MANAGING SOILBORNE PATHOGENS OF LEGUMINOUS CROPS IN MICHIGAN

By

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ABSTRACT

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Soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) are two of the most globally important leguminous crops. Yields can be reduced by high incidence of soilborne pathogens that cause seedling disease. Seed treatments are often used for management of soybean seedling disease, but profitability of seed treatment use remains unclear. To examine this in Michigan, seed treatments were evaluated at seven field sites each year from 2013 to 2015. Across sites in 2013, no seed treatment significantly improved partial returns relative to the non-treated control (NTC); across sites in both 2014 and 2015, the fungicide+insecticide+nematode protectant (FIN) treatment significantly reduced partial returns relative to the NTC. Seed treatment may control soybean seedling pathogens, but seed treatment use did not show improved profitability. Along with chemical control, genetic control is often used to reduce losses from root rot pathogens in common bean. Breeders have aimed to develop Pythium root rot-resistant bean varieties; however, relationships between common bean and most *Pythium* species remain uncharacterized. *Pythium* species (n=28) were tested in a growth chamber assay at 20°C and petri dish assay at 20°C and 26°C to describe their pathogenicity and virulence on two bean varieties from the Middle American and Andean gene pools. Root growth or disease severity was significantly impacted by 17 *Pythium* species, although results varied by bean variety, temperature, and assay used. Improved understanding of *Pythium* interactions with bean will help breeders and pathologists to control *Pythium*-induced seedling disease more effectively.

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INTRODUCTION

The production of leguminous crops is often challenged by numerous seedling pathogens (Bai et al. 2015). Due to the crucial role of leguminous grain crops in providing dietary protein for human and livestock consumption worldwide (Blair 2013; Broughton et al. 2003; Masuda et al. 2009), implementing improved control of these pathogens is important for establishing a resilient global food system. Aside from forage crops such as alfalfa, the two leguminous field crops planted on the greatest number of acres in the United States are soybean and dry edible bean (common bean) (National Agricultural Statistics Service 2015). In the current work, two different studies were conducted to improve the characterization between seedling diseases and these two leguminous hosts.

Chapter 1 is divided into two parts. Each part contains a review of the scientific literature that provides pertinent background information for each study.

In the first study, presented in Chapter 2, seedling disease management practices currently utilized in soybean production were evaluated. Use of soybean seed treatments for the management of seedling diseases has increased substantially within the past twenty years (Gaspar et al. 2014), despite ongoing uncertainty whether seed treatments consistently improve economic outcomes for soybean producers. Field and greenhouse trials were conducted to describe the effect of seed treatments on disease control, plant stand, yield, and consequent profitability. The results of the three-year study provide an updated basis for Michigan soybean growers to make seed treatment decisions.

In the second study, presented in Chapter 3, relationship between *Pythium* species and common bean are described. In growth chamber and petri dish assays, *Pythium* and

Phytopythium species collected from soybean and common bean were tested to determine their pathogenicity and virulence on common bean. The roles of common bean germplasm and temperature in host-pathogen interactions were also considered. By establishing a basis for comparative virulence among *Pythium* species, focused plant breeding can be conducted for *Pythium* species that cause the most serious disease symptoms. Improved understanding of *Pythium*-bean host interactions can also be used to inform management recommendations. This current study is the first to evaluate a large panel of North American *Pythium* species for their pathogenicity and virulence on bean.

CHAPTER 1. REVIEW OF LITERATURE

Part I. Historical Use and Efficacy of Seed Treatment in Soybean

Soybean production.

Soybean is the most extensively planted oilseed crop in the United States (National Agricultural Statistics Service 2015). Soybean is also of regional importance to the state of Michigan, contributing $600 million to Michigan's economy in 2010 as an export crop (National Agricultural Statistics Service 2011). In Michigan, more than 2 million acres of soybean were planted in 2014 (National Agricultural Statistics Service 2015). Soybean is an important crop for the United States, which produced nearly 110 billion kg of soybean in 2014 (National Agricultural Statistics Service 2015).

The economic benefit of soybean production in the Midwest continues to be challenged by several pathogens and pests. Soybean cyst nematode (SCN) and pathogens causing root rot and seedling disease have been cited as major causes of yield loss worldwide (Wrather and Koenning 2006; Koenning and Wrather 2010; Wrather et al. 2010; Hartman et al. 2011). Soybean aphid (*Aphis glycines*) is a soybean pest that was discovered in the Midwest in the early 2000s (Ragsdale et al. 2004). It is reported to have the potential to cause yield loss of up to 50% (Wang et al. 1994). Several chemistries for disease and pest control have been developed for use as a seed treatment that are used to target soybean pests and diseases (Munkvold 2009; Munkvold et al. 2014). Though seed treatment was applied to most of the soybean seed planted in the United States in 2014 (Gaspar et al. 2014), no study has demonstrated that seed treatment

significantly improves yield and profitability for the majority of environments evaluated (personal observation).

History of seed treatment in soybeans.

Seed-applied fungicides have been used on soybean for decades, though the earliest reports of seed treatment use on soybean use are unclear (Wall 1983). If not earlier, seed-applied pesticides were being tested for use on soybean in 1943 (Porter 1944). Seed-applied fungicides were first recommended to prevent reduced plant stand and yields caused by low quality seeds (e.g., seeds infected with seedborne pathogens such as *Diaporthe-Phomopsis* species) (Athow and Caldwell 1956; Wallen and Cuddy 1960; Chamberlain and Gray 1974). In the mid-1970s, TeKrony et al (1974) recommended seed-applied fungicide, even when seedborne pathogens were absent from seed, to improve emergence whenever soil temperatures following planting were below 16°C and seed germination was below 85%. An Iowa study later demonstrated that captan and combined carboxin-thiram significantly improved yields when applied to seed lots that were 50%-contaminated with *Phomopsis* species. However, seed treatments did not improve germination and emergence of damaged, aged, or under-sized seed (Wall et al. 1983). The performance of seed treatment on high-quality seed was unclear.

Seed treatments were also being tested for management of early season seedling diseases caused by soilborne pathogens. Soybean field surveys across the United States confirmed the prevalence of *Fusarium*, *Phomopsis*, *Phytophthora*, *Pythium*, and *Rhizoctonia* species in diseased soybean seedlings, focusing the development of improved disease management practices (Kilpatrick and Johnson 1953; Schenck and Kinloch 1974; Schlub and Lockwood 1981; Rizvi and Yang 1996). Some seed-applied fungicides that reduced seedborne disease also controlled *Rhizoctonia solani* and *Fusarium* species (Cox et al. 1976). For soybean seeds planted

into fields with a history of Phytophthora root rot, metalaxyl applied at 1.65 g kg^{-1} significantly improved plant stand by 25% and yield by 16% (Vaartaja et al. 1979). Other studies similarly found that seed-applied metalaxyl reduced disease severity and field losses in susceptible soybean cultivars planted where moderate *P. sojae* or *Pythium ultimum* pressure existed (Guy et al. 1989; Griffin 1990), but researchers found that high rates of metalaxyl occasionally caused yield reduction in the absence of pathogenic oomycete species (Schmitthenner 1985; Guy et al. 1989).

Seed-applied fungicides prior to the 1990s, such as captan, were primarily contact protectants with broad-spectrum toxicity to microbes (Edgington et al. 1980; Cohen and Coffey 1986). In 1980, approximately one third of registered fungicides were systemic in plant tissue, targeting only specific metabolic sites in certain fungi or oomycetes. For example, metalaxyl is a systemic fungicide that is able to be seed-applied for management of oomycete diseases, but not fungal diseases (Vaartaja et al. 1979; Wall 1983). Systemic chemistries could be used at much lower rates than previous chemistries, and were combined in seed treatments to control multiple fungal classes (Edgington et al. 1980). Though higher usage may have historically occurred in some states (Becker and Stockdate 1980), less than 10% of the soybean seed planted in the Midwest carried seed-applied fungicide in 1996 (Munkvold 2009). In-furrow fungicide applications were equally promoted for the control of soilborne pathogens through the 1990s (Anderson and Buzzel 1982; Schmitthenner 1985, 1999) and efficacy of in-furrow treatments tended to be higher than use of traditional seed treatments (Guy et al. 1989).

In the early 1990s, seed treatment was described to be most useful in reducing "the risk of nonuniform plant stands," but no clear effect on yield had been determined from previous studies (Sinclair 1993). Captan and metalaxyl were found to benefit yield for a *Phytophthora*

sojae-susceptible variety in a field where Phytophthora root rot likely reduced final plant stand, but yield improvements were generally not observed for varieties with resistance or in fields without stand reductions (Lueschen et al. 1991). Seed treatments were consequently recommended for low quality soybean seed infected with seedborne pathogens, seed planted under cool conditions that delayed germination and increased risk of seedling disease, or for seed planted at reduced populations (Sinclair 1993).

The soybean seed industry was reported to be reluctant to develop and utilize commercial seed treatment products due to the extra cost for soybean growers (Schmitthenner 1985). Because treated seed can only be used for planting in one growing season, seed dealers and soybean growers would experience economic risk because leftover seed could not be marketed (Sinclair 1993). Additionally, economic returns for soybean production in the North Central United States was above $10 per soybean acre for only three of the 15 growing seasons from 1981-1995 (Prentice 2001). During this time, however, the seed industry in the United States was changing. From 1960 to 1997, the real value of seed expenditures in the United States increased 2.5-fold, indicating that more farmers started purchasing commercial seed during this time period rather than saving seed from the previous year's crop (Fernandez-Cornejo 2004). Part of this shift may have been due to the introduction of glyphosate-resistant soybeans in 1996, which was followed with widespread adoption; within a decade, 90% of soybean acres were planted with glyphosate-resistant seed purchased from commercial sources (Fernandez-Cornejo et al. 2014). During this same time period that soybean growers began to consistently acquire seed from commercial sources, the strobilurin fungicides became commercially available as a seed treatment with activity against *Fusarium* spp., *Rhizoctonia solani*, and multiple oomycete species (Munkvold 2009).

