soybean seed treatments containing insecticides increased plant density, in fields in Michigan with a history of seedling disease pressure despite there being a lack of insect pressure. This observation perhaps indicates that the insecticide treatments were influencing soil-borne pathogens. Additionally, it is

uncertain if certain oomycete species are selected (i.e., filtered) based on the chemical applied to the seed due to inherent differences in sensitivity. Studies detailing the influence of seed treatment on oomycete assembly are lacking, but once performed will enhance our understanding of chemical control with seed treatments.

Conclusion

Oomycetes are essential players in soybean seedling loss and seed treatments containing anti-oomycete chemicals (i.e., oomicides) are the preferred method for protection of soybean seeds. However, due to the diversity of species present in soybean fields addressing problems such as resistance can be challenging questions to answer. To address these challenges the following objectives should be addressed, which outline the following chapters of this dissertation. First, the clarification of models and terms in dose-response analysis for plant pathology is an area that needs improving for future clarity and reproducibility. Secondly, the development of a new technique for oomicide sensitivity *in vitro* will improve the throughput necessary to screen hundreds of oomycete isolates quickly. Third, phenotype data gathered from the development of the new technique can be used for studies focused on the evolution and molecular mechanism of sensitivity in soybean associated oomycetes. Fourth, the isolation of and characterization oomycetes from oomicide treated roots will aid in understanding the efficacy of oomicides in reducing oomycete colonization and address resistance concerns. Lastly, using amplicon sequencing (metabarcoding) of oomycete communities will help diagnose the main oomycete players in problem fields and will help understand how oomycete communities are affected by different agronomic factors such as field location, soybean genotype, and soybean seed treatments.

Chapter 2

Significant influence of EC_{50} estimation by model choice and EC_{50} type

Source

This chapter has been published in *Plant Disease*: Noel, Z. A., Wang, J., & Chilvers, M. I. (2018). Significant influence of EC_{50} estimation by model choice and EC_{50} type. Plant Disease, 102: 708-714. DOI: 10.1094/PDIS-06-17-0873-SR

Abstract

The EC_{50} (effective control to 50% growth inhibition) is a standard statistic for evaluating dose response relationships. Many statistical software packages are available to estimate dose response relationships, but recently an open source package ("drc") in R, has been utilized. This package is highly adaptable, having many models to describe dose response relationships and flexibility to describe both hormetic relationships and absolute and relative EC_{50}. These models and definitions are generally left out of phytopathology literature. Here we demonstrate that model choice and type of EC_{50} (relative vs. absolute) can matter for EC_{50} estimation using data from *Pythium oopapillum* and *Fusarium virguliforme*. For some *Pythium oopapillum* isolates, the difference between absolute and relative EC_{50} was significant. Hormetic effects changed *Fusarium virguliforme* EC_{50} distributions leading to higher estimates when using four or three parameter log-logistic models. We recommend future studies pay careful attention to model selection and interpretation in EC_{50} estimation and clearly indicate which model and EC_{50} measure (relative vs absolute) was used. We provide guidelines for model choice and interpretation for those wishing to set up experiments for accurate EC_{50} estimation.

Chapter 3

A high-throughput microtiter fungicide sensitivity phenotyping platform for oomycetes using Z'-factor statistic

Source

This chapter has been published in *Phytopathology*: Noel Z.A., Rojas, J.A., Jacobs, J.L., Chilvers, M.I. A high-throughput microtiter fungicide sensitivity phenotyping platform for oomycetes using Z'-factor statistic. DOI: https://doi.org/10.1094/PHYTO-01-19-0018-R

Abstract

Current methods to quantitatively assess fungicide sensitivity for a diverse range of oomycetes are slow and labor intensive. Microtiter based assays can be used to increase throughput, however many factors can affect their quality and reproducibility, therefore efficient and reliable methods for detection of assay quality are desirable. The objective of this study was to develop and validate a robust high-throughput fungicide phenotyping assay based on spectrophotometric quantification of mycelial growth in liquid culture and implementation of quality control with Z'-factor and growth curves. The Z'-factor is a simple statistical parameter commonly used to assess the quality of high-throughput screening assays. EC50 and relative growth values were highly correlated in a side-by-side comparison to values obtained using the amended medium assay, and correctly distinguished resistant from sensitive isolates. To demonstrate utility, the sensitivity of 216 oomycete isolates representing four genera and 81 species to mefenoxam and ethaboxam was tested. The assay developed herein will enable high-throughput fungicide phenotyping at a population or community level.

Chapter 4

Convergent evolution of C239S mutation in *Pythium* spp. β-tubulin coincides with inherent insensitivity to ethaboxam and implications for other Peronosporalean oomycetes

Source

This chapter has been published in *Phytopathology*: Noel Z.A., Kyu H., Roth M.G., Chilvers, M.I. 2019. Convergent evolution of C239S mutation in *Pythium* spp. β-tubulin coincides with inherent insensitivity to ethaboxam and implications for other Peronosporalean oomycetes. DOI: https://doi.org/10.1094/PHYTO-01-19-0022-R

Abstract

Ethaboxam is a benzamide anti-oomycete chemical (oomicide) used in corn and soybean seed treatments. Benzamides are hypothesized to bind to β-tubulin thus disrupting microtubule assembly. Recently, there have been reports of corn and soybean associated oomycetes that are insensitive to ethaboxam despite never having been exposed. Here we investigate the evolutionary history and molecular mechanism of ethaboxam insensitivity. We tested the sensitivity of 194 isolates representing 82 species across four oomycete genera in the Peronosporalean lineage that were never exposed to ethaboxam. Eighty-four percent of isolates were sensitive to ethaboxam ($EC_{50} < 5$ μg ml^{-1}), whereas 16% were insensitive ($EC_{50} > 11$ μg ml^{-1}). Of the insensitive isolates, two different transversion mutations were present in the 239th codon in β-tubulin within three monophyletic groups of *Pythium* species. The transversion mutations lead to the same amino acid change from an ancestral cysteine to serine (C239S), which coincides with ethaboxam insensitivity. In a treated soybean seed virulence assay disease severity was not reduced on ethaboxam treated seed for an isolate of *Pythium aphanideramatum* containing the C239S mutation, but disease severity was reduced in the presence of mefenoxam or mefenoxam-ethaboxam treated seed. We queried publicly available β-tubulin sequences from other oomycetes in the Peronosporalean lineage to search for C239S mutations from other species not represented in our collection. This search resulted in other taxa that were either homozygous or heterozygous for C239S, including all species within the genus *Peronospora*. Evidence presented herein supports the hypothesis that the convergent evolution of C239S within Peronosporalean oomycetes occurred without a selection from ethaboxam yet confers insensitivity. We propose several evolutionary hypotheses for the repeated evolution of C239S mutation.

Chapter 5

Influence of soybean tissue and oomicide seed treatments on oomycete isolation

This chapter has been submitted to *Plant Disease*: Noel Z.A., McDuffee, D., Chilvers M.I. **Influence of soybean tissue and oomicide seed treatments on oomycete isolation.** Plant Disease. XX:XXX

Abstract

Soybean seedlings are vulnerable to a number of oomycete pathogens. Seed treatments containing the two anti-oomycete (oomicide) chemicals, metalaxyl-M (mefenoxam) or ethaboxam are used to protect against oomycete pathogens. This study aimed to evaluate the influence of these two oomicides on isolation probability of oomycetes from soybean taproot or lateral root sections. Soybean plants were collected between the first and third trifoliate growth stage from five Midwest field locations in 2016 and four of the same fields in 2017. Oomycetes were isolated from lateral root and taproot sections. In 2016, 369 isolation attempts were completed resulting in 121 taproot and 153 lateral root isolates. In 2017, 468 isolation attempts were completed, but 45 taproot and 120 lateral root isolates were collected. In some site-years, the probability of isolating an oomycete from a taproot or lateral root section was significantly different. Seed treatments containing a combination of ethaboxam and metalaxyl significantly reduced the probability of oomycete isolation from lateral roots in Illinois 2016, but not other locations, which may have been related to the heavy soil type (clay loam) combined with approximately 10 cm of rain two weeks after planting. Among the 439 isolates collected from the two years sampled, 24 oomycete species were identified, and community compositions were different depending on location and year. The five most abundant species were *Pythium sylvaticum* (28.9%), *Pythium heterothallicum* (14.3%), *Pythium ultimum* var. *ultimum* (11.8%), *Pythium attrantheridium* (7.9%), and *Pythium irregulare* (6.6%) which accounted for 61.7% of the isolates collected. Oomicide sensitivity was assessed for more than 300 isolates to ethaboxam and mefenoxam. There were large differences in ethaboxam sensitivity by oomycete species with EC_{50} ranging from < 0.01 to > 100 μg ml^{-1}, and a median of 0.65 μg ml^{-1}. Isolates with insensitivity to ethaboxam (> 12 μg ml^{-1}) belonged to the species *Pythium torulosum* and

Pythium rostratifingens but were sensitive to mefenoxam. Oomicide sensitivity to mefenoxam ranged from < 0.01 to 0.62 μg ml^{-1} with a median of 0.03 μg ml^{-1}. The mean EC_{50} of the five most abundant species to ethaboxam ranged from 0.35 to 0.97 μg ml^{-1} of ethaboxam or from 0.02 to 0.04 μg ml^{-1} of mefenoxam. No shift in sensitivity to mefenoxam or ethaboxam was observed due to soybean seed treatment or year relative to the non-treated seed controls. In summary, this study demonstrated that isolation of oomycetes from soybean can depend on the tissue, location, year, and seed treatment. Additionally, seed treatments containing mefenoxam or metalaxyl and ethaboxam can be effective to reduce the probability of oomycete isolation from soybean roots.

Introduction

Soybean seeds and seedlings are vulnerable to many soil-borne pathogens and protection of germinating and emerging seedlings is essential to prevent yield loss. Cultural techniques like tillage can reduce crop residue and, in some instances, help improve drainage to reduce pressure from some soil-borne pathogens like *Pythium* and *Phytophthora* but conservation tillage practices advocate leaving crop residue to maintain soil quality. Crop residue can harbor overwintering inoculum of oomycete pathogens which can attack germinating seeds and emerging seedlings (Pankhurst et al. 1995). Additionally, growers are increasingly planting earlier into cooler, wetter soils to lengthen the growing season, which exposes germinating seeds and emerging seedlings for a longer duration to conditions unfavorable for plant growth which may predispose the seed or seedling to oomycete pathogens. Soybean resistance to *Phytophthora sojae* has been intensively studied and deployed in resistance breeding (Dorrance and Grünwald 2009). However, partial resistance to *Phytophthora sojae* is not expressed in seeds or germinating seedlings so the use of a seed treatment is necessary to protect the young plant (Dorrance and McClure 2001; Dorrance et al. 2009). Moreover, few soybean varieties are bred for or are unknown to have resistance to other oomycete pathogens like *Pythium* or *Phytopythium* (Bradley 2008; Ellis et al. 2013; Kirkpatrick et al. 2006; Lin et al., 2018; Rosso et al. 2008; Rupe et al. 2011; Stasko et al. 2016). Soybean associated oomycete species vary in pathogenicity, virulence, and oomicide sensitivity (Broders et al. 2007; Matthiesen et. al. 2016; Noel et al. 2019a, b; Radmer et al. 2016; Zitnick-Anderson and Nelson 2015) and communities of these oomycetes can vary across location, temperature, and edaphic gradients (Broders et al. 2009; Rojas et al. 2017a, b; Zitnick-Anderson et al. 2017). The diversity of oomycete species can make management decisions difficult because they must be made considering the many pathogen

species present. Therefore, oomicides that are efficacious on the many pathogenic oomycete species present are essential management tools.

The phenylamide oomicide metalaxyl or its active stereoisomer metalaxyl-M (mefenoxam) are commonly used in seed treatments. Both metalaxyl and mefenoxam have the same mode of action and suppress oomycete growth by inhibiting ribosomal RNA synthesis, specifically RNA polymerase complex I (Davidse 1995). However, seed treatments generally contain other oomicides, one of which is ethaboxam, which was registered in 2014 in the U.S. for corn and soybean specifically for oomycetes. Ethaboxam is a benzamide (Kim et al. 1999), which disrupts microtubule formation (Uchida et al. 2005). Inter- and intraspecific variation in sensitivity among soybean associated oomycetes to mefenoxam and ethaboxam are documented (Broders et al. 2007; Matthiesen et. al. 2016; Noel et al. 2019a, b; Radmer et al. 2016). Therefore, the efficacy of a seed treatment may depend on the mode of action and rates of oomicides applied to the seed. The extent to which oomicide seed treatments reduce oomycete isolation from soybean lateral root or taproot is not well documented. Furthermore, the sensitivity of oomycetes isolated from soybean seed treated or seed not-treated with an oomicide is not well documented. Therefore, the objectives of this study were to (1) identify oomycetes associated with soybean taproot or lateral root sections from seed that was either non-treated, treated with mefenoxam, or treated with metalaxyl and ethaboxam to (2) understand the effect of soybean tissue on oomycete isolation, (3) understand the capacity seed treatments have in altering oomycete isolation, and (4) determine the sensitivity of isolated oomycetes to mefenoxam and ethaboxam to understand if shifts in sensitivity occurred due to seed treatment.

Materials and Methods

Field sites description. In 2016 non-treated soybeans (Navaho 1220RR24), soybeans treated with mefenoxam (Cruiser Maxx®), or soybeans treated with metalaxyl + ethaboxam (Intego Suite®) were planted in Illinois, Indiana, Iowa, Michigan, and Ohio (Table 5.1). Seed treatment formulations and application rates are described in Table 5.2. In 2017 soybeans with the same treatments as described above were planted in the same locations with the exception of Indiana which was not planted. In Illinois, Indiana, Iowa, and Ohio plots were planted in strips and whole plants were sampled in a stratified random method such that each treatment was broken into three equally spaced strata. In Michigan, plots were arranged in a completely randomized block design with six replicates and four to eight plants were collected randomly from each plot. Eight to twelve plants were collected from each stratum in Illinois, Indiana, Iowa, and Ohio, and four to six plants were collected from each plot in Michigan between the V1 (first trifoliate) and V3 (third trifoliate), which occurred 20-48 days post planting (Table 5.1). All plants were collected randomly and sent to Michigan State University for oomycete isolation. Soil was collected at each field site and submitted to the Michigan State University Soil and Plant Nutrient Laboratory for analysis of % sand, % silt, % clay, pH, cation exchange capacity (CEC), and % soil organic matter (% SOM) (Table 5.1).

Table 5.1. Field location and soil properties description.

Location[a]	Year	Coordinates	Planting Date	Date Sampled	Growth Stage	Soil Classification	% Sand	% Silt	% Clay	pH	CEC (meq 100 g^{-1})[b]	% SOM[c]
Michigan	2016	42.69 N, -84.49 W	20-May	16-Jun	V1-V2	Sandy Clay Loam	58.3	23.2	18.5	5.8	10.7	2.0
	2017	42.69 N, -84.49 W	24-May	15-Jun	V1-V2	Sandy Clay Loam	55.2	23.8	21.0	5.9	9.4	1.6
Iowa	2016	42.01 N, -93.79 W	23-May	19-Jul	V2-V3	Clay Loam	41.2	29.8	29.0	5.3	16.5	3.7
	2017	42.01 N, -93.79 W	1-Jun	23-Jun	V1-V2	Clay Loam	39.2	31.8	29.0	6.0	18.9	4.1
Illinois	2016	40.01 N, -88.24 W	9-Jun	30-Jun	V1-V2	Clay Loam	21.1	48.6	30.3	7.0	17.0	3.7
	2017	40.01 N, -88.24 W	23-May	21-Jun	V1-V2	Silty Clay Loam	13.2	49.8	37.0	6.9	21.0	3.7
Indiana	2016	40.67 N, -85.27 W	24-May	28-Jun	V2-V3	Silty Clay Loam	18.1	42.1	39.9	7.3	21.0	3.8
	2017	-	-	-	-	-	-	-	-	-	-	-
Ohio	2016	40.04 N, -84.09 W	3-Jun	7-Jul	V2-V3	Silt Loam	23.9	54.7	21.4	5.2	10.3	2.5
	2017	40.04 N, -84.09 W	1-Jun	21-Jun	V1-V2	Silt Loam	20.2	54.8	25.0	5.0	8.4	2.1

[a]Soybean were planted in 2016 and 2017 except for Indiana which was only planted in 2016

[b]CEC refers to the cation exchange capacity and is measured in the milliequivalents (meq) per 100 g soil

[c]SOM refers to the percent soil organic matter

Table 5.2. Chemical seed treatments and application rates used in this study

Trade name	Active ingredients	Target organism	Application rate (g a.i. 100 kg seed^{-1})
Intego Suite®	Ethaboxam	Oomicide	7.50
	Metalaxyl	Oomicide	2.00
	Clothianadin	Insecticide	50.00
	Ipconazole	Fungicide	2.50
Cruiser Maxx®	Thiamethoxam	Insecticide	50.00
	Mefenoxam	Oomicide	7.50
	Fludioxonil	Fungicide	2.50
	Sedaxane	Fungicide	2.50

Oomycete isolation. Oomycetes were isolated from one taproot and one lateral root section per plant. Isolation was performed as described in Rojas et al. (2017a). Briefly, roots were rinsed with tap water for at least 30 min to remove soil. Then a portion of lateral root tissue, approximately 3.8 to 5 cm, and a 1.5 cm portion of taproot were placed onto corn meal agar medium (CMA) amended with PARPB (pentachloronitrobenzene [PCNB] 50 mg L^{-1}, ampicillin 250 mg L^{-1}, rifampicin 10 mg L^{-1}, pimaricin 5 mg L^{-1}, and benomyl 10 mg L^{-1}) (Jeffers 1986). Coenocytic hyphae were hyphal tipped onto fresh CMA+PARPB medium to obtain pure oomycete cultures.

Oomycete identification. Oomycetes were identified to species by sequencing the internal transcribed spacer (ITS) region of the rDNA (Robideau et al. 2011). In 2016, five 3.7 mm plugs from pure oomycete cultures growing on CMA+PARPB, were transferred into 10% V8 broth containing 0.5 g $CaCO_3$ and grown for seven days. Mycelia were harvested and lyophilized overnight. Approximately 25 – 30 mg of lyophilized mycelial tissue was ground with 150 µl of

lysing matrix A (MP Biomedicals, Houston, TX) and a 4-mm diameter steel ball (SPEX SamplePrep, Metuchen, NJ) using a FastPrep FP120 (Thermo Fisher Scientific, Waltham, MA) and genomic DNA (gDNA) was extracted using the DNeasy plant Mini Kit (Qiagen Sciences Inc., Germantown, MA, USA) or OMEGA Mag-Bind Plant DNA Plus kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. In 2017, one 3.7 mm plug of pure oomycete cultures growing on CMA+PARPB were grown in 1 ml of 10% V8 broth in a 2 ml 96 deep-well plate. One well not containing a growing isolate was used as a negative control to assess any cross contamination. Porous sealing tape (Aera Seal™; RPI research products, Mount Prospect, IL) was applied to the 96 deep-well plate to allow gas exchange and avoid contamination. After seven days, the plate was frozen at -20ºC and lyophilized overnight. Following lyophilization, one sterile 4-mm diameter steel ball (SPEX SamplePrep, Metuchen, NJ) was added to each well along with 100 µl DNA extraction solution. The DNA extraction solution consisted of a final concentration of 0.5 M Tris, 0.25 M KCl, 1 x 10^{-3} M EDTA and pH 9.5-10. Lyophilized mycelium in extraction solution was ground within the 96 deep-well plate using a Geno/Grinder (SPEX SamplePrep, Metuchen, NJ) at 1000 rpm for 30 seconds. Following grinding, the 96 deep-well plate was centrifuged at 5,000 G for 1 minute then incubated at room temperature for 10 min, followed by incubation for 10 min at 95ºC. An aliquot of 300 µl of 3% bovine serum albumin (BSA) solution was added to each well of the 96 deep-well plate.

Polymerase chain reaction (PCR) consisted of a final concentration of 1X DreamTaq buffer containing 4 mM $MgCl_2$, 0.2 mM dNTP, 0.5 U of Dream Taq polymerase, 0.5 µM ITS6 and ITS4 primers and either 1 µl of 1:10 diluted genomic DNA or 2 µl of the extraction solution in 96 well plates. Thermal cycling conditions were as follows: 94ºC for 3 min, followed by 35 cycles of 94ºC for 45 sec, 55ºC for 45 seconds and 72ºC for 1 min, followed by a final extension

at 72ºC for 7 min. Successfully amplified ITS amplicons were determined by gel electrophoresis and purified by adding 5 µl containing 3 U exonuclease I and 0.5 U shrimp alkaline phosphatase (Thermo Scientific) and incubating at 37ºC for 45 min followed by an enzyme inactivation at 85ºC for 10 min. Amplicons were Sanger-sequenced via Macrogen USA (Rockville, Maryland) or at the Michigan State University Genomics core. Forward and reverse sequences were trimmed and assembled with Codon Code Aligner v4.2.7 and consensus sequences were compared against a curated set of oomycete sequences (Robideau et al. 2011; Levesque et al. 2004) using BLAST. Sequences with BitScore > 1000 and a sequence identity > 97% were given a species epithet (Rojas et al. 2017a). If an ITS sequence did not match any sequences in the curated set of oomycete sequences sufficiently, the ITS sequence was used in a BLAST search against the entire nucleotide database on NCBI to determine the closest match outside the curated database.

Oomicide sensitivity. Determination of the sensitivity of 313 oomycete isolates to ethaboxam and 360 oomycete isolates to mefenoxam was carried out using the high-throughput method of Noel et al. (2019). Briefly, technical grade ethaboxam was dissolved in 99.5% acetone. Mefenoxam was dissolved in dH_20 and filter-sterilized by passing the solution through a 0.22 µm filter. Isolates were tested in dilute V8 broth (dV8B: 82 ml V8 juice filtered through eight layers of cheesecloth, 0.5g $CaCO_3$, 918 ml distilled water) amended with ethaboxam or mefenoxam at the concentrations 0, 0.01, 0.1, 1, 10 and 100 µg ml^{-1} in 96-well flat bottom microtiter plates (Model 3620, Corning Inc., Kennebunk, ME). dV8B amended with 0.0995% (v/v) acetone or water was used for the ethaboxam or mefenoxam control, respectively. The optical density at 600 nm (OD_{600}) for each isolate was recorded at 24 and 48 hours. Z'-factor and growth curves were used for quality control metrics (Noel et al. 2019). Quality filtered optical density readings for

each isolate were transformed to percent relative growth by dividing the mean optical density of each oomicide concentration by the mean OD_{600} without oomicide, multiplied by 100. The EC_{50} (effective concentration to reduce OD_{600} by 50% when compared to the non-amended control) was estimated for each isolate.

Statistical Analyses. All statistical analyses were carried out in Rv3.5.2 (R Core Team 2018). A binomial generalized linear model was used to test the effect of location, year, tissue, and seed treatment on isolation success or failure. A permutational multivariate analysis of variance (PERMANOVA) on Jaccard distances (presence/absence), was used to test for differences in community centroids due to location, year, and interactions using the 'adonis2' function in the package 'vegan' (Oksanen et al. 2018). Dose response analysis was carried out with the 'drc' package (Ritz and Streibig, 2015). Percent relative growth was modeled against log transformed doses using a four-parameter log-logistic model. The absolute EC_{50} was estimated by solving for the concentration where 50% inhibition occurred (Noel et al. 2018). All code generated for statistical analysis was uploaded to GitHub (https://github.com/noelzach/Oomycete-Isolation-from-seed-treatments). All sequences were deposited into GenBank under the accession numbers MK794623-MK795061.

Results

Oomycete isolation. In 2016, 369 oomycete isolations were attempted from both taproot and lateral root sections across all the locations. In 2017, an additional 99 isolations were attempted from both taproot and lateral root sections for a total of 468 attempted oomycete isolations across all the locations. At least one oomycete was successfully isolated from 102 taproot sections or 129 lateral root sections in 2016. In 2017, oomycetes were successfully isolated from 31 taproot and 116 lateral root sections (Table 5.3). Multiple oomycete isolates were obtained from the

same plant (i.e., taproot and lateral root combined) 53 times (14.4%) in 2016 or 20 times (4.3%) in 2017 across all locations. From those instances multiple species were isolated 81.1% of the time in 2016 or 75.0% of the time in 2017. In contrast, multiple oomycete species were isolated from the same lateral root or taproot section 33 times (14.3%) in 2016 and 10 times (7.0%) in 2017. In total, 121 isolates were recovered from taproot sections and 153 isolates were recovered from lateral root sections in 2016. In 2017, 45 isolates were collected from taproot sections and 120 oomycete isolates from lateral roots. In Michigan 2016 and Ohio 2017 the success of isolating an oomycete from lateral root tissue was significantly greater ($P < 0.004$) than from taproot tissue (Table 5.4). This phenomenon contrasted with Iowa in 2016 as the success of isolating an oomycete from the soybean taproot was significantly greater than from lateral roots ($P = 0.001$). In other site years, the success of isolating an oomycete from root or taproot tissue from non-treated seed was not significantly different from each other (Table 5.4).

Seed treatment influence on oomycete isolation. Seed treatment significantly reduced the oomycete isolation rate from soybean roots in Illinois in 2016, but not in other location-year combinations (Fig. 1). In Illinois in 2016, the success of isolating oomycetes from non-treated roots or roots treated with mefenoxam was 67% or 42%, respectfully. The success of isolation from lateral root sections treated with ethaboxam and metalaxyl was significantly reduced to 8% when compared to lateral root sections from non-treated seed ($P = 0.039$) or lateral root sections from seed treated with mefenoxam ($P = 0.001$) (Fig. 5.1).

Table 5.3. Summary of oomycete isolation successes from stem or root tissue from all location-year-treatment combinations

		2016					2017				
			Successful Attempts		Number of oomycete isolates			Successful Attempts		Number of oomycete isolates	
Location	Treatment[a]	Attempted	Taproot	Lateral root	Taproot	Lateral root	Attempted	Taproot	Lateral root	Taproot	Lateral root
Michigan	NTC	24	1	17	1	24	36	3	8	3	8
	Ethaboxam + Metalaxyl	28	1	22	1	27	36	0	7	0	7
	Mefenoxam	29	2	14	2	16	36	0	11	0	11
Iowa	NTC	24	16	4	23	6	48	15	9	22	9
	Ethaboxam + Metalaxyl	24	20	5	25	5	48	6	11	9	11
	Mefenoxam	24	16	4	20	4	48	6	7	10	8
Illinois	NTC	24	12	16	13	22	36	0	11	0	11
	Ethaboxam + Metalaxyl	24	7	2	9	2	36	0	3	0	3
	Mefenoxam	24	8	10	8	10	36	0	9	0	9
Inidanna	NTC	24	7	9	7	9	-	-	-	-	-
	Ethaboxam + Metalaxyl	24	7	4	7	4	-	-	-	-	-
	Mefenoxam	24	4	4	4	5	-	-	-	-	-
Ohio	NTC	24	1	5	1	5	36	0	13	0	13
	Ethaboxam + Metalaxyl	24	0	6	0	6	36	0	12	0	12
	Mefenoxam	24	0	7	0	8	36	1	15	1	18
Total		369	102	129	121	153	468	31	116	45	120

[a]Treatments refer to the non-treated control (NTC), Intego Suite® (Ethaboxam + Metalaxyl), and Cruisermaxx® (Mefenoxam)

Table 5.4. Predicted probability of isolating oomycete from non-treated lateral root or taproot sections of soybean roots in each location-year combination.

		Tissue		
Location	Year	Lateral root	Taproot	*P*-value[b]
Michigan	2016	70.8 ± 9.3[a]	4.2 ± 4.1	< 0.001
	2017	22.2 ± 6.9	8.3 ± 4.6	0.107
Iowa	2016	16.7 ± 7.6	66.7 ± 9.6	0.001
	2017	18.8 ± 5.6	12.5 ± 4.8	0.278
Illinois	2016	66.7 ± 9.6	50.0 ± 10.2	0.251
	2017	30.6 ± 7.7	0.0 ± 0.0	0.980
Indiana	2016	37.5 ± 9.9	29.2 ± 9.3	0.112
	2017	-	-	-
Ohio	2016	20.8 ± 8.3	4.2 ± 4.1	0.114
	2017	41.7 ± 8.2	2.8 ± 2.7	0.003

[a]Values represent the % probabilities ± standard error predicted from a binomial generalized linear model of isolating an oomycete from a non-treated section of soybean lateral root or taproot.

[b]*P*-value represents the probability of rejecting the null hypothesis that the probability of isolating oomycetes from root or taproot tissue are the same ($\alpha = 0.05$).

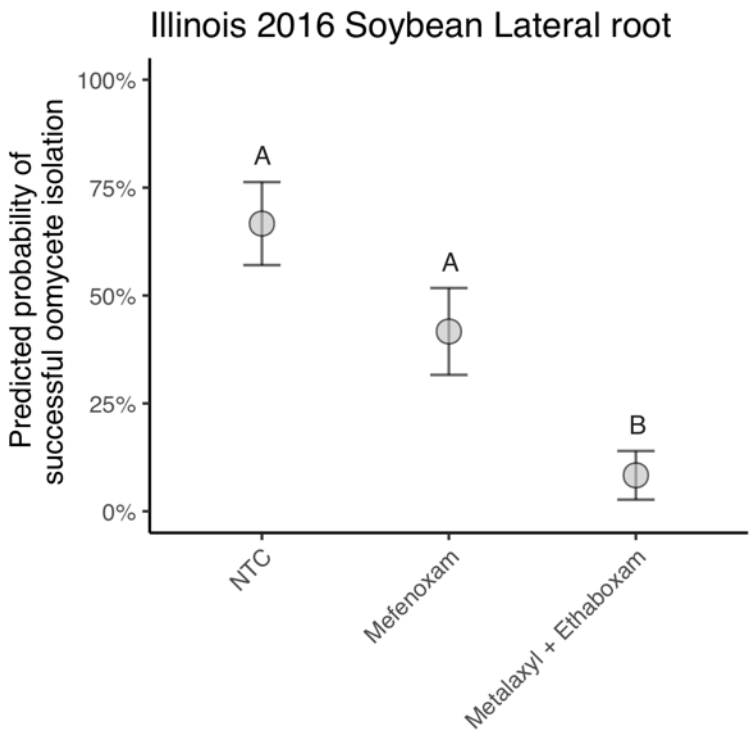

Figure 5.1. Oomycete isolation success from soybean lateral roots from non-treated seeds (NTC), mefenoxam treated seeds, or metalaxyl and ethaboxam treated seeds in Illinois 2016. Seed treatments containing ethaboxam and metalaxyl reduced the recovery of oomycete isolation from soybean lateral roots from Illinois in 2016 (n = 24) when compared to non-treated roots. Different letters above points indicate that the null hypothesis was rejected that the probability of isolation success was equal ($\alpha = 0.05$) with a Tukey P-value adjustment.