Several studies were conducted to evaluate the efficacy of commercial seed treatment formulations. For example, a field study in Illinois showed that even high-quality soybean seed planted into warm soil could experience a 6% improvement in seedling emergence from the use of seed-applied fungicide in one of two years, but yield was not affected by seed treatment in either year (Bradley et al. 2001). In greenhouse tests, soybean varieties with partial resistance to *P. sojae* exhibited improved emergence due to seed-applied metalaxyl and mefenoxam because partial resistance was not fully realized in emerging seedlings until unifoliates appeared (Dorrance and McClure 2001). Seed-applied fungicides were associated with reduced soybean stand loss and disease severity when soybean seedlings were challenged with *Rhizoctonia solani* isolates in a greenhouse study (Dorrance et al. 2003a). Poag et al (2005) developed a model which showed that a fungicide seed treatment investment of \$8.65 ha^{-1} enhanced profitability by \$43.71 ha^{-1} across sites and years – the first study to suggest that widespread use of fungicide seed treatment was profitable. However, the effect of seed-applied fungicides was not statistically significant for determining yield and was based on results from one soybean variety, creating the need for additional evaluation. A similar study in North Dakota assessed the efficacy of multiple seed-applied fungicides on one soybean variety. In the North Dakota study, only four of 14 sites experienced plant stand and yield benefit from seed treatment, though sites with improved stand were not always the same as those with improved yield (Bradley 2008a). A similar test in Michigan demonstrated that three of 16 sites experienced significant yield benefit from seed-applied fungicide. However, yield was significantly decreased at two additional sites where soybean had not been planted before, possibly due to interference between the seed-applied fungicide and *Rhizobium* inoculant (Schulz and Thelen 2008). In a regional study of sites with a history of Phytophthora root rot, sites in Ohio, South Dakota, and Ontario, Canada were

shown to have improved stand and yield from seed-applied metalaxyl or mefenoxam, though benefits from seed treatments on plant stand and yield were not observed at sites in Wisconsin, Nebraska, and Iowa (Dorrance et al. 2009a).

Innovation in coating techniques for seed-applied insecticides reduced the previous problem of insecticide phytotoxicity in field crops and improved insecticide efficacy (Turnblad and Chen 1998). Consequently, insecticidal seed treatments such as imidacloprid and thiamethoxam became more widely used in soybean production in the mid-2000s (DiFonzo 2006), controlling seedcorn maggot (*Delia platura*) and other pests such as soybean aphid (*Aphis glycines*) and bean leaf beetle (*Cerotoma trifurcate*) which can vector viral diseases (Munkvold et al. 2014). By the late 2000s, insecticides were recognized as a significant part of the soybean seed treatment industry for the United States (Munkvold 2009).

Following the registration of insecticidal seed treatments in 2004 for soybean, seed treatment formulations consisting of insecticide and fungicide-insecticide combinations soon became widely available (Myers and Hill 2014; Cox and Cherney 2011b). Seed-applied insecticides were shown to significantly reduce aphid populations and improve soybean yield in some instances where aphid pressure was high (McCornack and Ragsdale 2006; Magalhaes et al. 2009). In the absence of biologically significant aphid populations, however, a study demonstrated that use of seed treatment containing fludioxonil and either imidacloprid or thiamethoxam resulted in no significant gains in plant stand or seed yield (Cox et al. 2008). A different study from the same lab group used seed-applied fungicidal and insecticidal treatments on two commercial varieties of soybean in New York. Although overall plant stand and yield effects from seed treatment were statistically significant, the yield improvement was quite low, and the researchers concluded that equivalent economic returns could be obtained by increasing

plant populations of non-treated seed (Cox and Cherney 2011a). Esker and Conley (2012) observed that five of twenty environments exhibited significant yield improvements from seed treatment use, finding that seed treatment efficacy varied by soybean variety and environmental conditions. A similar study demonstrated that across two years, only one in four sites experienced significantly higher returns from use of combined seed-applied fungicides and insecticides (Cox and Cherney 2014). An on-farm study across Illinois and Indiana reported that fungicide-insecticide seed treatment resulted in yield improvements of 2% (Vossenkemper et al. 2016). Across sites and years in Wisconsin, seed treatment containing an insecticide was shown to provide 4% higher yield than non-treated seed or seed treated only with fungicides at normal planting populations (Gaspar et al. 2015).

Additional seed-applied products entered the seed treatment market to manage disease or promote growth of soybean and were evaluated to determine their benefits for soybean growers. For example, the combination of the seed-applied insecticide Poncho (clothianidin) and biological nematode protectant VOTiVO (*Bacillus firmus*) was registered for use on soybean in 2011 (Bayer CropScience, Research Triangle Park, NC) to protect yield losses against SCN. A study in Wisconsin testing fungicide, fungicide-insecticide, and fungicide-insecticide-nematicide combinations found that seed treatments that contained an insecticide resulted in significantly higher plant stands and yields than non-treated and fungicide-treated seeds (Gaspar et al. 2014); though treatments with either abamectin, a chemical nematicide, or Poncho/VOTiVO benefited yield outcomes at the most SCN-infested site over the three years of the study, the yield improvements attributed to nematode-antagonistic seed treatments were statistically significant at only four of the twenty-eight field sites evaluated. A Mississippi study found that use of seed-applied fungicides combined with inoculants and newly marketed proprietary growth-promoters

(lipo-chitooligosaccharides, LCOs) resulted in numerically higher yield than non-treated seeds, but significance of yield benefits from individual products were generally inconsistent from year to year (Golden et al. 2016). A region-wide study evaluated the benefits of high-input management techniques that included commercial seed-applied fungicides, Poncho/VOTiVO, and LCOs along with foliar applied fertilizers and pesticides. Yield was improved by at least one of the high-input systems tested relative to the non-treated seed at 33 of 53 sites across the three-year study; however, the specific effect of seed treatment components on yield was indeterminable due to the additional foliar treatments. Moreover, yield benefits from this regional study were not translated into higher profitability due to high input expenses (Marburger et al. 2016).

Despite the limited effectiveness of seed treatments in soybean to improve economic returns observed in the previously mentioned studies, more than 75% of soybean seeds in the United States were treated with a fungicide in 2013 (Munkvold et al. 2014). The soybean seed treatment industry has also grown in other major soybean-producing countries, such as Brazil, where more than 90% of soybeans were treated with a seed-applied fungicide in 2009 (Campo et al. 2009). The growth of the seed treatment industry has occurred internationally, with the seed treatment market doubling in value from 2002 to 2008 to reach the equivalent of more than $2 billion U. S. dollars (Munkvold 2009). As the seed treatment industry has continued to expand in the 2010s, it has remained unclear whether seed treatments ought to be broadly used across years and field sites or if they should be utilized only under certain climatic or edaphic conditions.

Potential determinants of seed treatment efficacy.

Though seed treatments have been shown to provide benefits to plant stand, yield, and economic outcomes for soybean producers at many field sites with a history of seedling disease

(Dorrance et al. 2009a), measurable factors that explain seed treatment efficacy. Soybean seed treatment efficacy may depend on pathogen sensitivity (Broders et al. 2007b; Dorrance et al. 2003b), host plant variety (Lueschen et al. 1991), soil chemical properties (Ware 2000), weather conditions such as temperature (Bradley 2008a), and other factors. Several hypotheses have been proposed to account for the observed variability in seed treatment efficacy and profitability, which will be discussed below.

One of the earliest explanations for the variability in seed treatment efficacy was related to soil moisture, with seeds planted into waterlogged soils being more likely to benefit from treatment (Ferriss et al. 1987). The yield response of one soybean variety to seed treatment was attributed to prolonged soil moisture in an additional study (Lueschen et al. 1991). However, flooding did not seem to elicit increased seed treatment efficacy in another study (Poag et al. 2005). Flooding has been shown to impact the recovery of certain seedling pathogens. For example, the incidence of *Pythium* species was significantly increased by flooding during seedling stages, whereas *Fusarium* and *Rhizoctonia* species were less frequently isolated after flooding events (Kirkpatrick et al. 2006b). Though high soil saturation may impact the microbial communities in soybean rhizospheres, seed treatment efficacy may not necessarily increase.

Another early hypothesis was that seed treatment is more effective in no-till systems than in conventional tillage systems (Guy and Oplinger 1989). However, other studies have shown that seed treatment efficacy is actually improved by tillage (Cox et al. 1976) or that tillage system does not impact seed treatment efficacy (Lueschen et al. 1991). Recent studies have observed no yield differences between non-treated seed and seed treated with a fungicide in no-till systems (Bradley et al. 2001; Wang et al. 2004). This hypothesis also does not account for differences in seed treatment efficacy observed among fields under the same tillage system.

Though tillage may impact seed treatment efficacy, other factors must also explain the circumstances in which seed treatment is most efficacious.

Previous work has shown that yield loss resulting from stand loss may be reduced by using seed treatment to manage stand-reducing pathogens and insects. Multiple studies have demonstrated that seed-applied fungicide use generally improves plant stand in controlled conditions (Dorrance et al. 2003a; Ellis et al. 2010; Urrea et al. 2013) and in field conditions (Golden et al. 2016). Results from greenhouse trials may not always transfer to the field, however, since emergence of non-treated seed under controlled conditions may not accurately represent field emergence (Urrea et al. 2013; TeKrony et al. 1974). Many field studies have shown either no effect or an inconsistent effect of seed-applied fungicide on plant stand (Lenssen 2013; Gaspar et al. 2015; Bradley et al. 2001). In contrast, seed-applied fungicide-insecticide combinations seem to result in relatively consistent plant stand improvements (Gaspar et al. 2014, 2015; Cox and Cherney 2011b; Esker and Conley 2012; Cox and Cherney 2014), though it is unclear why the addition of the insecticide tends to improve plant stand. Seed corn maggot is one of the primary soybean pests known to reduce plant stand in soils with high organic matter (Hammond 1991), but it is unclear if benefits from seed-applied insecticides are related to seed corn maggot incidence. Although sufficiently low plant stand due to pathogen or insect damage may result in yield loss, soybean plants can often compensate for evenly-distributed plant stand losses by bearing seed on additional branches (Stivers and Swearingin 1980; Ethredge et al. 1989; Cox and Cherney 2011a). Additionally, some studies reported instances where yield improvements occurred without plant stand improvements (Bradley 2008a; Schulz and Thelen 2008) and others observed plant stand improvements without yield improvements (Bradley et al. 2001; Bradley 2008a; Dorrance et al. 2009a; Golden et al. 2016). A study in Ohio also showed

that plant-stand reducing *Rhizoctonia solani* is not controlled well by seed-applied fungicides (Dorrance et al. 2003b). In anticipation of improved plant stand from use of fungicide-insecticide seed treatments, results from a study in Wisconsin indicated that growers should decrease planting populations to achieve optimal partial returns (Gaspar et al. 2015). Although many soybean growers still plant at populations above the recommended 370,000 seeds ha^{-1} in Michigan (Staton 2012) and may improve economic returns by reducing seeding rates, it remains unclear whether or not seed treatment will consistently improve plant stand, higher yields, and consequent higher profitability.