Oomycete species distributions. Twenty-four oomycete species were identified across all location-year combinations (Fig. 5.2A; Fig. 5.2B), Nineteen species were identified in 2016 and 18 species were identified in 2017, and 13 species were common to both years (Fig. 5.2A). The five most abundant species isolated across both years was *Pythium sylvaticum* (n = 127, 28.9%),

Pythium heterothallicum (n = 64, 14.3%), *Pythium ultimum* var. *ultimum* (n = 52, 11.8%), *Pythium attrantheridium* (n = 35, 7.9%), and *Pythium irregulare* (n = 29, 6.6%) (Fig. 5.2A). *Pythium sylvaticum* was the most abundant species in both years at 37.8% frequency in 2016 and 23.7% in 2017 (Fig. 5.2A) and was often present at Michigan and Ohio field sites (Fig. 5.2B; Fig. 5.3). *Pythium ultimum* var. *ultimum* was more abundant in 2016 (17.5%) compared to 2017 (2.5%) (Fig. 2A) and was often present at Michigan and Indiana field sites (Fig. 5.2B; Fig. 5.3). *Pythium irregulare* was isolated more frequently in 2016 (7.6%) than in 2017 (5.0%) and was often present in Michigan and Ohio field sites (Fig. 5.2B; Fig. 5.3). *Pythium heterothallicum* was often present in Iowa and the Illinois 2016 field sites (Fig. 5.2B; Fig. 5.3) and made up 14.6% and 15% of the isolates in 2016 and 2017, respectfully. *Pythium attrantheridium* was often present in Michigan and Ohio field sites (Fig. 5.2B; Fig. 5.3) and was isolated at a frequency of 4.7% in 2016 and 12.5% in 2017 (Fig. 5.2A).

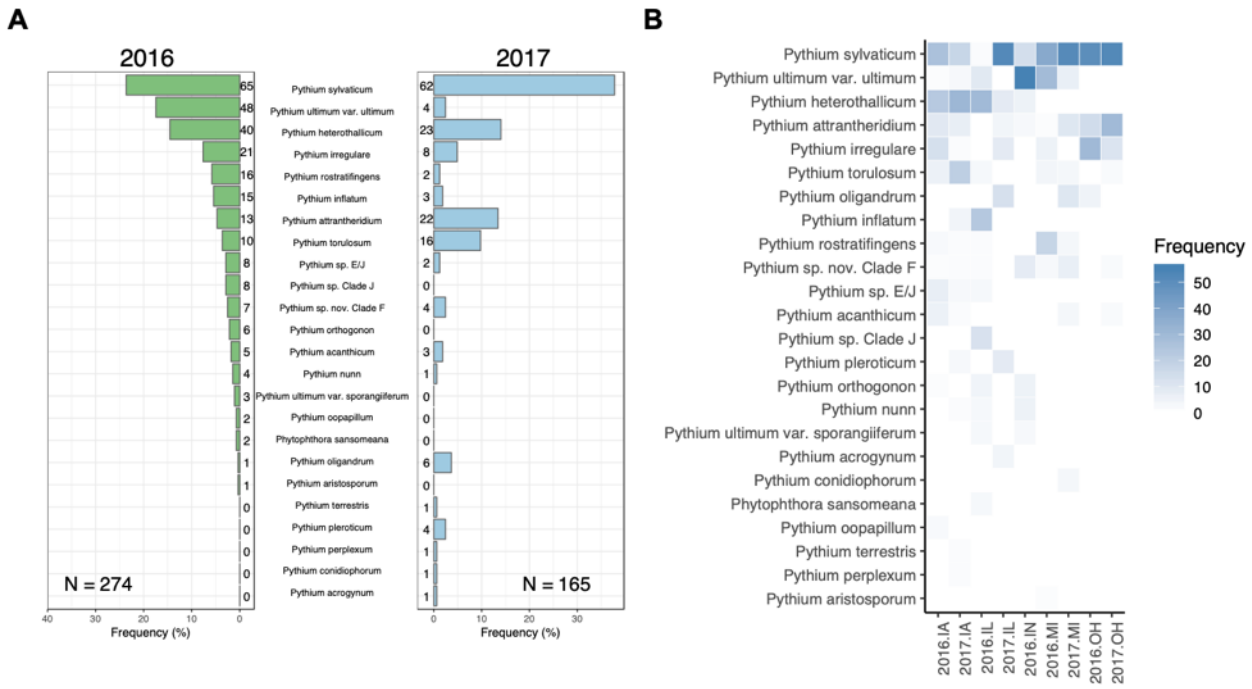

Figure 5.2. Oomycete species frequency distributions. (A) Frequency of each species in 2016 and 2017 across all locations. The number of isolates (n) is shown next to each species name. (B) Frequency in each location-year combination.

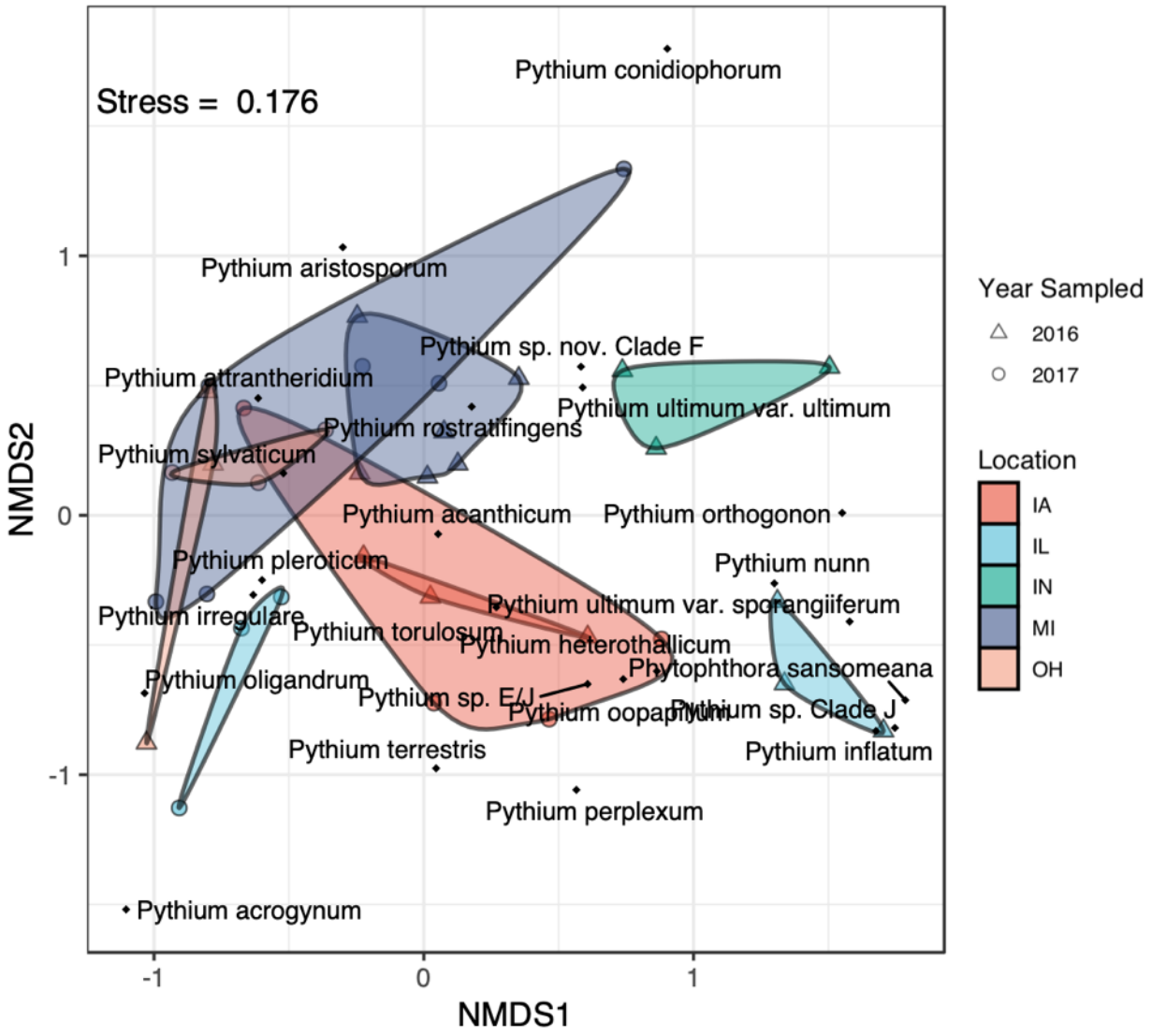

Figure 5.3. Non-metric multidimensional scaling (NMDS) ordination of Jaccard (presence-absence) distances (k = 2). Jaccard distances were calculated from the incidence of species summed across levels of seed treatment and isolation tissue (i.e., taproot or root tissue) to examine species associated with each location-year combination. Species appear closest to each respective location-year combination. Stress indicates how well the ordination reflects Jaccard distances.

Other species isolated less frequently included *Phytophthora sansomeana* (0.7% in 2016 and not isolated in 2017), *Pythium acanthicum* (1.8% in 2016 and 1.8% in 2017), *Pythium acrogynum* (not isolated in 2016 and 0.6% in 2017), *Pythium aristosporum* (0.4% in 2016 and not isolated in 2017), *Pythium conidiophorum* (not isolated in 2016 and 0.6% in 2017), *Pythium inflatum* (5.5% in 2016 and 1.9% in 2017), *Pythium nunn* (1.5% in 2016 and 0.6% in 2017), *Pythium oligandrum* (0.4% in 2016 and 3.7% in 2017), *Pythium oopapillum* (0.7% in 2016 and not isolated in 2017), *Pythium orthogonon* (2.2% in 2016 and not isolated in 2017), *Pythium perplexum* (not isolated in 2016 and 0.6% in 2017), *Pythium pleroticum* (not isolated in 2016 and 1.9% in 2017), *Pythium rostratifingens* (5.5% in 2016 and 1.2% in 2017), *Pythium* sp. Clade J (2.9% in 2016 and not isolated in 2017), *Pythium* sp. Clade E/J (2.9% in 2016 and 1.2% in 2017), *Pythium* sp. Clade F (2.5% in 2016 and 2.5% in 2017), *Pythium terrestris* (not isolated in 2016 and 0.6% in 2017), *Pythium torulosum* (3.6% in 2016 and 9.3% in 2017), and *Pythium ultimum* var. *sporangiiferum* (1.9% in 2016 and not isolated in 2017) (Fig. 5.2A). Community composition (presence/absence) was significantly different based on location and year ($P < 0.001$) (Fig. 5.3).

Three taxonomic groups named *Pythium* sp. Clade E/J, *Pythium* sp. Clade J, and *Pythium* sp. nov. Clade F, were not assigned to a species level. Sequences assigned to *Pythium* sp. Clade E/J were found in Iowa and Illinois and ITS sequences had an 85-86% identical match to *Pythium marisipium* CBS 773.81 when compared to sequences from the curated set of oomycete sequences. However, when used in a BLAST search against sequences outside of this database they had a 99% match to *Pythium* sp. UZ182 (AB468789.1) (Uzuhashi et al. 2010). *Pythium* sp. UZ182 was closely related to species within *Pythium* clades E or J in a multigene phylogeny (Uzuhashi et al. 2001). *Pythium* sp. Clade J was only found in Illinois in 2016 and had a 90-91%

match to *Pythium perplexum* CBS 674.85 in the curated database and a 98-99% identity ITS sequences identified as *Pythium nodosum, Pythium perplexum*, or *Pythium yorkensis* using BLAST search. *Pythium* sp. nov. Clade F was isolated from all locations and had a 98-100% match to ITS sequences from *Pythium* sp. CAL_2011F (HQ643829, HQ643777, HQ643800).

Oomicide sensitivity. For ethaboxam, almost three-quarters (76.6%) of isolates had an EC_{50} < 1 μg ml^{-1} and 12.0% of isolates had an EC_{50} between 1 μg ml^{-1} and 9 μg ml^{-1} (Fig. 4A). The rest of the isolates (11.4%) tested against ethaboxam had an EC_{50} ranging from 12.08 μg ml^{-1} to > 100 μg ml^{-1} (Fig. 4A). The EC_{50} for *Pythium torulosum* ranged from 4.88 μg ml^{-1} to > 100 μg ml^{-1} and *Pythium rostratifingens* isolates ranged from 30.91 μg ml^{-1} to > 100 μg ml^{-1} (Fig. 4A). All five of the most abundant species were sensitive to ethaboxam with mean EC_{50} ranging from 0.35 to 0.97 μg ml^{-1} (Table 5.5). The ethaboxam EC_{50} of the most abundant species, *Pythium sylvaticum*, ranged from < 0.01 to 2.17 μg ml^{-1}, and 98% of *Pythium sylvaticum* isolates tested (n = 96) had an EC_{50} less than 1 μg ml^{-1} (Table 5.5). The mean EC_{50} of *Pythium ultimum* var. *ultimum* was 0.97 ± 0.149 and ranged from 0.136 to 6.660 μg ml^{-1}, but 88% of isolates tested had an EC_{50} less than 1.5 μg ml^{-1} of ethaboxam (Table 5.5). All *Pythium heterothallicum* (n = 27) and *Pythium irregulare* (n = 22) isolates tested had EC_{50} values < 1 μg ml^{-1}. For *Pythium attrantheridium*, the EC_{50} distribution to ethaboxam ranged from 0.02 to 2.77 μg ml^{-1}, though 26 of the 27 isolates tested had an EC_{50} less than 1 μg ml^{-1} (Table 5.5). For mefenoxam, 69.7% of isolates tested across all species tested had an EC_{50} less than 0.5 μg ml^{-1} and the maximum EC_{50} estimated was 0.62 μg ml^{-1} (Fig. 5.4B). The most abundant species, *Pythium sylvaticum*, had an EC_{50} ranging from 0.01 μg ml^{-1} to 0.18 μg ml^{-1} of mefenoxam and the mean EC_{50} of the 108 isolates tested was 0.04 ± 0.002 (Table 5.5). The mean EC_{50} for the four other most abundant species, (*Pythium*

heterothallicum, *Pythium ultimum* var. *ultimum*, *Pythium attrantheridium,* and *Pythium irregulare*) was ≤ 0.06 µg ml^{-1} mefenoxam (Table 5.5).