Some seed treatments, in addition to affecting pathogen or pest populations, may also induce resistance in soybean hosts. For example, neonicotinoid insecticides have been shown to increase plant growth and induce systemic acquired resistance (SAR) in multiple host plants (Ford et al. 2010; Elbert et al. 2008). Insecticidal seed treatments may stimulate the SAR salicylic acid-signaling pathways, which often improves plant resilience to some abiotic and biotic factors (Miura and Tada 2014; Senaratna et al. 2000). One study has shown that SAR induction contributes to improved soybean resistance to the root rot pathogen *Phytophthora sojae* (Han et al. 2013). However, other studies have demonstrated that seed-applied insecticides used independently do not consistently improve yields and partial profits – particularly in the absence of insect pressure – and may have unintended negative consequences such as mortality of generalist insect predators (Seagraves and Lundgren 2012; Johnson et al. 2009). The chemical and biochemical interactions among seed treatments, soybean hosts, and various soil factors may require additional study before the activity of seed-applied pesticides on soybean emergence and yields can be characterized clearly.

Planting date has also been proposed as a factor that affects seed treatment efficacy, particularly as it relates to the early-season temperature effects on soybean emergence (Bradley et al. 2001; Bradley 2008a). An early source reported no effect of planting date on seed treatment efficacy (Wall 1983), and a more recent study investigating the role of seed treatments and planting dates on soybean yield did not report an interaction between these factors (Cox et al. 2008). Early planting has been recommended for maximizing soybean yield in recent decades (De Bruin and Pedersen 2008), but the effect of planting date on seed treatment efficacy has yet to be described for early-planted soybean systems.

Due to the complexity of soybean systems, describing the factors that influence seed treatment efficacy and consequent profitability remains a challenge. A seed treatment may exhibit higher efficacy due to variation in pathogen sensitivity to seed treatment chemistries (Ellis et al. 2010; Dorrance et al. 2003b), effects of soil characteristics such as and bulk density and soil organic matter (Ware 2000), variability in host plant response to seed treatment (Lueschen et al. 1991; Esker and Conley 2012), or perhaps even due to impacts of herbicide applications on the populations of *Pythium* species and other microbes (Descalzo et al. 1998; Meriles et al. 2006). Though interactions in field systems may be complex, identifying multiple factors that are able to quantifiably describe seed treatment efficacy still holds utility for informing management decisions by growers, product development strategies for industry, and future research emphases in academia.

Part II. Previous Characterization and Management of Pathogenic *Pythium* Species on Common Bean (*Phaseolus vulgaris*)

Common bean production.

Common bean (*Phaseolus vulgaris*) is the most important food legume internationally in terms of production and direct human consumption (Broughton et al. 2003). Varieties of common bean are generally either from the Andean or Middle American gene pools, a distinction based on the dual geographical centers for common bean domestication as well as several physiological differences (Debouck et al. 1993). Nearly 70% of common bean production occurs in Latin America and East Africa, where it is grown as a staple (Broughton et al. 2003; CGIAR 2016). In four East African countries, pulses such as common bean provide at least 20% of per capita protein intake (Akibode and Maredia 2011). Nearly 60% of common bean production in Africa comes from four countries in East Africa – Tanzania, Uganda, Kenya and Rwanda (FAO 2014) – countries that are considered to be at high risk for the development of seedling and root disease of common bean due to climatic and terrestrial conditions (Farrow et al. 2011). Though bean producers use chemical control to prevent soilborne diseases (Lennox and Alexander 1981), damping-off and root rot pathogens persist as a significant production issue in the United States and Canada (Kelly et al. 1998).

In the state of Michigan, common bean is raised for the snap bean processing industry and as a dry edible bean (National Agricultural Statistics Service 2011). Snap bean production is a relatively small industry in Michigan, accounting for about 15,000 acres planted in 2014 (USDA-NARS 2014). However, Michigan ranks second nationally for the production of dry beans and in 2010 was the top producer of multiple dry bean classes – navy bean, cranberry

bean, and black bean – on a total of 235,000 acres (National Agricultural Statistics Service 2011). Dry beans in Michigan are typically grown in rotation with other field crops, such as corn, sugar beet, soybean, or wheat. Yields of dry beans grown in Michigan and other parts of the developed world are regularly around 1.7 metric tons ha^{-1}, more than twice the yields in developing countries (Akibode and Maredia 2011). This disparity in bean yield may be related to many genetic or environmental factors, such as drought, soil fertility, suboptimal planting conditions, weeds, weather events, or soilborne pathogens (Lobell et al. 2009; Beebe 2012). Yield loss of 70% or more due to *Pythium* species and other soilborne pathogens has been previously reported in common bean (Singh and Schwartz 2010; Nzungize et al. 2012).

Description of* Pythium-*host interactions.

Pythium is a genus of filamentous saprotrophs, myco-parasites, and plant pathogens (Adhikari et al. 2013). *Pythium* species are grouped into subgenus categories called clades based on phylogenetic differences with distinct morphological characteristics (Lévesque and De Cock 2004). *Pythium* Clade K was recently reclassified as a new genus, *Phytopythium* (Abad et al. 2010; de Cock et al. 2015), but for the purposes of this review, both genera will be collectively referred to as *Pythium*. Where species names are listed, the abbreviation "*Py.*" will be used for *Pythium* and "*Phy.*" for *Phytopythium*. There are more than 150 species of *Pythium* recognized (Senda et al. 2009), although more than 300 possible species have been proposed (Schroeder et al. 2013). *Pythium* species reproduce both sexually and asexually and may be either homothallic or heterothallic, depending on the species (Lévesque and De Cock 2004).

Many *Pythium* species have been found to cause damping-off or root rot either independently or in a complex with other soilborne pathogens (Johnson and Doyle 1986). Studies have demonstrated that the severity of seedling disease may be elevated by pathogen

complexes with *Fusarium* and *Rhizoctonia* (Wong et al. 1984; Pieczarka and Abawi 1978a). In saturated field conditions, *Pythium* incidence has been shown to rise whereas true fungi incidence tends to decrease (Li et al. 2014), indicating that complexes with true fungi may be less likely to form under certain field conditions. Instead, different *Pythium* species may cause more aggressive disease symptoms by forming synergistic complexes with one another (Kobriger and Hagedorn 1984). Whether or not root rot species complexes are formed, some *Pythium* species, such as *Pythium ultimum*, can cause disease across a wide host range of plants (Okubara et al. 2014; Robertson 1976).

Many of the plant species that are known hosts of *Pythium*-induced diseases are important cereal crops, such as corn, wheat, and rice (Broders et al. 2007a; Dewan and Sivasithamparam 1988; Higginbotham et al. 2004; Paulitz and Adams 2003; Van Buyten and Höfte 2013; Zhang and Yang 2000). However, many specialty crops also are susceptible to multiple *Pythium* species (Mazzola et al. 2002; Moorman et al. 2002; Munera and Hausbeck 2016; Petkowski et al. 2013; Tewoldemedhin et al. 2011; Weiland et al. 2013), as are leguminous crops, such as soybean, peanut, and common bean (Bates et al. 2008; Kirkpatrick et al. 2006b; Li et al. 2014; Matthiesen et al. 2016; Nzungize et al. 2011; Wei et al. 2010; Wheeler et al. 2005; Zitnick-Anderson and Nelson 2015). *Pythium* species have been considered a leading cause of root rot development in the major bean growing regions of North America, Latin America, and East Africa (Hoch and Hagedorn 1974; Rusuku et al. 1997; Pfender 1981).

Previous studies conducted with different bean varieties and temperatures have identified *Pythium* species that are pathogens of common bean (Pieczarka and Abawi 1978c; Pfender 1981; Sippell and Hall 1982). Recent papers describing the pathogenicity and virulence of *Pythium* species have expanded the number of known *Pythium* spp. pathogenic on common bean with

updated taxonomy (Li et al. 2014; Nzungize et al. 2011). At present the USDA Fungal Database currently has recognized 34 *Pythium* species as being associated with common bean (USDA-ARS 2016), including *Pythium aphanidermatum*, *Py. aristosporum*, *Py. arrhenomanes*, *Py. butleri*, *Py. catenulatum*, *Phytopythium chamaehyphon*, *Py. conidiophorum*, *Py. cryptoirregulare*, *Phy. cucurbitacearum*, *Py. debarynaum*, *Py. diclinum*, *Py. dissotocum*, *Py. folliculosum*, *Phy. helicoides*, *Phy. indigoferae*, *Py. intermedium*, *Py. irregulare*, *Py. lutarium*, *Py. macrosporum*, *Py. mamillatum*, *Py. myriotylum*, *Py. oligandrum*, *Py. pachycaule*, *Py. paroecandrum*, *Py. perplexum*, *Py. rostratifingens*, *Py. rostratum*, *Py. solare*, *Py. spinosum*, *Py. sulcatum*, *Py. sylvaticum*, *Py. torulosum*, *Py. ultimum* (including vars. *sporangiiferum* and *ultimum*), and *Phy. vexans*. Though these species are known to form associations with common bean, some of these species, such as *Py. perlexum,* was not reported to cause seedling disease in bean (Li et al. 2014). The pathogenicity and comparative virulence of many *Pythium* species remains unclear.

Pathogenicity and virulence of *Pythium* species may not be well-characterized because virulence of oomycete pathogens has been reported to vary in different environmental conditions (Hendrix and Campbell 1973a). Growth and reproduction of pathogenic *Pythium* species varies depending genetic differences of host and pathogen (De Cock and Lévesque 2004) as well as temperature, pH, and many other environmental factors (Hendrix and Campbell 1973a; Lumsden et al. 1975; Nelson and Craft 1991). For example, growth of *Pythium ultimum*, *Phytopythium vexans*, and *Pythium irregulare* has been previously reported to respond to changes in temperature (Cantrell and Dowler 1971; Pieczarka and Abawi 1978c). For *Pythium* species responsive to temperature, however, optimal temperatures for growth and virulence may not be the same (Hendrix and Campbell 1973b). Moreover, the virulence of all pathogenic *Pythium*

species does not change uniformly in regard to temperature. For example, *Py. irregulare* has been shown to induce comparatively more severe disease symptoms at temperatures <20°C than at temperatures ≥20°C across several host species (Wei et al. 2010; Ben-Yephet and Nelson 1999; Wong et al. 1984; Stovold 1974; Biesbrock and Hendrix Jr 1970; Sippell and Hall 1981; Roncadori and McCarter 1972). *Pythium aphanidermatum* has exhibited the opposite effect, causing increasingly severe disease as temperature increases (Wei et al. 2010; Gold and Stanghellini 1985; Thomson et al. 1971). Multiple other studies have demonstrated that the virulence of some other *Pythium* species, *Py. ultimum* or *Py. lutarium,* are not responsive to temperature (Wei et al. 2010; Matthiesen et al. 2016). However, other studies have demonstrated that *Py. ultimum* loses virulence as temperatures approach 26°C (Thomson et al. 1971; Pfender 1981). Although the growth and virulence of some *Pythium* species have not been observed to respond to temperature, lack of observed temperature response may have resulted if assay conditions were not ideal for observing pronounced temperature responses or if the virulence of certain isolates within a *Pythium* species do not respond uniformly to temperature changes. Even within a single *Pythium* species, instances have been recorded in which either some isolates cause disease symptoms and others do not (Augspurger and Wilkinson 2007; Abad et al. 1994) or the aggressiveness among isolates is significantly different (Higginbotham et al. 2004; Olson et al. 2016; Wei et al. 2010). Though differences in virulence among isolates are observed, many factors may contribute to this variability, such as differences in inoculum density (Raftoyannis and Dick 2002; Sippell and Hall 1981) or dissimilar preferences for environment conditions, such as temperature (Hendrix and Campbell 1973a; Martin and Loper 1999; Nelson and Craft 1991).

Though panels of 10 or fewer *Pythium* species have been tested to determine their pathogenicity and virulence on common bean in North America (Kobriger and Hagedorn 1984; Olson et al. 2016; Pieczarka and Abawi 1978c), virulence has not been compared among a large panel of *Pythium* species to determine which species cause the most severe disease in bean production. Previous studies with different experimental conditions may confound comparisons among *Pythium* species. By specifying a subset of the key *Pythium* species that cause disease on common bean under planting conditions, control strategies can be developed and evaluated respective to these species.

Management of* Pythium *disease on common bean.

Management of Pythium root rot comes with inevitable challenges. Growers need to make disease management decisions before planting without knowing whether or not disease pressure will be high in a given year (Abawi and Corrales 1990). Thus, Pythium damping-off is often managed proactively with fungicide-treated seed (Ramos and Ribeiro Jr 1993). Alternative methods of disease management that aim to predict the risk of *Pythium*-induced disease are not yet fully developed. For example, molecular techniques have been developed that can identify the presence of oomycete DNA from plant and soil samples to estimate inoculum density in a soil sample (Lievens et al. 2006; Catal et al. 2013; Tambong et al. 2006). Most authorities have not agreed on a single reliable pre-season risk assessment tool that could be used to guide management decisions. Since recovered oomycete DNA from soil samples may not be part of a living cell (Steffan et al. 1988) or may be inhibited under certain edaphic and management conditions (Bulluck et al. 2002; Johnson and Doyle 1986; Lumsden et al. 1976), various preventative management strategies for *Pythium* seedling disease remain important in bean

production, such as cultural, biological, chemical, and genetic approaches (Abawi and Corrales 1990).

Diverse cultural management practices have been utilized to try to diminish *Pythium*-induced seedling disease and root rot. For example, steam pasteurization or solarization of soil has been utilized to temporarily sterilize surface soil (Hendrix and Campbell 1973a). Adjusting the planting date has been shown to affect the incidence of Fusarium root rot (Naseri and Mousavi 2013) and may impact incidence of disease caused by certain *Pythium* species as well. Adjusting planting date may reduce disease severity because of differences in soil moisture and temperature among planting dates (Hwang et al. 2015, 2000). Under field conditions, planting common bean at 50mm depth or shallower has been demonstrated to reduce root rot severity and increase root growth relative to seed planted at 75mm or deeper (Naseri and Mousavi 2013; O'Brien et al. 1991). Additional findings indicated that shallow tillage also may have benefits for root health (O'Brien et al. 1991). Treatment with glyphosate was found to increase *Pythium* spp. populations in laboratory and field conditions (Meriles et al. 2006; Descalzo et al. 1998), so modifying herbicide chemistries and application rates may reduce risk of *Pythium*-induced seedling disease. However, cultural disease management practices are just one of multiple strategies for integrated disease management (Abawi and Corrales 1990).

Commercial biological control strategies for *Pythium* seedling disease management have been developed for use in common bean production and have been evaluated for field activity against root rot pathogens (Keinath et al. 2000). Studies have shown that *Pythium* species can be sensitive to bacterial antagonism, whether due to the production of antifungal compounds or competition (Tedla and Stanghellini 1992; Howell and Stipanovic 1980; Walker et al. 1998). Some *Bacillus* species have been effectively developed as a commercial seed treatment to protect

against root rot and seedling disease induced by *Pythium* and *Fusarium* species (Mao et al. 1997; de Jensen et al. 2002; Keinath et al. 2000). Additionally, some *Pythium* species, such as the mycoparasitic *P. oligandrum* and *Py. nunn* (Lévesque and De Cock 2004; Elad et al. 1985), have been shown to colonize the host rhizospheres and parasitize pathogenic *Pythium* species, reducing disease incidence and improving plant biomass in multiple crops (Al-Rawahi and Hancock 1997; Paulitz et al. 1990; Zhu et al. 2015). *Pythium oligandrum* also has been registered for use as a biocontrol agent for root rot management in multiple field crops (Milofksy 2007). Alternatively, certain isolates of non-pathogenic, non-mycoparasitic *Pythium* have been shown to actively colonize host root systems while improving host growth, suggesting that some *Pythium* species may have a role in either enhancing plant development or inhibiting colonization by pathogenic soil biota (Mazzola et al. 2002; Bahramisharif et al. 2014). Though biological disease management strategies are not utilized extensively for *Pythium* root rot, these practices may become more feasible as technologies continue to develop.

Common bean producers regularly utilize different forms of chemical control to prevent losses from Pythium root rot. Previous studies have demonstrated limited benefits from soil fumigation (Hendrix and Campbell 1973a; Kerr and Steadman 1973; Navarro et al. 2008). In-furrow fungicide applications have been more readily implemented for reducing seedling diseases in common bean (Elwakil and Mossler 1999; Bost 2005; O'Brien et al. 1991). The most economical and direct chemical control method for oomycete pathogens are seed treatments of metalaxyl or mefenoxam that have become standard in commercial production ever since seed-application of fungicide was first shown to be highly successful for control of *Pythium* species on common bean (Locke et al. 1983; Papavizas et al. 1977; Abawi and Corrales 1990). Seed-applied fungicides for the control of *Pythium*-induced seedling disease and root rot is used on

more than 75% of bean seeds in parts of the United States (Fuchs and Hirnyck 2007). Though seed-applied fungicide application has proven to be effective in the control of soilborne seedling pathogens (Keinath et al. 2000), successful control is not always achieved (de Jensen et al. 2002; Trutmann et al. 1992). Inconsistent chemical control may be due to multiple factors, such as chemical insensitivity in some *Pythium* populations (Cook and Zhang 1985; Brantner and Windels 1998; Moorman and Kim 2004; Taylor et al. 2002; Olson et al. 2016) or complex interactions between seed-applied chemistries and non-target micro-organisms (Monkiedje et al. 2002). Disease control methods other than chemical control are needed for organic production (Roberts et al. 2014). Though seed-applied fungicides may be profitable for bean production in developing countries (Trutmann et al. 1992), alternatives may still be needed where the initial expense of chemical inputs is unaffordable or reliable products are difficult to attain. Limitations associated with use of chemical control of *Pythium*-induced disease may be best addressed by integrating chemical control with other management strategies, such as host resistance.

Plant breeding also has been utilized to reduce the economic impact of *Pythium*-induced disease of common bean by developing resistant varieties. Breeders have identified patterns between enhanced root rot resistance and easily identifiable phenotypic traits, such as seed pigmentation (Lucas and Griffiths 2004) and seed size (Schneider and Kelly 2000; Li et al. 2014). Other studies have associated resistance to damping-off and root rot pathogens with a specific bean gene pool, indicating that Middle American varieties are generally more resistant to damping-off and root rot pathogens than Andean varieties (Schneider et al. 2001a; Román-Avilés and Kelly 2005; Beebe et al. 1981). However, each of these views may be overly simplistic, as phenotypic markers can become separated from resistance traits (Zaumeyer and

Meiners 1975), especially if certain root rot resistance traits in common bean are associated with combinations of genes (Román-Avilés and Kelly 2005).

Though previous correlations between phenotypic traits and observed root rot resistance may explain part of the resistance patterns observed in common bean varieties, particularly for root rot genes controlled by one dominant trait (Namayanja et al. 2014), current approaches to breeding have depended increasingly on molecular techniques (Moose and Mumm 2008). Within the last decade, plant breeders have increasingly utilized quantitative trait loci mapping, marker-assisted selection, and sequencing to more effectively understand the genes that confer root rot resistance in common bean (Navarro et al. 2008; Miklas et al. 2006; Hagerty et al. 2015). Host mechanisms for resistance to *Fusarium solani* have been described well in recent studies (Chowdhury et al. 2002; Schneider et al. 2001b; Navarro et al. 2009). However, developing *Pythium* resistant bean varieties that maintain commercial quality has proven to be difficult (Navarro et al. 2008). By clearly explaining the interactions of pathogenic *Pythium* species with bean hosts, breeders may be further equipped to develop new, commercially acceptable bean varieties with comprehensive root rot resistance and that is effective under diverse environmental conditions.