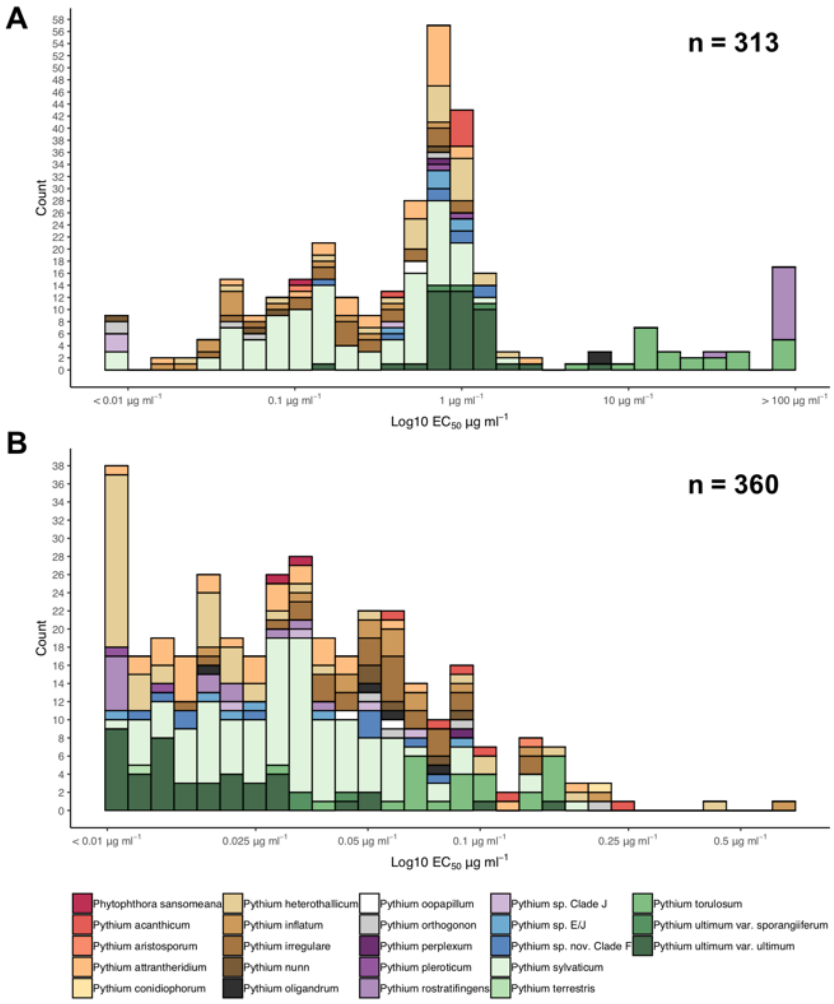

Figure 5.4. EC_{50} (effective concentration to reduce OD_{600} by 50% when compared to the non-amended control) distribution for (A) ethaboxam (n = 313 isolates) and (B) mefenoxam (n = 360). Colors of bars represent species. The x-axis was plotted on the log scale, and labels were back-transformed for visualization.

Table 5.5. Mean EC_{50} estimates for the five most abundant species to ethaboxam and mefenoxam

		Ethaboxam		Mefenoxam	
Species	Number of isolates collected	Number of isolates tested	$EC_{50} \pm SE$[a]	Number of isolates tested	$EC_{50} \pm SE$
Pythium sylvaticum	128	98	0.37 ± 0.037	108	0.04 ± 0.002
Pythium heterothallicum	64	27	0.73 ± 0.083	47	0.04 ± 0.009
Pythium ultimum var. ultimum	52	42	0.97 ± 0.149	43	0.02 ± 0.004
Pythium attrantheridium	33	27	0.56 ± 0.102	31	0.03 ± 0.005
Pythium irregulare	29	22	0.35 ± 0.064	27	0.06 ± 0.006

[a]Means with different letters denote that the null hypothesis was rejected that the mean EC_{50} was the same in a one-way analysis of variance (ANOVA) and Tukey *P*-value adjustment ($\alpha = 0.05$).

Discussion

The objectives of this study were to isolate and identify oomycete isolates associated with taproot or lateral root sections, to understand the extent to which seed treatments containing the oomicides mefenoxam (CruiserMaxx®) or metalaxyl and ethaboxam (Intego Suite®) reduce the recovery of oomycetes from taproot or lateral root sections, and to investigate the variation in sensitivity to mefenoxam and ethaboxam of the recovered isolates. Notably, this study demonstrated that the success of isolating oomycetes from soybeans depended on location, year, tissue, and seed treatment. Additionally, the distribution of oomycete species was dependent on location and year. Inter- and intraspecific variation in sensitivity to ethaboxam and mefenoxam was present, but interspecific variation in ethaboxam sensitivity was greater than that of mefenoxam. No isolate was insensitive to both mefenoxam and ethaboxam.

The purpose of performing isolations from soybean roots from seed that had been non-treated or treated with either mefenoxam or metalaxyl and ethaboxam was to first determine if fewer oomycetes would be isolated from those seed treated roots compared to non-treated seed roots. This hypothesis was supported in this study for only Illinois in 2016 lateral roots, but not other location-year combinations. Illinois had heavier soils compared to most other locations in this study with 37.0% and 30.3% clay, and 49.8% and 48.6% silt (Table 1). According to the PRISM Climate Group database (Prism Climate Group, Oregon State University, 2016), the Illinois location in 2016 experienced approximately 10 cm rain two weeks after planting compared to less than 7.3 cm at other locations. This trend of precipitation did not occur in Illinois 2017 in which only 1.7 cm rain fell two weeks after planting, which might be one of the reasons a difference in isolation was not observed in 2017. The weather conditions in Illinois combined with the soil texture in 2016 may have increased favorable conditions for oomycete

growth and this likely resulted in increased colonization on non-treated roots compared to roots treated with mefenoxam or ethaboxam and metalaxyl.

No increase in ethaboxam insensitive isolates was detected from isolates recovered from the ethaboxam and metalaxyl combination treated seed. Additionally, no shift was detected in oomicide sensitivity when the combination treatment was used in the sample plots for a successive year. Due to the known spectrum of ethaboxam activity, it is recommended that the product is used in combination with metalaxyl/mefenoxam. To test if the use of ethaboxam alone would create a shift in oomycete species or oomicide sensitivities, additional studies could be conducted where ethaboxam is used as a standalone treatment for oomycetes. Instead, future studies could use ethaboxam only seed treatment to address this question. The isolates with insensitivity to ethaboxam collected in this study corroborates previous studies that demonstrated that *Pythium rostratifingens* and *Pythium torulosum* are insensitive to ethaboxam (Noel et al. 2019a,b; Rojas et al 2019). It is hypothesized that a C239S substitution in the β-tubulin protein sequence is a causal mutation resulting in inherent differences in ethaboxam sensitivity (Noel et al. 2019b). Several *Pythium rostratifingens* isolates used in this study contained the C239S substitution (*data not shown*). *Pythium rostratifingens* and *Pythium torulosum* are not thought to be highly aggressive to soybean (Matthiesen et al. 2015; Rojas et al. 2017a). In contrast, the five most abundant species recovered in this study are recognized as highly aggressive species to soybean seeds or roots (Broders et al. 2007; Rojas et al. 2017a) and are considered sensitive to ethaboxam and mefenoxam.

No shift in isolate sensitivity to mefenoxam was detected from isolates recovered from mefenoxam containing seed treatments, even when used for two consecutive years. This observation may also indicate that there is little selection pressure or that the isolates had

colonized soybean tissues after the seed treatment had degraded and was no longer active. Seed treatments are generally thought to be active for approximately 2-3 weeks post planting. In situations where mefenoxam resistance is problematic, seed treatments containing ethaboxam have been effective in reducing infection from metalaxyl or mefenoxam resistant *Pythium ultimum* isolates in Pacific Northwest chickpea (*Cicer arietinum*) and hard red spring wheat (*Triticum avesticum* L.) fields (Chen and Van Vleet 2016; White et al. 2019).

There is a lack of information regarding the spatiotemporal activity of seed treatments given different biotic and abiotic factors. Mefenoxam can influence the functional diversity of the microbial community. For example, Demanou et al. (2006) found that *Nitrosospira*-like bacteria were major contributors to nitrification activity in mefenoxam applied soil. Monkiedje and Spiteller (2005) found that distinct microbial communities were capable of degrading metalaxyl and mefenoxam at different rates. Similarly, Park et al. (2002) concluded that ethaboxam was efficiently broken down by the soil fungus *Cunninghamella elegans*. Thus, seed treatments may be more or less active depending on the microorganisms in the soil and how quickly the chemistries are broken down. Additionally, seed treatment chemistries can differ in translocation in the plant or in the soil profile. Camargo et al. (2019) demonstrated that the concentration of mefenoxam in above ground soybean tissue (i.e. leaves and flowers) was below 2 ng g^{-1} when applied as a seed treatment. Therefore, it is difficult to accurately assess the selection pressure posed by seed treatments in specific soil profiles and environmental conditions.

Oomycete communities in this study were significantly structured by location and year, which corroborates previous studies (Broders et al. 2009; Rojas et al. 2017a, b). Along with Rojas et al. (2017a) *Pythium sylvaticum* was found to be the most abundant species in Michigan,

Illinois. However, the second most abundant species in Rojas et al. (2017) was *Pythium oopapillum* which was not frequently isolated in this study. However, *Pythium oopapillum* was only isolated in Iowa in this study, which corroborates Rojas et al. (2017) since about 50% of the species composition in Iowa consisted of *Pythium oopapillum* isolates.

Intriguingly, this study demonstrated that multiple species coexist on the same plant at high frequencies, but less frequently from the same taproot or lateral root section. Multiple oomycete species are known to colonize the same soybean plant (Broders et al. 2009; Schmitthenner and Bhat 1994), but this has not been documented in detail as it was in this study. This could indicate that competition among different oomycete species for resources excludes two species from coexisting on a single taproot or lateral root piece, but that competition for resources in different plant tissues is less severe. In future studies it will be important to study co-occurrence and spatiotemporal species distribution in more depth with additional isolation strategies or amplicon sequencing. Amplicon sequencing enables a greater depth of sampling which could aid in the power to detect differences in species distributions and co-occurrence patterns.

The results of this study demonstrated that the success of isolating an oomycete from soybean roots can depend on the tissue, location, and seed treatment. Our results also corroborate previous studies showing that oomycete communities on soybean roots are largely structured by location, and therefore, the decision of seed treatment for protection against oomycete pathogens depends on the unique combination of oomycete species present, the soil conditions in the field, and the weather conditions. Furthermore, due to the species present in fields and differences in sensitivity to ethaboxam and mefenoxam seed treatments, seed treatments containing both chemistries are important for management of oomycete species associated with soybean root.

Chapter 6

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

This chapter has been submitted to *Applied Soil Ecology*: Noel Z.A., Chang, H.X., and Chilvers, M.I. **Variation in soybean rhizosphere oomycete communities from Michigan fields with contrasting disease pressures.**

Abstract

Although oomycete species can contribute to significant losses in soybean plant density, root mass and yield, they are often underrepresented in high-throughput sequencing studies. In this study, soybean oomycete rhizosphere communities were characterized over two years from locations with and without historical disease pressure. The goals of this research were to examine the effect of location, soybean genotype, and seed treatment on oomycete communities. Soybean oomycete rhizosphere communities were dominated by *Pythium*, but community composition differed depending on the location and year. *Pythium ultimum var. ultimum* was the most abundant oomycete OTU accounting on average for more than 30% relative abundance in high disease pressure sites. However, sites without historical disease pressure were not devoid of oomycete plant pathogens indicating that historical disease pressure may be due to an imbalance of species, rather than simply the presence or absence of highly pathogenic species. High-disease pressure sites contained more oomycete taxa and were less even in 2015. There was no substantial evidence of seed treatment or genotype impacting oomycete community composition or diversity, however, plant density and root biomass increased with the addition of neonicotinoid insecticides. Overall, this study represents an improvement of our understanding of oomycete communities in soybean rhizosphere and the impacts of agronomic factors on oomycete diversity.

Introduction

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

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

In previous studies, over 80 oomycetes species belonging to genera *Phytophthora*, *Pythium*, *Phytopythium*, and *Aphanomyces* were identified to be associated with soybean (Rojas et al. 2017a,b; Broders et al. 2009; Zitnick-Anderson and Nelson 2015). While some oomycetes such as *Phytophthora sojae* or *Pythium ultimum* are well known to be pathogenic, others are weakly pathogenic or may be mycoparasitic or entomopathogenic (Martin and Loper 1999, Paul

et. al. 1999, Su et al., 2001; Sholte et al. 2004, Ribeiro and Butler, 1995) suggesting potential complex and multi-kingdom interactions. Therefore, studying the oomycete community and its association with disease severity and agricultural practices will provide information for improved oomycete disease management.

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

It has been recognized that location and edaphic factors are drivers of oomycete diversity (Rojas et al. 2017ab; Broders et al. 2009; Zitnick-Anderson and Nelson 2015, Taheri et al. 2017). However other agronomic factors have not been examined in detail. For example, soybean

genotypes have been shown to recruit different taxa of beneficial bacterial (Mendes et al., 2014). Additionally, there is inter- and intraspecific variation in sensitivity to anti-oomycete chemicals (oomicides) used within soybean seed treatments (Broders et al. 2007; Matthiesen et. al. 2016; Noel et al. 2019; Radmer et al. 2017; Weiland et al. 2014) suggesting the possibility of a specific oomycete lineages being selected or counter-selected in the soybean rhizosphere in the presence in different oomicide-genotype combinations. Moreover, because soybean seed treatments usually contain oomicides along with many other active ingredients such as fungicides, nematicides, or insecticides, the likelihood of these chemicals influencing seedling rot diseases or shaping the structure of oomycete communities is considerable. For example, soybean seed treatments have been observed to be more effective and consistent in field sites in Allegan county of Michigan, where heavy oomycete damage has been observed (Rossman et al. 2018). Herein, this study aimed to provide a profile of the oomycete community present in soybean rhizosphere soils and compare the structure of oomycetes communities between high disease pressure fields in Allegan county and low disease pressure field sites in Ingham county of Michigan. To accomplish this, we used next generation amplicon sequencing to characterize oomycete communities between these two counties in two years. We investigated the association between oomycetes community and disease severity as well as seed treatments and soybean genotype. Additionally, we identified the taxonomic difference of the oomycete that links to the disease pressures between these sites.

Materials and Methods

Experimental design and field setup. Field experiments were set up in two locations, Allegan county (with high disease stress) and Ingham county (with low disease stress) of Michigan, in two years (2015 and 2016). In each location-year combination, a complete randomized factorial

design with four soybean genotypes, four seed treatments, and six replicates was set up in plots (6.10 m by 3.05 m), which resulted in a total of 96 plots in each of four location-year combinations. Full seed treatment formulations and application rates were described in Rossman et al. (2018). In brief, seed treatments used in this study were generalized based on the target pests, herein abbreviated as non-treated control (NTC), fungicides (F), fungicides plus insecticides (FI), and fungicides plus insecticides plus a biological control nematode protectant (FIN) (Table S.6.1). The fungicide component contained the oomicides metalaxyl or mefenoxam. Soybean seeds were planted 3.8 cm below ground, in six rows with 38 cm row spacing, and at a seeding rate of 395,000 seeds ha^{-1}. In all locations, the crop planted in the previous growing season was corn. The coordinates, plating dates, plant sampling dates, and precipitation occurring two weeks after planting for each location-year combination along with bulk soil texture and nutrient levels for each location-year as characterized by the MSU Soil and Plant Nutrient Laboratory were documented (Table 6.1).