CHAPTER 2. PROFITABILITY AND EFFICACY OF SOYBEAN SEED TREATMENT IN MICHIGAN

Introduction

Seed-applied fungicides were utilized on 10% of United States soybean seed planted in 1996 (Munkvold 2009). Seed treatment was recommended when soybean seed quality was reduced due to age or seedborne disease, when varieties displayed susceptibility or partial resistance to seedling diseases, or when seed was planted in favorable conditions for seedling and root disease (Dorrance and McClure 2001; Lueschen et al. 1991; Guy et al. 1989; Wall et al. 1983; TeKrony et al. 1974; Edje and Burris 1971; Sinclair 1993). However, 75% of soybean seed was treated in 2013 (Munkvold et al. 2014). This change in seed treatment use could be due to earlier planting dates and reduced tillage practices that may increase the risk of plant stand loss (Esker and Conley 2012; Dorrance et al. 2009b). Seed treatments currently may contain fungicides, insecticides, and nematicides and are more frequently utilized in soybean to manage early-season disease and insect pressure than in previous decades (Munkvold et al. 2014).

Soybean cyst nematode (SCN), root rots, and seedling diseases are cited as major causes of yield loss throughout the soybean-producing regions of the United States (Wrather et al. 2001; Wrather and Koenning 2006; Koenning and Wrather 2010) and the rest of the world (Wrather et al. 2010). Seedling diseases and root rots are caused by true fungi such as *Rhizoctonia solani* and *Fusarium* species or oomycetes such as *Phytophthora sojae* and *Pythium* species (Arias et al. 2013; Farias and Griffin 1990; Rizvi and Yang 1996; Schlub and Lockwood 1981; Tachibana et al. 1971; Schmitthenner 1985). Seedcorn maggot (*Delia platura*) can reduce soybean plant stand

(Miller and McClanahan 1960) and soybean aphid (*Aphis glycines*) can substantially reduce yield when present at populations above 675 aphids plant^{-1} at growth stages R3-R5 (Ragsdale et al. 2007). Seed treatment active ingredients are used in soybean production to manage the previously mentioned pathogens and pests and resulting loss of plant stand and yield.

In several previous studies, fungicide seed treatments improved emergence relative to non-treated seed, which has generally been attributed to control of soybean seedling pathogens (Bradley et al. 2001; Dorrance et al. 2003a; Dorrance and McClure 2001). In most field studies, however, significant improvements in plant stand resulting from the use of seed-applied fungicides have been found at fewer than 50% of sites (Bradley et al. 2001; Bradley 2008a; Guy et al. 1989; Schulz and Thelen 2008). In two Wisconsin studies, seed treatments have been found to cause no significant stand count improvement in some years (Esker and Conley 2012; Gaspar et al. 2014). However, seed treatments containing combined fungicides and insecticides have been shown to improve plant stand from 3% to 17% across field sites depending on year and seed treatment formulation (Esker and Conley 2012; Gaspar et al. 2014). However, it remains unclear if this benefit of seed treatment on plant stand is consistent across states and years. If stand loss drops below 247,000 plants ha^{-1}, economic returns of a soybean grower could be negatively affected by reducing yield or necessitating replanting of a site (Gaspar and Conley 2015; Lee et al. 2008). However, the evidence that seed treatments improve economic outcomes across growing conditions by protecting plant stand remains largely anecdotal.

Multiple studies demonstrated that seed-applied fungicides result in significant yield improvements in fewer than 30% of field sites (Bradley 2008b; Schulz and Thelen 2008; Cox et al. 2008). Similarly, a recent study in Wisconsin indicated that the probability of breaking even (recovering the cost of the seed treatment by experiencing increased yield) may be equivalent

between seeds with fungicide and those without (Gaspar et al. 2015). Significant yield responses to seed-applied insecticides and combined fungicide-insecticide applications were observed at fewer than 40% of sites (Bradshaw et al. 2008; Esker and Conley 2012; Gaspar et al. 2014). In addition to fungicides and insecticides, seed-applied nematode protectants combined with fungicide and insecticide resulted in significantly higher yield than non-treated seed in fewer than 20% of sites across a three-year study (Gaspar et al. 2014). Findings from previous studies indicate that seed treatments have variable efficacy and economic benefit across growing conditions. Consequently, it remains important to evaluate whether or not current commercial seed treatments are profitable for soybean production under Michigan growing conditions.

The objectives of this study were to 1) assess the efficacy of various seed treatment components and 2) to compare the impact of multiple commercially available seed treatments on yield and profitability in soybean.

Methods

Field Study.

Seeds of four soybean varieties varying in SCN susceptibility were planted in 2013, 2014, and 2015. Soybean varieties used differed slightly by site and year (Table 1), but all varieties were resistant to Phytophthora root rot. Resistance to other seedling and root rot pathogens was not described. Variety names were not specified due to the data being proprietary.

Table 1. Soybean varieties used to evaluate seed treatments, with "X" denoting their use in the greenhouse trial or in a given year of the field trial, respectively. Variety names are not specifically mentioned due to the information being proprietary.

Variety Name	SCN Resistance	2013	2014	2015	Greenhouse
Asgrow-1	None	X	X	X	X
Asgrow-2	PI 88788	X	X	X	X
Pioneer-1	None	X			
Pioneer-2	PI 88788	X			
Pioneer-3	PI 88788		X	X	
Pioneer-4	Peking		X	X	

Soybean varieties either had no SCN resistance or SCN resistance conferred by the soybean line PI 88788 (Eppes and Hartwig 1972) or Peking (Ross and Brim 1957).

Planting dates ranging from May 7 to June 9 at seven field sites that were part of the Michigan Soybean Performance Trials (Table 2). Plots were arranged in a randomized complete block design with four replications in 2013 and six replications in 2014 and in 2015. In 2013, seeds were planted with a custom-built, six-row planter with seed units (John Deere, Moline, IL). In 2014 and 2015, seeds were planted with a six-row Almaco custom-built precision vacuum planter with a Seed Pro 360 controller (Almaco, Nevada, IA) and John Deere seed units. Seeds were planted 3.8 cm deep in 38 cm rows with a seeding rate of 395,000 seeds per hectare. Each plot was 6.1 m long and was trimmed to 4.3 m prior to harvest.

Soybean seed treatments evaluated in the study included i) non-treated control [NTC], ii) fungicide [F], iii) fungicide and insecticide [FI], and iv) fungicide, insecticide, and a biological

Table 2. Geographic and edaphic characteristics, planting dates, and harvest dates of soybean field site sites included in the 2013, 2014, and 2015 seed treatment profitability field study.

Year, County	Coordinates	Soil texture	pH	CEC meq 100 g^{-1}	SOM (%)	Clay (%)	Planting date	Harvest date
2013								
Allegan	42.68 N, -86.02 W	Sandy Loam	5.9	7.4	1.5	6.0	05/07	10/12
Hillsdale	41.78 N, -84.61 W	Silt Loam	6.4	13.0	2.2	18.7	05/15	10/11
Ingham	42.69 N, -84.50 W	Loam	6.6	10.2	2.5	14.9	06/07	11/09
Lenawee	41.94 N, -83.81 W	Clay Loam	6.5	29.9	4.8	34.3	05/17	10/01
Saginaw	43.40 N, -83.87 W	Clay Loam	7.0	7.9	3.1	17.0	05/16	10/09
Sanilac	43.47 N, -82.82 W	Clay Loam	6.9	13.2	3.4	22.4	05/27	10/23
St Joseph	42.04 N, -85.32 W	Sandy Loam	6.0	5.4	1.4	7.3	05/08	10/29
2014								
Allegan	42.69 N, -86.03 W	Sandy Loam	7.3	7.0	2.2	11.0	06/06	11/03
Hillsdale	41.83 N, -84.70 W	Loam	7.2	10.4	3.9	26.6	05/23	10/26
Ingham	42.69 N, -84.49 W	Loam	6.2	12.0	4.2	21.0	06/07	11/09
Lenawee	41.93 N, -83.82 W	Clay Loam	6.2	14.1	3.8	33.0	06/09	11/05
Saginaw	43.40 N, -83.84 W	Clay Loam	7.8	18.4	3.7	34.6	05/29	10/23
Sanilac	43.48 N, -82.81 W	Clay Loam	7.4	11.1	3.4	27.6	05/31	10/30
St Joseph	42.04 N, -85.33 W	Sandy Loam	7.5	8.0	2.2	10.3	05/12	10/27
2015								
Allegan	42.70 N, -86.01 W	Sandy Loam	6.8	8.7	3.4	17.9	05/29	10/19
Hillsdale	41.84 N, -84.70 W	Sandy Clay Loam	6.6	2.3	3.9	24.0	05/21	10/13
Ingham	42.69 N, -84.50 W	Loam	7.1	9.4	3.2	18.0	05/23	10/27
Lenawee	41.94 N, -83.81 W	Silty Clay Loam	6.5	16.2	4.3	38.0	05/22	10/15
Saginaw	43.41 N, -83.88 W	Clay Loam	7.6	18.1	4.0	36.0	05/14	10/12
Sanilac	43.47 N, -82.80 W	Loam	7.2	9.0	3.3	20.0	05/18	10/17
St Joseph	42.00 N, -85.43 W	Loamy Sand	6.8	9.1	2.2	9.6	05/07	10/22

In 2013, pH, cation exchange capacity (CEC), and percent soil organic matter (SOM) were estimated using the NRCS Web Soil Survey Online Maps; in 2014 and 2015, the same information was determined from soil tests conducted by the MSU Soils lab.

nematode protectant [FIN]. Active ingredients and application rates varied by company (Table 3). Asgrow seed was treated by agitating seeds and respective treatments in a 5-gallon pail until seed was uniformly coated. Pioneer seed was commercially treated in a custom drum applicator. The same seed chemistries were applied in all three years of the study. Climate data for the field

sites were collected using the PRISM Climate Group database (Prism Climate Group, Oregon State University 2016).

Table 3: Soybean seed treatments evaluated in field studies in 2013, 2014, and 2015 and a greenhouse study.

	Asgrow			Pioneer		
	Trade Name	**Active Ingredients**	**Application Rate**	**Trade Name**	**Active Ingredients**	**Application Rate**
			mL kg^{-1} seed			mL kg^{-1} seed
Fungicide	Accerelon DX-109®	pyraclostrobin 18.4%	0.43	Evergol Energy	prothioconazole 7.18% penflufen 3.59% metalaxyl 5.74%	0.60
	Acceleron DX-309®	metalaxyl 28.35%	0.27	ApronMaxx RTA®	mefenoxam 1.10%, fludioxonil 0.73%	3.25
	Acceleron DX-612®	fluxapyroxad 28.7%	0.17	PPST 2030	biological control *Bacillus* spp.	1.21
Insecticide	Acceleron IX-409®	imidacloprid 48.7%	1.43	Gaucho® 600 Flowable	imidacloprid 48.7%	0.97
Insecticide-Nematode Control	Poncho-VOTiVO	clothianadin 40.3% *Bacillus firmus* I-1582 8.1%	1.46	Poncho-VOTiVO	clothianadin 40.3% *Bacillus firmus* I-1582 8.1%	0.63

Asgrow and Pioneer indicate seed company names. Application rates refer to the amount of commercial seed treatment applied by trade name. Percentage of active ingredient present within the commercial formulation is listed after the name of each active ingredient. Seed treatments evaluated included non-treated seed and treated seeds containing fungicide, a fungicide and insecticide, or a fungicide, insecticide, and nematode biocontrol agent. All treated seeds contained the fungicide formulations respective to each company. ApronMaxx RTA application rate is estimated from labeled rates due to proprietary information.

Soil characteristics in 2013 were estimated using the NRCS-USDA Web Soil Survey online database (Natural Resources Conservation Service 2013). The four center rows of each six-row plot were harvested using an Almaco 4-row combine with weight buckets and a HarvestMaster (Juniper Systems, Logan, UT) harvest system; harvest mass and moisture was determined. Grain yield was adjusted to 13% moisture.

In 2014, Approximately 25, 15 cm-deep soil subsamples were collected in a zigzag pattern from each site within three weeks of planting. Subsamples from within each site were mixed thoroughly to form a composite soil sample for that site. Soil composite samples were submitted both to the MSU Soil and Plant Nutrient Laboratory for soil analysis and to the MSU

Plant Diagnostics Laboratory for SCN analysis. If SCN was found in a site's composite sample, early-season and late-season subsampling of each plot was conducted to determine the reproductive factor (late-season eggs/mean early-season eggs) and fecundity (late-season eggs/mean late-season cysts) of SCN. Plant stand was determined at growth stages VC-V1 (Fehr et al. 1971) by counting all of the living, emerged plants in two of the four center rows. From the four center rows of each plot in 2014, distances from the soil surface to the apical tip of ten arbitrarily selected plants were recorded at growth stages V2/V3 at all sites except Hillsdale, where plants were measured at growth stage V1. In late June, fifty soybean plants were scouted from the two outside rows in the control plots at each site to evaluate aphid pressure. Sites that had aphids present in ≥25% of control plots were revisited to determine aphid populations in each plot; in two of the four center rows, the apical tip and nearest trifoliate leaf of 25 consecutive plants were examined for aphids, and populations were recorded individually by plant. Plant stand, plant height, and aphid counts were determined in rows that were harvested at the end of the season. Yield was collected using the same method as in 2013.

In 2015, soil sampling was conducted using the same method as in 2014, except that soil samples were collected within one week of planting. SCN samples, plant stands, and aphid counts were likewise determined using the same method as in 2014. Root dry weight was measured for ten consecutive emerged seedlings from each plot by washing the roots, separating roots and shoots, and drying the roots at 49°C ± 11°C until dry weights stabilized. Yield was collected using the same method as in 2013 and 2014.

A survey of fungi and oomycetes associated with soybean seedlings was conducted in 2015. At each site, two seedling samples displaying stunting or other signs of poor fitness were taken from three randomly-selected reps of each NTC treatment and kept cool on ice for

transportation; samples were stored at 4°C until processed. Roots were washed in tap water within 24 hours and lesioned root pieces about the size of a pinhead were plated onto semi-selective media, including both water agar amended with metalaxyl (300 μg/mL) and streptomycin (15 μg/mL) (WMS) and corn meal agar amended with pimarcin, ampicillin, rifampicin, pentochloronitrobenzene (Jeffers and Martin 1986) and benomyl (10 μg/mL) (CMA-PARPB). WMS and CMA-PARPB were used to isolate true fungi and oomycetes, respectively. Isolates were transferred to fresh media to obtain crude fungal cultures. Oomycete isolates were transferred to a broth of 10% filtered V8 juice amended with ampicillin (250 mg/mL), and fungal isolates were transferred to potato dextrose broth (Neogen Corporation, Lansing, MI). Isolates were incubated in the dark at room temperature until a layer of hyphal tissue formed on the broth surface. The mycelia was aseptically transferred to a 2.0 mL microcentrifuge tube, lyophilized, and ground in a cell disruptor system (Thermo Savant FastPrep 120, GMI, Ramsey, MN). Fungal isolate DNA was obtained via a phenol chloroform extraction modified from (Al-Samarrai and Schmid 2000) and was used for PCR. For oomycetes, PCR for the 25 μL samples used 20.1 μL of sterile filtered water, 2.5 μL of 10x DreamTaq Buffer (ThermoFisher Scientific, Waltham, MA, USA), 0.2 μL of 25 mM dNTP, 0.5 μL of 10 μM primers ITS4 and ITS6 (Cooke and Duncan 1997; White et al. 1990), 0.2 μL of 5U μL^{-1} DreamTaq Polymerase, and 1 μL of 20-200 ng μL^{-1} DNA. PCR parameters to amplify DNA samples included the following: 94°C for 3 min; 35 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 1 min; 72°C for 7 min; and hold at 4°C. For fungi, PCR was performed one of two ways. For amplification of the translation elongation factor 1α (TEF-1α) gene region, 25 μL samples included 14.2 μL of sterile filtered water, 5 μL of 5x Phusion Buffer, 0.2 μL of 25 mM dNTP, 1.25 μL of 10 μM primers EF1 and EF2 (O'Donnell et al. 1998), 0.25 μL of 5U μL^{-1} Phusion Polymerase (New England Biolabs, Ipswich, MA,

USA), and 1 μL of 20-200 ng μL^{-1} DNA. PCR parameters to amplify TEF-1α included the following: 98°C for 30 s; 35 cycles of 98°C for 10 s, 60°C for 15 s, 72°C for 30 s; 72°C for 5 min; and hold at 4°C. If amplification of the TEF-1α region was unsuccessful, amplification of the internal transcribed spacer (ITS) region was conducted using 25 μL samples that included 20.1 μL sterile, filtered water, 2.5 μL 10x DreamTaq Buffer, 0.2 μL of 25 mM dNTP, 0.5 μL of 10 μM primers ITS4 and ITS5 (White et al. 1990), 0.2 μL of 5U μL^{-1} DreamTaq Polymerase, and 1 μL of 20-200 ng μL^{-1} DNA. PCR parameters to amplify DNA samples included the following: 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; 72°C for 10 min; and hold at 4°C. Gel electrophoresis was used to verify successful amplification of DNA with a 0.5x strength Tris-borate buffer with EDTA (TBE buffer), for which 10x TBE buffer included 86.4g Tris base, 44g boric acid, and 32 mL of 0.5M EDTA (pH 8.0). DNA was purified with 20U μL^{-1} exonuclease I and 1U μL^{-1} shrimp alkaline phosphatase (EXO-SAP) (Dugan et al. 2002) and incubated at 37°C for 45 min and 85°C for 5 min. Samples were loaded into 96-well plates with 3 μL of primer, 3 μL of sterile H_2O, and 6 μL of PCR product and sent to Macrogen USA (Macrogen, Rockville, MD) for sequencing. DNA sequences from Macrogen were aligned using Unipro UGENE open source software (Okonechnikov et al. 2012). Aligned sequences were matched to oomycete or fungal DNA sequences using a curated oomycete database (Rojas et al. in press; Robideau et al. 2011) and Mycobank, respectively.

Greenhouse study.

The root rot-susceptible soybean variety 'Sloan' and the two Asgrow varieties with respective seed treatments from the field study were planted in 1 L plastic pots containing vermiculite. The susceptible soybean variety 'Sloan' was included to evaluate the seedling disease susceptibility of Asgrow 1 and Asgrow 2. Inoculation treatments included a non-

inoculated control and inoculum from representative isolates of one of six known seedling pathogens – *Pythium sylvaticum* (INSO_1-10C), *Phytophthora sojae* (V-SDSO2_1-53), *Fusarium oxysporum* (F_14-26), *Fusarium solani* (F_14-7), and *Rhizoctonia solani* anastomosis groups (AG) 2-2 (RS_14-17) and 4 (R09-08). The oomycete and *Fusarium* isolates were obtained from the Chilvers lab culture collection. *Rhizoctonia solani* isolates were obtained from Linda Hanson's lab where they were identified using the methods of Shen et al. (1991) with confirmation by primers specific to AG 2-2 (Salazar et al. 2000) or PCR restriction length fragment polymorphism (Guillemaut et al. 2003). Treatments were replicated five times in the greenhouse experiment, which was repeated; this resulted in ten total replicates per treatment.

Inocula for the *P. sojae* and *Py. sylvaticum* isolates were prepared by growing out oomycete species on corn meal agar amended with 10 mg/mL pimarcin, 250 mg/mL ampicillin, 10 mg/mL rifampcin, 5 mg/mL pentochloronitrobenzene (Jeffers and Martin 1986) and 50% benomyl (CMA-PARPB) until the mycelial growth nearly filled the petri dish. Mushroom spawn bags (Mycosupply, Pittsburgh, PA) were filled with a mixture of 500 mL distilled water and 1250 mL of millet (John A. Van Den Bosch Company, Holland, MI, USA). Moistened millet was autoclaved for three hours at 121°C. Once the sterile millet had cooled to room temperature, colonized agar from three petri dishes were cut into squares (about 0.5 cm x 0.5cm) and added to mushroom spawn bags. The bags containing the *Py. sylvaticum* and *P. sojae* were sealed and kept at room temperature for 2-4 weeks, agitating the bags by hand every two days, until millet was visibly colonized.