Table 6.1 Field location, soil properties, and weather description.

						Soil Texture			Soil Nutrients[ab]			Weather summary two weeks after planting	
Year	Location	Coordinates	Planting Date	Date Sampled	Soil Class.	% Sand	% Silt	% Clay	pH	CEC	SOM	Sum precip. (mm)	Min. ave. temp. (ºC)
2015	Allegan	42.70 N, -86.01 W	29-May	16-Jun	Sandy Loam	56.4	25.7	17.9	6.8	8.7	3.4	46.61	12.13
	Ingham	42.69 N, -84.50 W	23-May	9-Jun	Loam	46.3	35.7	18.0	7.1	9.4	3.2	48.8	11.8
2016	Allegan	42.70 N, -86.01 W	19-May	13-Jun	Loam	42.4	36.5	21.1	5.5	11.6	3.0	9.05	12.12
	Ingham	43.05 N, -84.40 W	20-May	9-Jun	Sandy Loam	66.4	17.4	16.2	5.5	10.6	3.0	9.88	13.33

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

Sample collection. For each location-year combination, three measurements were taken for disease stresses, including plant density, root biomass, and yield. The four middle rows in each six-row plot were harvested for yield quantification at the end of the season, and plant density was measured by counting the number of emerged soybeans in two of the four harvested rows of each plot at the first trifoliate growth stage. Meanwhile, rhizosphere samples were collected from two side rows (non-harvested rows), and ten random emerged plants (excluding plants within 2.74 meters of either end of a row) were collected in each non-harvested row. Loosely adhering soil was shaken from the roots, and these twenty plants were pooled to represent a plot and stored together on ice and transported to the lab for processing the following day. Root tissue was determined based on the soil line, and ten random roots were cut, washed with tap water, and oven dried before measuring the root biomass. The remaining ten roots were used for rhizosphere soil collection. Rhizosphere soil was washed from roots by vortexing for 15 seconds in a 50ml tube with 35ml 10mM NaCl solution (Shakya et al., 2013). Roots were removed from the tube, and the solution was centrifuged for 10 minutes at 3500 rpm to pellet rhizosphere soil. The supernatant was decanted, and the rhizosphere pellet was frozen at -20ºC then lyophilized and stored in sterile coin envelopes with desiccants before DNA extraction.

Oomycetes ITS1 amplification and sequencing. For rhizosphere soil samples, total DNA was extracted from 0.35 g of lyophilized rhizosphere soils using the Qiagen PowerMag® Soil DNA Isolation Kit (Toronto, ON, Canada) following the manufacturer's recommendations. A DNA extraction negative control and artificial oomycetes community (Rojas et al. 2019) containing the genomic DNA of 15 oomycete species mixed equivalently and adjusted to a final concentration of 0.05 ng μL^{-1} were included in polymerase chain reaction (PCR) amplification for internal

transcribed spacer 1 (ITS1) of oomycetes using a three-step PCR program modified based on the protocol from Lundberg et al. (2013) which uses primers with frameshifts to increase nucleotide diversity and instead of a Phix spike-in. In the PCR step one, samples were amplified using primers ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS7 (5'-AGCGTTCTTCATCGATGT-3') (Cooke et al. 2000) with an annealing temperature of 59ºC, which preferentially amplifies oomycetes ITS1 while minimizes fungal ITS amplification (Sapkota and Nicolaisen 2015). In the PCR step two and step three, ITS1 amplicons were amplified with frameshift primers and then with a ten bp barcode plus Illumina adapters, respectfully. All PCR steps contained a final concentration of 1X DreamTaq buffer (ThermoFisher Scientific, USA), 0.2mM dNTP, 0.8mg ml^{-1} bovine serum albumin (BSA), 0.2 µM primers and 1 U DreamTaq Polymerase (ThermoFisher Scientific, USA) and 2 µl DNA template for the PCR step one and step two. The PCR step three contained 4 µl aliquots from PCR step two. Thermal cycling conditions for PCR step one were as followed: 95ºC for 5 min followed by 15 cycles of 95ºC for 15 seconds, 59ºC for 30 seconds and 72ºC for 30 seconds followed by a final extension at 72ºC for 7 min. Thermal cycling conditions for PCR step two were as followed: 95ºC for 5 min followed by ten cycles of 95ºC for 20 seconds, 57ºC for 30 seconds and 72ºC for 35 seconds followed by a final extension at 72ºC for 7 min. Thermal cycling conditions for PCR step three were as follows: 95ºC for 5 min followed by ten cycles of 95ºC for 20 seconds, 63ºC for 50 seconds and 72ºC for 1 minute 20 seconds followed by a final extension at 72ºC for 7 min. PCR products were normalized using SequalPrepTM Normalization Plate Kit (ThermoFisher Scientific, USA), pooled then concentrated 20:1 with Amicon® Ultra 0.5 mL filters (EMDmillipore, Germany). The amplicon library was purified, and size selected with Agencourt AMPure XP magnetic beads at 0.6X sample to bead volume (Beckman Coulter,

USA) and subsequently paired-end sequenced on an Illumina MiSeq using the v2 500 cycles kit (Illumina, USA).

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

(2011) and LéVesque and De Cock (2004). Samples with less than 1000 reads were dropped from analysis due to low sequencing coverage.

Statistical Analysis. Data were imported into R 3.2.2 (R core team 2016) and analyzed using the packages 'phyloseq' 1.24.2 (McMurdie and Holmes, 2013) and 'vegan' 2.5.3 (Oksanen et al. 2018). All samples were rarefied to the minimum reads per sample (i.e., 1522 reads) before α-diversity analysis (Fig. S.6.1). Alpha-diversity was estimated for each sample, and only OTUs observed more than once were used before estimating α-diversity. OTU richness (S), Shannon diversity (H′), and Plieou's evenness (J) were used as α-diversity metrics. Non-metric multidimensional scaling (NMDS) (k = 2) was performed on Bray-Curtis distances to examine differences in beta-diversity (Bray and Curtis, 1957). A permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis distances, was used to test for differences in community centroids due to location, year, seed treatment, soybean genotype, and all interactions using the 'adonis2' function in the package 'vegan'. Differences in community multivariate dispersion were tested with the 'betadisper' function in the R package 'vegan'. Stepwise model building using plant density, root biomass, and yield as input variables in each location-year combination was used to select a constrained model for input into distance-based redundancy analysis (db-RDA) to examine the variation in Bray-Curtis distances due to plant density, root biomass, and yield. A Monte-Carlo permutation test was used to test the significance of constrained factors within db-RDA. Indicator species analyses was performed using the package 'indicspecies' 1.7.6 to identify OTUs significantly associated with covariates.

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

Results

Overview of experimental design and factors. A two-year field study in a high disease stress location (Allegan county of Michigan) and in a low disease stress location (Ingham county of Michigan) was established to understand the association of location, soybean genotype, seed treatments, and disease severity with oomycete rhizosphere communities. Among three disease severity measurements, plant density was the most consistent indicator as it was lower in Allegan than Ingham in both years, especially for 2015 where Allegan had on average 17.62 plants m^{-2} compared to Ingham which had on average 31.72 plants m^{-2} (Fig. 6.1A). Root biomass and yield reflected this tendency, but the reduction of root biomass and yield in Allegan was more evident in 2015 than 2016 (Fig. 6.1B and 6.1C).

When the seed treatments were applied, plant density and root biomass in Allegan was significantly higher for the FIN treatment compared to the NTC for all genotypes tested for both years. However, no significant difference in either plant density or root biomass was observed when F was applied in Allegan alone, which indicated the influence of FI or FIN interaction was more important in determining the outcome of plant density and root biomass. There was no significant improvement in plant density, root weight, or yield due to seed treatment in Ingham regardless of the soybean genotype (Table S.6.2).

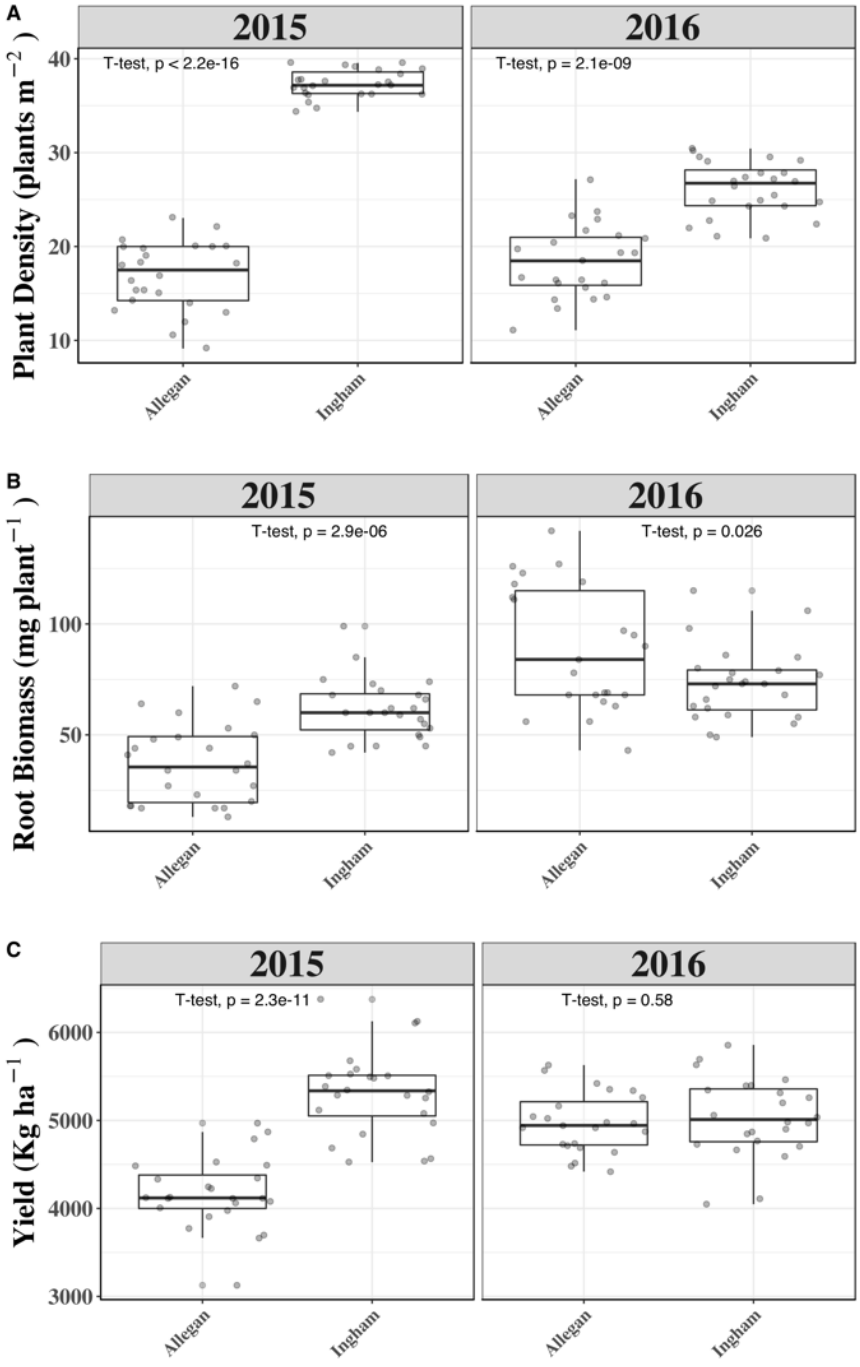

Figure 6.1. The effect of location tested within year on plant density (A), root biomass (B) and yield (C) of plants from non-treated seed across all genotypes. T-test P value is shown within each figure.

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

The most abundant oomycete genus was *Pythium* at 86.3% across the rhizosphere samples. *Phytophthora* comprised of 3.2% and the genera *Lagenidium*, *Apodachlya*, *Albugo*, *Plasmopara*, *Phytopythium*, *Peronospora*, *Hyaloperonospora*, *Brevilegnia*, *Plectospira*, and *Achlya* together comprised of 1.8% across rhizosphere samples, and 8.7% of the OTUs were not confidently assigned to an oomycete genus (Fig. 6.2A). *Pythium* clade I (including important pathogenic species like the *Pythium ultimum* species complex, and *Pythium heterothallicum*) was the most abundant clade in Ingham 2015, Allegan 2015, and Allegan 2016 making up 66.3%, 55.0%, and 44.4% across the rhizosphere samples, respectively. In Ingham 2016, *Pythium* clade F (including important pathogen species like *Pythium irregulare* and *Pythium sylvaticum*) was most abundant making up 41.1% of the reads (Fig. 6.2B). The most abundant OTU was identified as *Pythium ultimum* var. *ultimum* (OTU1 in *Pythium* clade I) and was found in Ingham 2015, Allegan 2015, and Allegan 2016 (Fig. 6.2C), while an unidentified *Pythium* species (OTU2 in *Pythium* clade F) was the most abundant in Ingham 2016 (Fig. 2C). Other frequently

observed OTUs were identified as *Pythium heterothallicum* (OTU 3 and 7 in *Pythium* clade I), which was present in all location-year combinations.

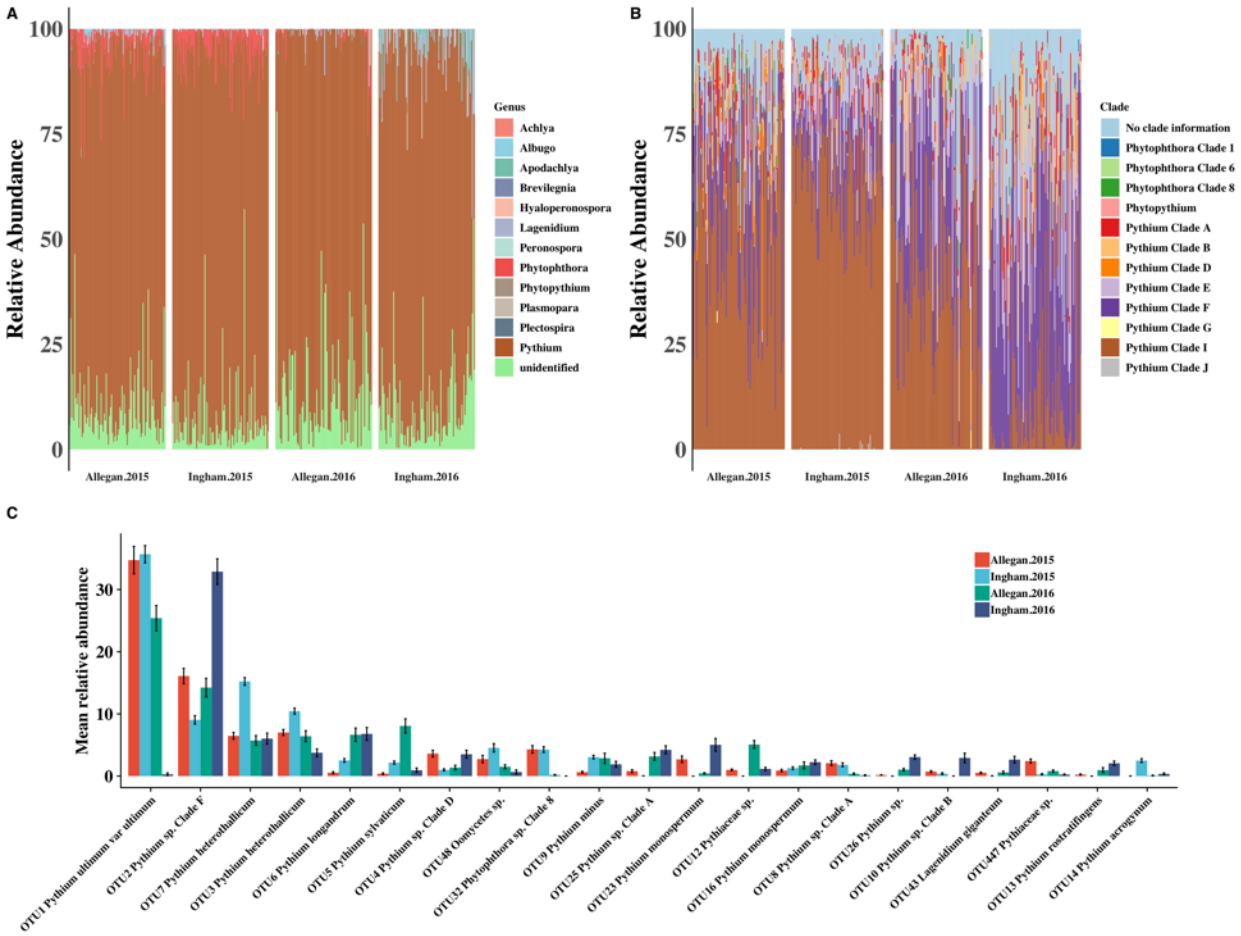

Figure 6.2. (A) Genus-level relative abundance of oomycete communities for each year-location combination. (B) Clade-level relative abundance of oomycete OTUs in the genera *Pythium*, *Phytophthora*, and *Phytopythium*. (C) Mean relative abundance of OTUs where the OTU was observed greater than 2% mean relative abundance at least one site.

α-diversity analysis for Allegan and Ingham. In order to understand the oomycete communities, α-diversity was estimated for each year-location combination. There was no significant difference in Shannon index (H′) between locations (Fig. 6.3A); however, when the diversity was broken down into Plieou's evenness (J) and richness (S), richness was significantly higher in Allegan than Ingham in 2015 ($P < 0.0001$) (Fig. 6.3B) whereas the evenness was significantly lower in Allegan than Ingham in 2015 ($P < 0.01$) (Figure 6.3C). Additionally, there were no significant differences in α-diversity metrics due to genotype or seed treatment within any location-year combination.

β-diversity analysis and identification of unique oomycete communities in Allegan. Rhizosphere communities were highly clustered based on location and year, and the interaction contained significantly different centroids ($P < 0.001$) and multivariate dispersion ($P < 0.001$) (Fig. 4A). Similar to α-diversity, neither soybean genotypes nor seed treatment influenced β-diversity. Most oomycetes OTUs were associated with multiple year-location combinations; however, there were 21 OTUs uniquely associated with Allegan 2015 (Fig. 6.4B; Table 6.2). The 21 OTUs, unique to Allegan 2015 included OTUs identified as *Pythium ultimum var. ultimum*, *Pythium heterothallicum*, *Pythium irregulare*, and *Laganidium giganteum*, and *Pythiaceae* sp. which added up to 5.61% relative abundance (Table 6.2).

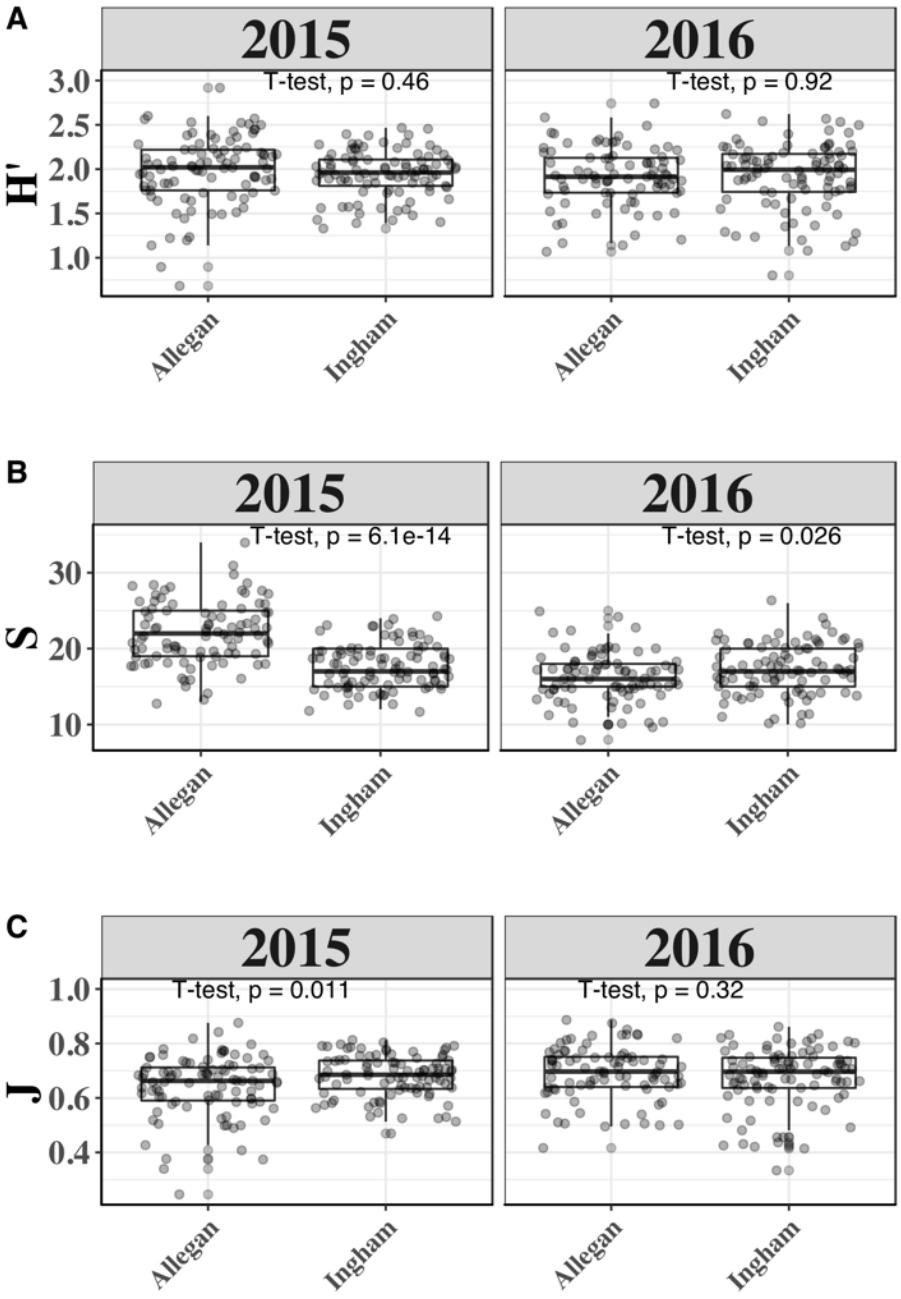

Figure 6.3. Influence of location on oomycete alpha diversity within year as estimated by (A) Shannon diversity index (*H*') and (B) richness (S) and (C) Plieou's evenness (J). T-test *P*-value is shown within each figure.

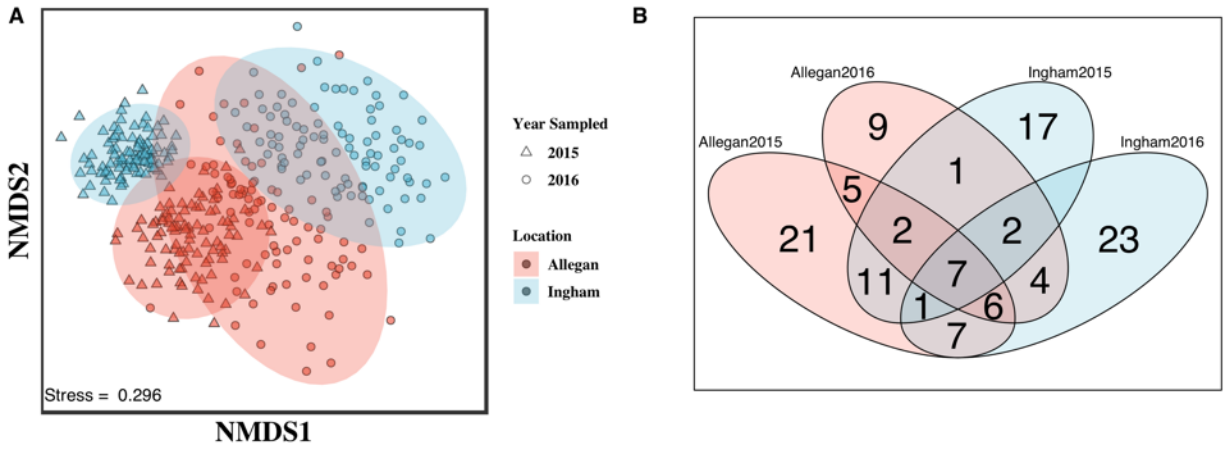

Figure 6.4. Between sample diversity of oomycete rhizosphere communities. (A) Non-metric multidimensional scaling ordination of soybean rhizosphere oomycete communities based on log-transformed and Wisconsin double standardized Bray-Curtis distances. Point shapes represent year (2015 or 2016) sampled and color represents location (Ingham or Allegan). Ellipses represent 95% confidence interval of a multivariate normal distribution for each year-location combination. Stress indicates how well the ordination reflects Bray-Curtis distances. (B) Venn-diagram or the number of OTUs significantly associated with each year-location combination.

Table 6.2. Operational taxonomic units (OTUs) significantly associated uniquely with Allegan 2015

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

Focusing on Allegan 2015, a model selection in the distance-based redundancy analysis (db-RDA) pointed out a significant association between oomycetes community and plant density ($P < 0.001$) and root biomass ($P < 0.005$), but not yield based on Monte-Carlo permutation tests. However, only 3.89% of the total variation in oomycete communities could be attributed to plant density and root biomass. Rhizosphere samples from plots with increased plant density and root biomass were associated with positive db-RDA1 scores. Rhizosphere samples from plots with increased root biomass were more associated with positive db-RDA2 scores, whereas samples with increased plant density were more associated with negative db-RDA2 scores (Fig. 6.5).

Among plots in Allegan 2015, OTU18 *Pythium* sp. nov (Clade B) was significantly associated with higher plant density and OTU41 *Pythium ultimum var. ultimum* (Clade I) was significantly associated with higher root biomass. On the other hand, OTU135 Saprolegniaceae sp. was significantly associated with lower plant density and OTU71 Oomycete sp. was significantly associated with lower root biomass (Table 6.3). Among these OTUs identified from indicator species analysis, OTU41 and OTU71 were also found to be unique to Allegan 2015 (Table 6.2), which indicates their potential importance in the association between oomycetes communities and disease severity at Allegan.

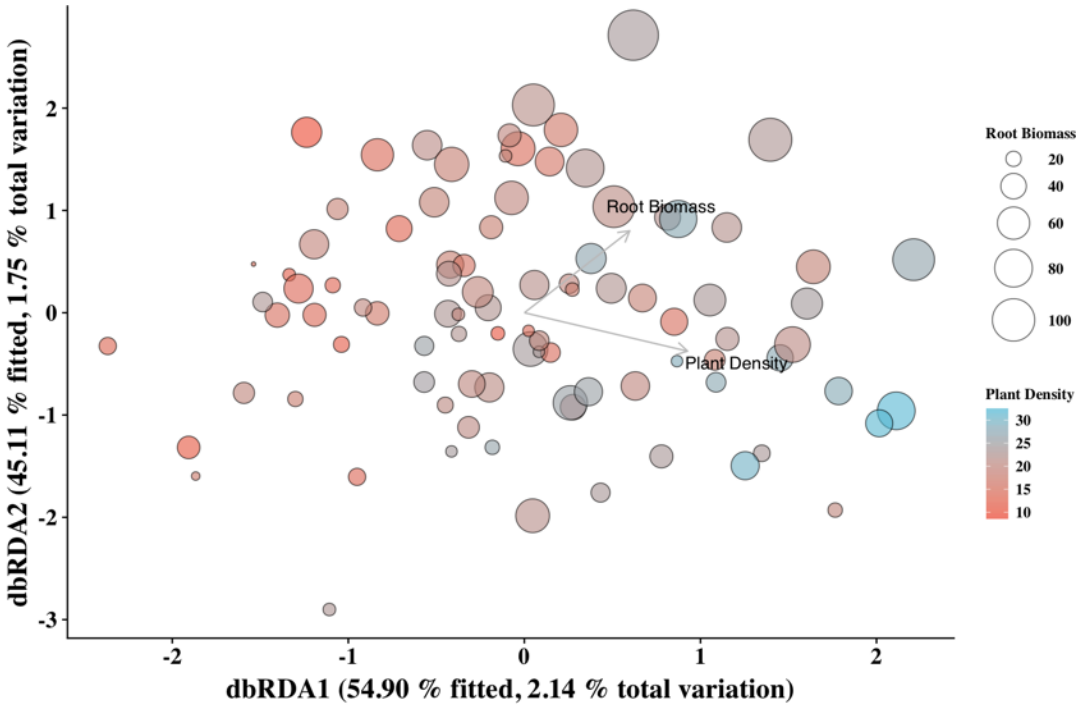

Figure 6.5. Association of oomycete communities with plant density and root biomass in Allegan 2015. Distance based redundancy analysis (db-RDA) of rhizosphere oomycete communities based on log-transformed and Wisconsin double standardized Bray-Curtis distances in Allegan 2015. Variation in root biomass and plant density were significantly associated with oomycete community composition based on a Monte Carlo permutation test. Arrows represent direction of increasing root biomass and plant density. Points represent plots sampled are scaled to the mean root biomass per plant and colored by mean plant density.

Table 6.3. Operational taxonomic units (OTUs) significantly associated with high or lower than average plant density or root biomass in Allegan 2015

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

Discussion

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

In all location-year combinations, oomycete communities in soybean rhizosphere samples were dominated by *Pythium*. Notably, this included important pathogenic species like *Pythium ultimum* var. *ultimum* and *Pythium heterothallicum* and putatively beneficial oomycetes. For example, OTU4 was identified as a *Pythium* sp. in clade D with a 100% match to *Pythium oligandrum* and had 3.62 and 3.51% mean relative abundance in Allegan 2015 and Ingham 2016. *Pythium oligandrum*, *Pythium acanthicum*, and *Pythium periplocum* are well-known soil-dwelling antagonists of fungi and oomycetes (Martin and Loper 1999; Paul et al. 1999; Ribeiro and Butler, 1995). An OTU identified as *Lagnenidium gigateum*, an entomopathogenic oomycete, was also observed in soybean rhizospheres.