Inocula for the *F. solani* and *F. oxysporum* isolates were prepared by growing out *Fusarium* species on Nash-Snyder Medium (Nash and Snyder 1962) for two weeks at room temperature. Sorghum (John A. Van Den Bosch Company, Holland, MI, USA) was soaked in

distilled water overnight and then approximately 1.7 kg hydrated sorghum was placed into mushroom spawn bags. Six bags of sorghum were autoclaved for five hours at 121°C. Once sterile sorghum had cooled to room temperature, colonized agar from three petri dishes were cut into squares, blended aseptically to homogenize, and added to each bag. Bags were sealed and incubated at room temperature for 2-3 weeks. The inoculum was air-dried for one week, and one hundred inoculated sorghum grains were plated onto PDA to verify >75% growth of the *Fusarium* species.

Inocula for the two *Rhizoctonia solani* isolates were prepared by growing out isolates on chloramphenicol-amended PDA (100 μg/mL). Barley (Discount Seeds, Inc., Watertown, SD, USA) was soaked in distilled water overnight; approximately 1.7 kg was transferred to mushroom spawn bags the following day and about 23 kg of barley was autoclave sterilized for about eight hours at 121°C. Once sterile barley had cooled to room temperature, colonized agar from two petri dishes was cut into squares and used to inoculate each bag of barley. Bags were sealed and incubated at room temperature for 1-2 weeks. One hundred inoculated barley kernels were placed onto PDA to verify inoculum viability was above 75%. Inoculated barley kernels were ground into fine particle sizes using a Wiley Mill (Thomas Scientific, Swedesboro, NJ) for AG 2-2 and a Geno/Grinder (SPEX SamplePrep, Stanmore, UK) for AG 4.

For inoculated treatments, inoculum was mixed in a 4 L plastic bag with 800 mL medium vermiculite and poured into pots. Ten seeds were placed into the pot and covered with 200 mL medium vermiculite. The amount of inoculum varied by the type of pathogen: 20 g, 18 g, and 0.6 g of the inocula for the oomycetes, *Fusarium* species and *Rhizoctonia solani* isolates, respectively. At 20 days after planting (dap), emergence was recorded. At 21 dap, soybean

seedlings were harvested, roots were washed, and roots and shoots were separated. Seedlings were bagged, dried at 38°C until root weight stabilized, and weighed.

Statistical analysis.

In both the field study and greenhouse study, mean statistical differences were calculated using proc mixed from SAS statistical software, version 9.3 (SAS Institute Inc., Cary, NC, USA). Multiple comparisons were made using the Tukey adjustment option in either the SLICE or LSMEANS statements. Pearson correlations were conducted using the rcorr function in R.

For comparisons made across varieties and sites in the field study, data values were normalized by subtracting data means of the four (2013) or six (2014 and 2015) NTC replicates from each individual observation within the same year, site, and variety; normalized values represented the net effect of a seed treatment relative to the zeroed NTC. Partial returns were determined using the net yield effect of seed treatment and the following prices that correspond approximately to current market prices (Chilvers, personal communication): a soybean market price of \$0.37 kg^{-1}, F application cost of \$9.88 ha^{-1}, FI application cost of \$24.71 ha^{-1}, and FIN application cost of \$49.42 ha^{-1}. For statistical analysis of field measurements, seed treatment was generally regarded as the sole fixed effect while soybean variety, field site, soybean variety x seed treatment, field site x seed treatment, and replicate nested within field site were regarded as random effects. Interaction terms were dropped if they were non-significant for explaining a particular parameter. Comparisons also were made among varieties to determine the impact of soybean variety on the net seed treatment effect. For this analysis, variety, seed treatment, and their interaction were treated as fixed effects while site, site x seed treatment, and replicate nested within site were treated as random effects. The BY statement of proc mixed was used to evaluate seed treatment efficacy within each location.

Maximum likelihood estimation (MLE) was performed to develop a model for predicting the probability that use of treated seed will result in a sufficient yield increase to compensate for the input cost. The response ratios (RR) and cost relative yield (CRY) were calculated from observed data from the field study as described by (Esker and Conley 2012). The difference between RR and CRY was changed to binomial data. That is, if the difference was <0, i.e. seed treatment did not break even, it received rank 0; if the difference between RR and CRY was ≥0, i.e. seed treatment broke even or resulted in positive gains, it was ranked 1. Using the lme4 package in R (Bates et al. 2015), model selection for MLEs was optimized by regarding year, site, and replicate nested within site as random effects and including the following additional significant fixed effects that returned the lowest Akaike information criterion (AIC) and Bayesian information criterion (BIC) values: soybean variety, soybean market price, degrees longitude of field site, the mean of daily low temperatures of the 5 weeks following planting at each field site, and seed treatment. Using the plogis function (R Core Team 2015), the predicted probabilities of breaking even and their 95% confidence intervals were determined from the fit values generated from the model.

In the greenhouse study, pathogen, soybean variety, and seed treatment, and all significant 2-way interactions were treated as fixed effects; experiment and replicate nested within experiment were treated as random effects. For making comparisons, data was normalized by subtracting data means of the non-inoculated, non-treated control from individual observations within the same pathogen treatment, variety, and experiment. The SLICE statement was used to split data by significant fixed effects for mean comparisons.

Results

Field study.

Overall effects of seed treatments between the two seed companies were not significantly different from one another for all parameters tested ($p>0.05$), so results were pooled into four categories – NTC, F, FI, and FIN – across the different seed treatment active ingredients and application rates used between the two companies.

Across sites and varieties in 2013, seed treatment was found to have a significant overall effect on yield, with the FI treatment resulting in higher yields than the NTC; however, the effects of seed treatment on partial returns were not significant (Table 4).

Table 4. Yield and partial returns of soybean by seed treatments in 2013, across sites and soybean varieties

Seed Treatment	Yield ---Mg ha^{-1}---		Partial Returns ---$ ha^{-1}---	
NTC	4.21	b	-0.36	ns
F	4.28	ab	13.86	ns
FI	4.44	a	50.86	ns
FIN	4.42	a	19.00	ns

Seed treatment – F: fungicide, FI: fungicide & insecticide, FIN: fungicide, insecticide, and nematode biocontrol. Values within a site marked with the different letters are significantly different by Tukey's HSD, α=0.05. Values followed by 'ns' are not significantly different.

In 2014, SCN was detected in early-season composite soil samples from the Hillsdale site. Upon early-season subsampling of all plots, however, SCN populations were only observed in one plot, preventing further analysis. Several other parameters, however, were impacted by seed treatment use in 2014 (Table 5). Plant stand was significantly higher for FIN-treated seed than the NTC at the Allegan and Ingham sites, but significantly lower than the NTC at the Lenawee site. Plant heights of seedlings in FI and FIN plots were significantly lower than the

Table 5. Plant stand at VC-V1 and plant height at V1-V2 growth stages by field site county and seed treatments in 2014, across soybean varieties

Site, Treatment	Plant stand ---plants m^{-2}---	Plant height ---% of NTC---
Allegan		
NTC	26.45b	100.0ns
F	28.53b	99.9ns
FI	29.28b	99.2ns
FIN	35.09a	103.3ns
Hillsdale		
NTC	30.73ns	100.0a
F	30.15ns	96.8ab
FI	30.98ns	93.2b
FIN	32.13ns	93.9b
Ingham		
NTC	29.60c	100.0ns
F	30.72bc	103.6ns
FI	33.41ab	106.3ns
FIN	34.97a	104.9ns
Lenawee		
NTC	31.50a	100.0ns
F	31.62a	102.9ns
FI	31.37ab	100.4ns
FIN	30.33b	101.2ns
Saginaw		
NTC	34.67ns	100.0ns
F	35.09ns	103.8ns
FI	34.99ns	101.8ns
FIN	34.50ns	101.0ns
Sanilac		
NTC	31.33ns	100.0ns
F	32.23ns	95.7ns
FI	31.93ns	93.7ns
FIN	32.22ns	95.8ns
St Joseph		
NTC	25.82ns	100.0ns
F	25.12ns	96.0ns
FI	27.24ns	101.3ns
FIN	26.51ns	104.1ns

Seed treatment – F: fungicide, FI: fungicide & insecticide, FIN: fungicide, insecticide, and nematode biocontrol. Values within a site marked with the different letters are significantly different by Tukey's HSD, α=0.05. Values followed by 'ns' are not significantly different.

NTC at the Hillsdale site. Soybean aphid was found in the Ingham site control plots; though the numbers were far below the approximately 250 aphids plant^{-1} action threshold (Ragsdale et al. 2011, 2007), significant differences were still observed between NTC and the FI seed treatment at growth stage V3, 34 days after planting (p=0.0427). Seed treatment did not have a significant effect on yield in 2014. However, FIN significantly reduced partial returns relative to the F and NTC treatments (Table 6).

Table 6. Yield and partial returns of soybean by seed treatments in 2014, across sites and soybean varieties

Seed Treatment	Yield ---Mg ha^{-1}---		Partial Returns ---$ ha^{-1}---	
NTC	4.49	ns	0	a
F	4.45	ns	-21.51	a
FI	4.49	ns	-26.23	ab
FIN	4.46	ns	-58.73	b

Seed treatment – F: fungicide, FI: fungicide & insecticide, FIN: fungicide, insecticide, and nematode biocontrol. Values within a site marked with the different letters are significantly different by Tukey's HSD, α=0.05. Values followed by 'ns' are not significantly different.

SCN was detected in the Saginaw composite soil sample in 2015, exceeding the one-cyst per field sample action threshold (Niblack 2005). The SCN reproductive rate of all treated seed was numerically higher than the NTC, though the overall seed treatment effect was non-significant (p=0.4127). The effect of seed treatment on SCN fecundity was likewise insignificant (p=0.9608). The FIN seed treatment significantly improved plant stand relative to the NTC at the sites in Allegan, Sanilac, and St Joseph counties (Table 7). Though there was no significant overall effect of seed treatment on root dry weight (p=0.8874), the interaction between seed treatment and soybean variety were significant (p=0.0147). Though the interaction between seed treatment and field site were significant in describing the net effect of seed treatments on root dry weight relative to the NTC (p=0.0286), no seed treatment significantly improved root dry weight

Table 7. Plant stand and root dry weight of soybean at growth stages VC/V1 in 2015, by seed treatment, across soybean varieties.