The observation of *Pythium* dominance in the soybean rhizosphere corroborates observations of other culture-based and culture-independent metabarcoding studies where *Pythium* was dominant in agricultural soils (Rojas et al. 2017ab; Broders et al. 2009; Taheri et al. 2017; Coince et al. 2013; Vannini et al. 2013; Sapkota and Nicolaisen 2015; Singer et al. 2016; Bakker et al. 2017; Riit et al, 2016; Duran et al. 2018; Coffua et al., 2016; Sapp et al., 2018; Schlatter et al. 2017). Historically, soybean breeding efforts have primarily focused on *Phytophthora sojae* because of its gene-for-gene interaction with soybean R gene products (Dorrance and Grunwald 2009). There have been studies focused on *Pythium* resistance breeding (Rosso et al. 2008; Rupe et al. 2011; Kirkpatrick et al. 2006; Lin et al. 2018; Sasko et al. 2016; Ellis et al. 2013) but genetic resistance is not known to be or is not intentionally applied in the field as it is for *Phytophthora sojae*.

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

have increased favorable conditions for oomycete growth and stressed germinating seeds. The same weather trends did not occur in 2016, when both locations received less than 10 mm rain two weeks following planting.

There were 21 OTUs found to be unique to Allegan 2015 significantly associated with the oomycete community based on indicator species analysis. Notably, OTU41, identified as *Pythium ultimum* var. *ultimum* was significantly associated with higher than average plant density in Allegan 2015, perhaps indicating that increased resource availability provided by increased plant density and root mass is attractive to some oomycete taxa. Interestingly, OTU1 was also identified as *Pythium ultimum* var. *ultimum* and was the most abundant in Allegan 2015 but was associated with both high and low plant density. Based on this observation it could be hypothesized that with increased niche space provided by increased plant density allowed for multiple *Pythium ultimum* var. *ultimum* genotypes to coexist.

The results of the db-RDA indicated that although small, some variation in plant density and root biomass was attributed to oomycete community composition in Allegan 2015. Other edaphic factors such as soil pH and soil temperature could also explain why disease pressure was not observed in Ingham field sites, as these factors can influence pathogenicity (Martin and Loper 1999; Rojas et al. 2017a). It is possible that disease stress did not merely result from the presence or absence of pathogens; instead, it depends on the evenness of pathogens with the possibly of facilitative interactions between oomycetes to other organisms. An observation to support this statement is plant density, and root biomass was significantly higher in plots with the (FIN) treatment compared to the non-treated control (NTC) but not for the F or FI seed treatments (Table S.6.2). On the other hand, there was no significant improvement in plant density, root weight, or yield due to seed treatment in Ingham regardless of the soybean

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

Interestingly, two OTUs identified as *Lagnidium giganteum* were unique to Allegan 2015 (Table 6.4). Members of the *Lagnidium* genus are known pathogens of animal hosts and the presence of these isolates along with the observation of increased plant stand with insecticides is intriguing. Facilitation of plant death by pathogenic oomycetes may be influenced by the presence of insects or nematode damage allowing more accessible entry into plant tissue (Graham and McNeill, 1972; Willsey et al., 2017). Furthermore, neonicotinoid insecticides can induce systemic acquired resistance (SAR) and prime plant defenses (Ford et al. 2010). Insect larval root feeding injury, presumably from seedcorn maggot (*Delia platura*) has been observed in Allegan field sites, but extensive insect surveys were not conducted since incidence was not above an economic threshold (Rossman et al. 2018). Additional study using metagenome sequencing may reveal other pests or organisms in Allegan, and analyses on multiple organisms together with oomycetes may improve the explanation of variance.

Conclusions

Oomycetes are important drivers of community assembly but are often overlooked and an understudied portion of the plant microbiome (Agler et al. 2016). This study represents a 2-year field survey of oomycete communities from a location previously observed to have high disease pressure compared to one that did not. Interestingly and unexpectedly, seed treatments and plant genotype did not have a substantial impact on oomycete community structure, despite their improvement to plant density and root biomass in Allegan. Oomycete communities were different based on location, but field sites without historical disease pressure had pathogenic

oomycetes. Therefore, we hypothesize that possible dominance of pathogenic oomycete species, oomycete interactions with edaphic factors, weather conditions at planting, and possible interactions with other soil-dwelling organisms are responsible for the disease pressure observed in Allegan. In conclusion, this study improves our understanding of oomycete diversity in soybean rhizosphere which will aid in recommendations for plant breeders and oomicide recommendations. Future studies are encouraged to integrate oomycetes with fungal, bacteria and soil fauna datasets for understanding disease resistant factors in the plant microbiome.

APPENDIX

APPENDIX

Table S.6.1. Adapted from Rossman et al. (2018). Chemical seed treatment information, 2015-2016

Abreviation[a]	Asgrow			Pioneer		
	Trade Name	Active Ingredients	Application Rate	Trade Name	Active Ingredients	Application Rate
F	Accerelon DX-109®	pyraclostrobin	0.43 ml kg seed^{-1}	Evergol Energy	prothioconazole, penflufen, metalaxyl	0.60 ml kg seed^{-1}
	Acceleron DX-309®	metalaxyl	0.27 ml kg seed^{-1}	ApronMaxx RTA®	mefenoxam, fludioxonil	2.09 ml kg seed^{-1}
	Acceleron DX-612®	fluxapyroxad	0.17 ml kg seed^{-1}	PPST 2030	Biological component	1.21 ml kg seed^{-1}
+I	Acceleron IX-409®	imidacloprid	1.43 ml kg seed^{-1}	Gaucho® 600 Flowable	imidacloprid	0.97 ml kg seed^{-1}
+IN	Poncho-Votivo	clothianadin, *Bacillus firmus* I-1582	1.46 ml kg seed^{-1}	Poncho-Votivo	clothianadin, *Bacillus firmus* I-1582	0.63 ml kg seed^{-1}

[a]F, +I, and +IN refers to the base mix of fungicides and oomicides applied to all treated seed, the insecticide of the fungicide-insecticide combined seed treatment, and the insecticide-nematistat of the fungicide-insecticide-nematistat treatment, respectively.

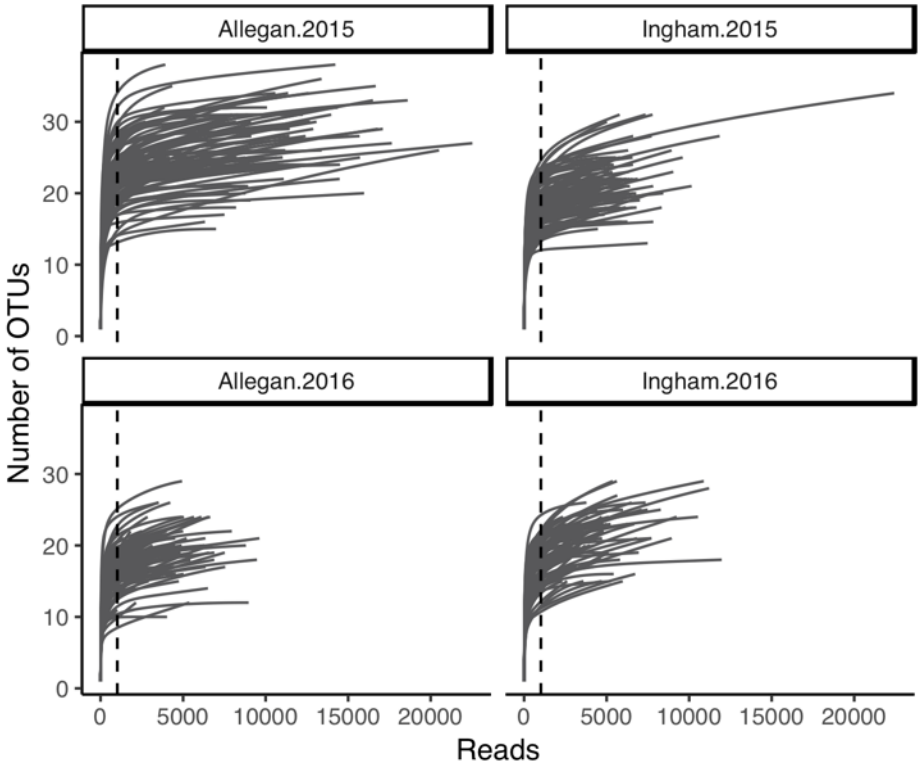

Figure S.6.1. Rarefaction curves for rhizosphere samples for all location-year combinations. Dashed line indicates the minimum number of reads of all samples.

Table S.6.2. Effect of seed treatment on plant density, root biomass, and yield for each location, year, genotype combination

Location	Year	Genotype	Seed Treatment[a]	Plant Density (plants m^{-2}) Mean ± SE[b]	Root Biomass (mg $root^{-1}$) Mean ± SE	Kg ha^{-1} Mean ± SE
Allegan	2015	AG2431	NTC	18.41 ± 1.25 b	33.50 ± 9.20 b	4133.70 ± 73.09 b
Allegan	2015	AG2431	F	18.01 ± 1.16 b	41.00 ± 6.12 b	4123.61 ± 186.02 b
Allegan	2015	AG2431	FI	20.91 ± 1.43 ab	53.50 ± 10.63 b	4410.55 ± 99.30 b
Allegan	2015	AG2431	FIN	25.51 ± 1.56 a	88.17 ± 13.52 a	4861.13 ± 171.05 a
Allegan	2015	AG2433	NTC	15.72 ± 1.79 c	42.33 ± 7.47	4318.64 ± 193.24
Allegan	2015	AG2433	F	18.99 ± 0.69 bc	25.67 ± 5.52	4139.30 ± 279.67
Allegan	2015	AG2433	FI	22.86 ± 1.36 ab	27.00 ± 4.52	4367.96 ± 108.21
Allegan	2015	AG2433	FIN	25.58 ± 0.96 a	28.33 ± 5.23	4230.09 ± 193.02
Allegan	2015	P26T76R	NTC	17.90 ± 1.46 b	32.00 ± 4.19	4027.22 ± 193.13
Allegan	2015	P26T76R	F	18.22 ± 2.31 b	36.17 ± 5.95	4225.61 ± 252.18
Allegan	2015	P26T76R	FI	22.25 ± 1.45 ab	34.83 ± 12.66	4489.01 ± 165.40
Allegan	2015	P26T76R	FIN	25.18 ± 2.16 a	50.50 ± 6.75	4465.47 ± 106.43
Allegan	2015	P92Y12	NTC	15.43 ± 1.46 b	40.83 ± 8.27	4215.52 ± 193.09
Allegan	2015	P92Y12	F	17.43 ± 1.53 ab	40.00 ± 8.17	4300.70 ± 146.98
Allegan	2015	P92Y12	FI	19.09 ± 1.85 ab	43.17 ± 8.72	4354.51 ± 125.98
Allegan	2015	P92Y12	FIN	22.86 ± 1.28 a	51.67 ± 5.48	4410.55 ± 162.86
Allegan	2016	AG2431	NTC	21.01 ± 1.54 b	94.83 ± 11.38	5042.71 ± 125.11
Allegan	2016	AG2431	F	21.96 ± 1.59 b	96.83 ± 7.25	5074.09 ± 97.71

Table S.6.2 (cont'd)

Allegan	2016	AG2431	FI	22.83 ± 1.22	b	93.40 ± 7.79		5238.86 ± 261.61
Allegan	2016	AG2431	FIN	31.78 ± 0.51	a	97.00 ± 7.41		5412.59 ± 166.9
Allegan	2016	AG2433	NTC	15.94 ± 1.52	b	64.50 ± 7.23	b	4899.24 ± 153.54
Allegan	2016	AG2433	F	17.65 ± 0.46	b	82.40 ± 10.17	b	4800.38 ± 253.39
Allegan	2016	AG2433	FI	20.33 ± 1.97	b	71.00 ± 9.01	b	4833.67 ± 87.57
Allegan	2016	AG2433	FIN	31.91 ± 0.88	a	98.20 ± 11.06	a	5031.72 ± 70.80
Allegan	2016	P26T76R	NTC	16.85 ± 1.09	b	93.33 ± 10.77		4985.54 ± 165.15
Allegan	2016	P26T76R	F	17.30 ± 1.74	b	81.40 ± 8.28		5135.29 ± 108.62
Allegan	2016	P26T76R	FI	19.57 ± 1.28	b	91.75 ± 8.32		5103.01 ± 120.91
Allegan	2016	P26T76R	FIN	29.82 ± 0.77	a	101.33 ± 12.69		5099.87 ± 122.67
Allegan	2016	P92Y51	NTC	20.13 ± 1.60	b	106.20 ± 12.48		4948.33 ± 155.92
Allegan	2016	P92Y51	F	19.61 ± 1.70	b	91.00 ± 7.57		5194.47 ± 103.26
Allegan	2016	P92Y51	FI	21.04 ± 0.73	b	96.80 ± 8.85		5250.96 ± 156.43
Allegan	2016	P92Y51	FIN	30.47 ± 0.27	a	96.00 ± 7.21		5382.33 ± 110.88
Ingham	2015	AG2431	NTC	38.19 ± 0.42		52.17 ± 3.70		5380.08 ± 87.72
Ingham	2015	AG2431	F	35.43 ± 0.95		61.67 ± 4.24		5677.11 ± 214.83
Ingham	2015	AG2431	FI	35.36 ± 0.47		67.00 ± 5.90		5540.37 ± 107.33
Ingham	2015	AG2431	FIN	34.49 ± 0.83		59.83 ± 4.21		5204.11 ± 402.00
Ingham	2015	AG2433	NTC	37.61 ± 0.61		59.32 ± 4.62		5275.84 ± 251.60
Ingham	2015	AG2433	F	38.26 ± 0.35		55.00 ± 2.58		5440.61 ± 165.44
Ingham	2015	AG2433	FI	37.07 ± 0.39		43.50 ± 3.48		5197.38 ± 148.45
Ingham	2015	AG2433	FIN	37.17 ± 0.32		46.50 ± 2.81		5356.55 ± 164.03

Table S.6.2 (cont'd)

Ingham	2015	P26T76R	NTC	37.46 ± 0.57	68.17 ± 6.80	5280.33 ± 240.81
Ingham	2015	P26T76R	F	37.68 ± 0.32	67.33 ± 4.50	5474.24 ± 118.09
Ingham	2015	P26T76R	FI	38.73 ± 0.41	77.00 ± 5.28	5120.05 ± 209.28
Ingham	2015	P26T76R	FIN	37.46 ± 0.44	68.33 ± 6.68	5131.25 ± 142.75
Ingham	2015	P92Y12	NTC	36.09 ± 0.55	67.33 ± 5.10	5329.65 ± 224.05
Ingham	2015	P92Y12	F	37.07 ± 0.45	72.00 ± 3.04	5524.67 ± 172.19
Ingham	2015	P92Y12	FI	37.07 ± 0.20	74.33 ± 2.84	5306.11 ± 231.93
Ingham	2015	P92Y12	FIN	36.41 ± 0.39	76.67 ± 6.55	5684.96 ± 220.09
Ingham	2016	AG2431	NTC	26.16 ± 1.67	69.50 ± 5.10	4956.4 ± 129.04
Ingham	2016	AG2431	F	26.49 ± 1.88	66.83 ± 7.33	5056.16 ± 100.78
Ingham	2016	AG2431	FI	26.59 ± 0.9	68.50 ± 7.32	4879.06 ± 60.97
Ingham	2016	AG2431	FIN	28.26 ± 1.05	71.17 ± 7.74	4705.33 ± 229.17
Ingham	2016	AG2433	NTC	26.92 ± 0.65	61.00 ± 3.45	4940.71 ± 224.48
Ingham	2016	AG2433	F	28.15 ± 1.34	68.17 ± 6.57	5182.81 ± 131.36
Ingham	2016	AG2433	FI	27.36 ± 1.68	61.17 ± 3.86	5398.02 ± 93.94
Ingham	2016	AG2433	FIN	28.26 ± 1.58	52.83 ± 3.68	5196.26 ± 223.52
Ingham	2016	P26T76R	NTC	24.75 ± 1.42	83.00 ± 9.00	5334.13 ± 172.22
Ingham	2016	P26T76R	F	26.67 ± 1.74	72.83 ± 6.75	5429.4 ± 143.98
Ingham	2016	P26T76R	FI	25.69 ± 1.80	80.33 ± 5.43	5293.78 ± 180.03
Ingham	2016	P26T76R	FIN	27.10 ± 1.78	81.00 ± 6.18	5380.08 ± 175.57
Ingham	2016	P92Y51	NTC	26.59 ± 0.83	79.67 ± 5.54	4909.33 ± 187.85

Table S.6.2 (cont'd)

Ingham	2016	P92Y51	F	28.70 ± 1.26	85.83 ± 9.91	5096.51 ± 102.07
Ingham	2016	P92Y51	FI	24.93 ± 2.29	71.33 ± 6.97	4789.4 ± 184.63
Ingham	2016	P92Y51	FIN	28.55 ± 2.06	64.00 ± 2.91	4974.34 ± 196.41

[a]Seed treatment abreviations (Table 1) are as followed: NTC = Non-treated control, F = Fungicide only, FI = Fungicide and Insecticide, FIN = Fungicide and Insecicide and nematicide biological control agent.

[b]Means followed by the same letters within columns are not significanlty different, Analysis of Variance (ANOVA), followed by separation with Tukey's honest signficant differnce, ($\alpha = 0.05$)

Chapter 7

Conclusions and Impacts

Conclusions

Seed treatment chemicals are an essential management tool for seedling diseases caused by oomycetes. Over 80 oomycete species are known to be associated with soybeans in North America and over half of them are pathogenic at the seed or seedling level. Therefore, my research focused on understanding the impact of soybean seed treatments on oomycete communities.

There have been concerns of increasing mefenoxam insensitivity in soybean associated oomycetes. Based on the results obtained using a newly developed technique and statistical considerations for assessing fungicide sensitivity, we have generated sensitivity data for over 500 oomycete isolates from 84 different oomycete species collected from across the Midwest. From these data we found no substantial evidence that sensitivity to mefenoxam has shifted compared to the sensitivity reported about fifteen years ago. In contrast, there was substantial evidence that the variation in sensitivity to the recently introduced chemical ethaboxam is inherently related to differences between species in β-tubulin protein structure specifically at the 239^{th} residue, which supports the hypothesis that the insensitivity reported here is inherent rather than acquired. These data are important for appropriate use of seed treatment chemicals and for understanding the breadth of activity for ethaboxam. The breadth of activity of ethaboxam covers the most abundant species associated with soybeans, and the species that were insensitive to ethaboxam were sensitive to mefenoxam. Therefore, we conclude that a seed treatment containing both mefenoxam and ethaboxam is an essential management tool for oomycetes in a soybean production system. This conclusion was supported by the observation that seed treatments containing both ethaboxam and metalaxyl (which contains mefenoxam) was effective to reduce oomycete recovery from soybean lateral roots.

Additionally, studies herein demonstrated that that different soybean fields had considerable influence over oomycete diversity, indicating that a grower's field may have a different consortium of species than the neighbor's field. This is vitally important information to know since recommendations for management, such as seed treatment or deployment of soybean varieties, may not be universally applied to each grower's field depending on the species present. Moreover, these data demonstrate that the decision on the use of seed treatment chemical for protection of oomycete pathogens depends on the consortia of species present in a field. This promotes the idea that in future work "prescription" based seed treatment recommendations can be made for growers depending on the unique conditions of that field. Ultimately, these data are useful to devise new management strategies for oomycete associated oomycetes, with the overall goal of improving farmer's livelihoods by providing effective disease control.

Impacts

Results from studies presented herein have immediate impact on recommendations for management of oomycetes with soybean seed treatments. The statistical considerations for accurate EC_{50} estimation (Chapter 2) enable more reproducible research to be carried out. Since this paper was published in 2018 it has been downloaded 113 times and has one citation so far. I have also held an internal Chilvers lab lecture about this paper. I have also been contacted by at least three individuals wanting help with analysis. With the high-throughput assay developed (Chapter 3), researchers have the ability to test the sensitivity of oomycete isolates quickly and accurately at a population or community level. Which will enable quicker decision making and more robust recommendations than previously available. This assay has been taught to at least three other individuals in the Chilvers lab and to other researchers at Iowa State University.

The results of the C239S evolution (Chapter 4) have been communicated to researchers, industry professionals, extension professionals, and funding bodies both domestically and internationally. In fact, I had the pleasure of communicating these results at the Oomycete Molecular Genetic Network meeting in Tia'an China where I learned that another research group in China was also working on ethaboxam resistance. We agreed to publish our findings in the same issue of Phytopathology, so that equal credit is given for the discovery.

The results of chapters 5 and 6 really demonstrated that location is a huge driver of oomycete diversity using both culture dependent and culture independent methods. I have also provided more information on the current status of oomycete sensitivity to mefenoxam in a soybean pathosystem, which indicated that sensitivity does not appear to have changed in the past fifteen years. This observation corroborates fungicide resistance theory that low exposure to mefenoxam and low dispersal of the pathogen would lead to a low incidence of practical resistance issues. These results have been communicated to industry professionals and funding bodies on the current recommendations for soybean seed treatments.

Finally, chapter 6 indicated that oomycete diversity was different in fields with and without a history of seedling disease pressure, but that sites without disease history were not devoid of pathogenic species. Furthermore, seed treatments and genotype had little influence on oomycete diversity despite the improvement of plant density with the usage seed treatment in high-disease pressure sites. This could indicate that other soil micro- or macrooganisms are responsible or are acting together with oomycetes to exacerbate seedling disease. These results have been communicated to researchers through presentations at meetings. In a continuation of this work (not included in this dissertation) I have collected soybean roots from the 2017 and 2018 growing seasons and have constructed and sequenced amplicon libraries of bacteria, fungi,

and oomycetes to further explore the interkingdom interactions on soybean roots. These data are currently being analyzed.

I have included a figure about the information flow of my research and how knowledge gained with the studies presented herein have been disseminated (Fig. 7.1).

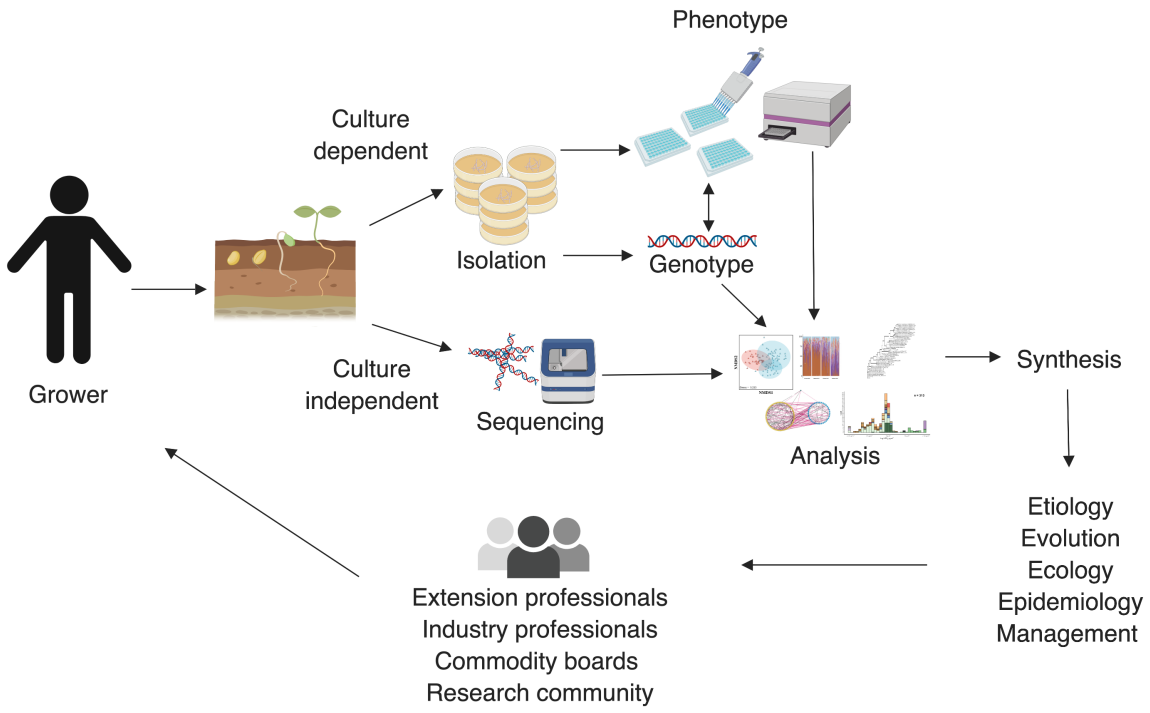

Figure 7.1. Information flow of the results in this dissertation.

List of publications or intended publications and description of contributions from the duration of my PhD

1. **Noel, Z.A.** and Chilvers, M.I. 2019. Microbial interkingdom interactions in soybean roots with different soybean seed treatments. *Manuscript in preparation.*

ZAN and MIC designed the study. ZAN collected samples, prepared and sequenced libraries, and is currently analyzing data.

2. **Noel, Z.A.** McDuffee, D., and Chilvers M.I. 2019. Influence of soybean tissue and oomicide seed treatments on oomycete isolation. *Submitted to Plant Disease.*

ZAN, DM, and MIC designed the study. ZAN and DM collected samples. ZAN processed samples, analyzed data. ZAN and MIC wrote manuscript. DM and MIC edited the manuscript.

3. **Noel, Z.A.**, Chang, H.X., Chilvers M.I. 2019. Variation in soybean rhizosphere oomycete communities from Michigan fields with contrasting disease pressures. *Submitted to Applied Soil Ecology*

ZAN and MIC designed study. ZAN sampled, constructed and sequenced amplicon libraries. ZAN analyzed data. ZAN and MIC wrote manuscript. HXC and MIC edited the manuscript.

4. Sang, H., Chang, H.X., Wang, J., Rojas, J.A., **Noel, Z.A.**, and Chilvers, M.I. 2019. Gene expression profiling and effector characterization of *Sclerotinia sclerotiorum* during infection of pea. *Submitted to Applied Environmental Microbiology*

HS, HXC, JW, AJR and MIC designed study. HS, HXC, ZAN performed and guided experiments. HS and MIC wrote manuscript. HXC, JW, AJR, ZAN, and MIC edited the manuscript.

5. *McCoy, A.G., *Roth, M.G., *Shay, R., ***Noel, Z.A.**, Jayawardana, M. A., Longley, R.W., Bonito, G., and Chilvers, M.I. 2019. *Submitted to Phytobiomes*.

AGM, MGR, RS, MAJ, GB, and MIC planned experiments. AGM, MGR, RS, and MAJ and collected and processed samples. RWL performed PCR and constructed libraries. AGM submitted raw sequences to Sequence Read Archives. MGR and ZAN analyzed data and generated figures. AGM, RS, MGR, ZAN, and MAJ wrote the manuscript. * indicates co-first authors.

6. McCoy, A.G., **Noel, Z.A.,** Sparks, A., and Chilvers, M.I. 2019. R Code Resource for Pathotype Analysis in Phytophthora Stem and Root Rot of Soybean, *Phytophthora sojae. In preparation for Plant Disease.*

AGM and ZAN wrote R code with contributions from AS. AGM wrote the manuscript. AGM, ZAN, AS and MIC edited the manuscript.

7. Roth, M.G., **Noel, Z.A.**, Wang, J., Byrne, A.M., Chilvers, M.I. Assessment and utilization of risk factors in predicting soybean yield and sudden death syndrome development. *Phytopathology:* doi.org/10.1094/PHYTO-02-19-0040-R

MGR, JW, and MIC designed the experiment, MGR, JW, FW, and AMB collected data, MGR and ZAN analyzed data and generated figures, MGR, ZAN, and MIC wrote the manuscript.

8. **Noel, Z.A.**, Sang, H., Roth, M.G., Chilvers, M.I. 2019. Convergent evolution of C239S mutation in *Pythium* spp. β-tubulin coincides with inherent insensitivity to ethaboxam and implications for other Peronosporalean oomycetes. Phytopathology 10.1094/ PHYTO-01-19-0022-R.

ZAN and MIC designed study. ZAN performed all experiments, collected data, and analyzed data. HS and MGR provided insight and guidance for experimental procedures. ZAN wrote the manuscript with contributions from HS, MGR, and MIC.

9. Chilvers, M.I., McCoy A.M., Byrne A.M., Cornett, A.J., Chang, H.-X., **Noel, Z.A.**, Koeman S., Effects of fungicides on the management of tar spot of corn in Michigan, 2018. *Plant Disease Management Reports*

ZAN analyzed data. MIC wrote manuscript.

10. **Noel Z.A.**, Rojas, J.A., Jacobs, J.L., Chilvers, M.I. A high-throughput microtiter fungicide sensitivity phenotyping platform for oomycetes using Z'-factor statistic. DOI: https://doi.org/10.1094/PHYTO-01-19-0018-R

ZAN and MIC designed the study. AJR and JLJ provided significant insight for experimental procedures. ZAN wrote the manuscript with contributions from AJR, JLJ and MIC.

11. Rojas, J. A., Witte, A., **Noel, Z. A.**, Jacobs, J. L., and Chilvers, M. I. 2019. Diversity and characterization of oomycetes associated with corn seedlings in Michigan. Accepted for publication pending minor revision Phytobiomes: doi.org/10.1094/PBIOMES-12-18-0059-R

AJR and MIC designed study. AJR, AW, and JLJ performed experiments. ZAN provided guidance on experimental procedures and AJR and ZAN analyzed data. AJR and MIC wrote manuscript. AJR, JLJ, ZAN, and MIC edited manuscript.

12. Chang, H-X., **Noel, Z.A.**, Sang, H., Chilvers, M.I. 2018. Annotation resource of tandem repeat-containing effectors in fifty fungi. *Fungal Genetics and Biology* 119:7-19

HXC and MIC designed study. HXC performed all experiments. HXC, ZAN, and HS provided guidance on experimental procedures.

13. Noel, Z. A., Wang, J., and Chilvers, M. I. 2018. Significant influence of EC_{50} estimation by model choice and EC_{50} type. *Plant Disease* 102:708–714

ZAN, JW, and MIC designed study. ZAN and JW performed experiments and analyzed data. ZAN wrote the manuscript. JW and MIC edited the manuscript.

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