Site, Treatment	Plant stand ---plants m^{-2}---	Root dry weight ---in g---
Allegan		
NTC	16.8c	0.37ns
F	18.1c	0.36ns
FI	21.2b	0.40ns
FIN	24.6a	0.55ns
Hillsdale		
NTC	35.1ns	0.66ns
F	35.5ns	0.71ns
FI	35.5ns	0.67ns
F+IN	35.3ns	0.66ns
Ingham		
NTC	37.1ns	0.62ns
F	36.9ns	0.64ns
FI	36.9ns	0.65ns
FIN	36.2ns	0.63ns
Lenawee		
NTC	35.7ns	0.70ab
F	35.1ns	0.83a
FI	35.5ns	0.73ab
FIN	35.2ns	0.66b
Saginaw		
NTC	33.3ns	0.76ns
F	33.0ns	0.75ns
FI	33.0ns	0.76ns
FIN	33.8ns	0.68ns
Sanilac		
NTC	24.7c	0.26ns
F	26.3bc	0.27ns
FI	27.5b	0.26ns
FIN	32.3a	0.26ns
St Joseph		
NTC	22.6b	0.70ns
F	22.5b	0.70ns
FI	20.4c	0.68ns
FIN	27.3a	0.74ns

Seed treatment – F: fungicide, FI: fungicide & insecticide, FIN: fungicide, insecticide, and nematode biocontrol. Values within a site marked with the different letters are significantly different by Tukey's HSD, α=0.05. Values followed by 'ns' are not significantly different.

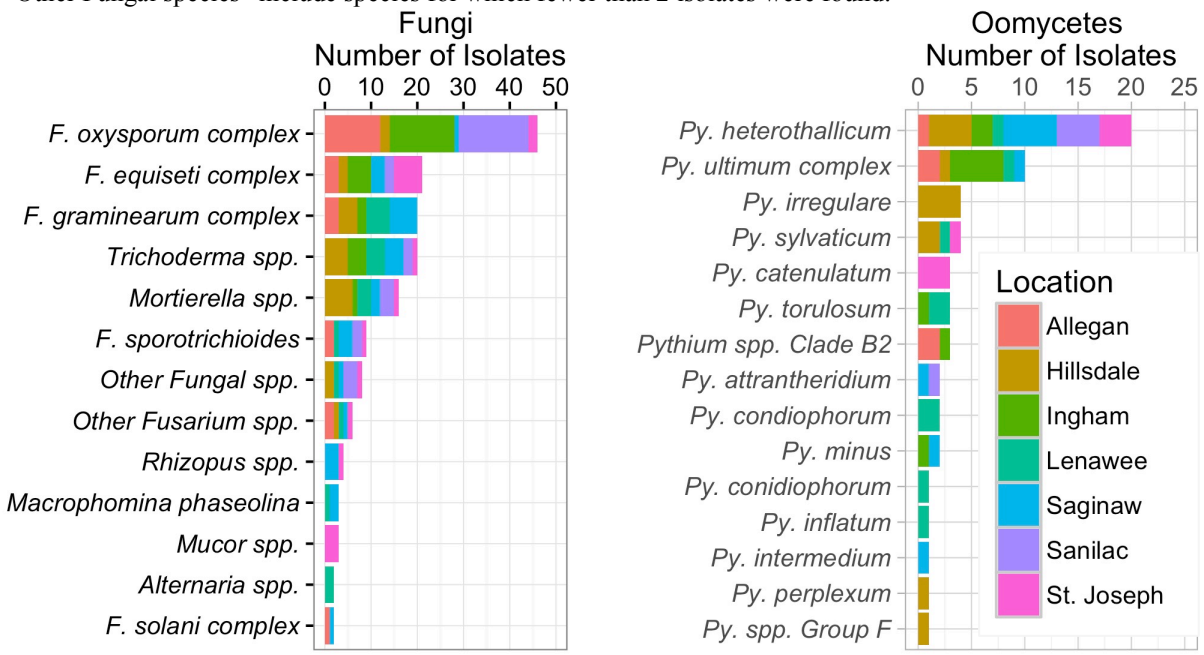
Table 8. Yield and partial returns of soybean by seed treatments in 2015, across sites and soybean varieties

Seed Treatment	Yield ---Mg ha^{-1}---	Partial Returns ---$ ha^{-1}---
NTC	4.79ns	0.14a
F	4.84ns	8.86a
FI	4.80ns	-18.65ab
FIN	4.82ns	-37.62b

Seed treatment – F: fungicide, FI: fungicide & insecticide, FIN: fungicide, insecticide, and nematode biocontrol. Values within a year and site marked with the different letters are significantly different by Tukey's HSD, α=0.05. Values followed by 'ns' are not significantly different.

relative to the NTC at any site (Table 7). Aphids were observed in ≥25% of control plots of the Ingham, Saginaw, and Sanilac sites. Similar to 2014, aphid populations were biologically insignificant in 2015, and observed populations were still far below action thresholds (Ragsdale et al. 2007). Nonetheless, significant differences in aphids per plant were found between the FI treatment and the NTC at two of the three sites. The effect of seed treatment on yield was non-

Figure 1. Histogram of soybean-associated fungi and oomycete isolates collected from non-treated soybean seedlings at seven field sites in 2015. Oomycete species (top), fungal species (bottom) listed from greatest to least number of isolates. *Pythium* spp. Clade B2 refers to closely related *Pythium* species that could not be differentiated using the ITS region. "Other *Fusarium* species" includes *Fusarium* species for which two or fewer isolates were found. *F. solani* is shown on the chart due to its previous association with root rot in soybean. "Other Fungal species" include species for which fewer than 2 isolates were found.



significant. Use of the FIN seed treatment in 2015 resulted in significantly lower partial returns than the F and NTC treatments (Table 8). Across field sites in 2015, a total of 58 oomycete

Table 9. Soybean yield by year, location, and seed treatment, across varieties.

Site, Treatment	2013	2014	2015
	--- Yield, Mg ha^{-1}---		
Allegan			
NTC	2.71b	4.27ns	4.17b
F	3.15ab	4.24ns	4.20b
FI	3.61a	4.34ns	4.41ab
FIN	3.52a	4.42ns	4.49a
Hillsdale			
NTC	4.18ns	3.54ns	4.45ns
F	4.32ns	3.60ns	4.45ns
FI	4.35ns	3.51ns	4.43ns
FIN	4.37ns	3.51ns	4.45ns
Ingham			
NTC	4.14ns	4.47ns	5.32ns
F	4.15ns	4.40ns	5.53ns
FI	4.19ns	4.57ns	5.29ns
FIN	4.19ns	4.47ns	5.34ns
Lenawee			
NTC	5.02ns	4.89ns	4.57ns
F	5.06ns	4.76ns	4.55ns
FI	5.23ns	4.82ns	4.70ns
FIN	5.22ns	4.76ns	4.57ns
Saginaw			
NTC	3.47ns	5.07ns	5.36ns
F	3.40ns	5.02ns	5.45ns
FI	3.49ns	5.01ns	5.22ns
FIN	3.42ns	5.00ns	5.28ns
Sanilac			
NTC	4.78ns	3.89ns	3.72ns
F	4.83ns	3.69ns	3.75ns
FI	4.99ns	3.80ns	3.68ns
FIN	4.85ns	3.71ns	3.62ns
St Joseph			
NTC	5.13ns	5.30ns	5.91ns
F	5.02ns	5.47ns	5.98ns
FI	5.17ns	5.34ns	5.90ns
FIN	5.30ns	5.37ns	6.01ns

Seed treatment – F: fungicide, FI: fungicide & insecticide, FIN: fungicide, insecticide, and nematode biocontrol. Values within a year and site marked with the different letters are significantly different by Tukey's HSD, α=0.05. Values with 'ns' are not significantly different.

isolates and 160 fungal isolates were collected and identified (Figure 1). The most frequently isolated oomycete species was *Pythium heterothallicum* (34%). Isolates from the *Pythium ultimum* complex were isolated second-most frequently (17%). Most of the true fungi isolated were *Fusarium* species (63%). *Fusarium oxysporum* complex isolates were the most frequently isolated fungal species (29%), though several other *Fusarium* species were also isolated. In addition, *Trichoderma* species represented more than 12% of isolates collected.

Across both 2014 and 2015, all sites, and all varieties, FIN seed treatment was found to significantly improve plant stand by nearly 8% ($p=0.0141$); no other seed treatment significantly improved stand. Across all years, sites, and varieties, the net effect of seed treatment on yield and consequent partial returns were non-significant ($p=0.2685$). When analyzed by year and site, significant improvements in yield were observed at two of 21 field sites in the three-year study with application of FIN (Table 9). Across years, field site county significantly influenced seed treatment effects on yield and partial returns. The FI and FIN treatments resulted in significantly higher yield than the NTC at the Allegan sites across years and varieties ($p=0.0008$ and $p<0.0001$, respectively), and partial returns from use of the FI treatment were significant ($p=0.0347$), though FIN partial returns were not ($p=0.0675$). The FIN treatment resulted in significantly lower partial returns than the NTC at sites in Lenawee, Saginaw, and Sanilac counties ($p=0.0032$, $p=0.0002$, $p=0.0015$, respectively). Due to the relatively consistent effect of field site on seed treatment partial returns, correlation tests were conducted between partial returns and respective weather and soil characteristics from the 21 sites over the three-year study, including soil pH, cation exchange capacity (CEC), soil organic matter (SOM), clay content, sand content, degrees longitude, mean low temperatures for the first five weeks after planting, mean rainfall for the first two weeks after planting, and planting date. For each seed treatment,

partial returns were significantly correlated with at least one factor, though no factor was significant in explaining partial returns for all three seed treatments (α=0.05) (Table 10).

Based on the maximum likelihood estimation model, FIN treatment was predicted to be less likely to result in break-even scenarios than the other seed treatments across years and

Table 10. Pearson's correlations between partial returns from use of each seed treatment and site characteristics, including soil pH, cation exchange capcity (CEC), percent soil organic matter, percent sand content, percent clay content, degrees longitiude, mean low temperature for the five weeks following planting, mean rainfall for the two weeks following planting, and planting date.

Site Characteristics	F		FI		FIN	
	Correlation Coefficient	p-value	Correlation Coefficient	p-value	Correlation Coefficient	p-value
Soil pH	-0.13	0.5767	-0.55	0.0096	-0.46	0.0379
CEC	-0.07	0.7526	-0.04	0.8798	-0.15	0.5256
Soil Organic Matter	-0.36	0.1053	-0.35	0.1156	-0.55	0.0099
Sand Content	0.41	0.0679	0.30	0.1910	0.54	0.0118
Clay Content	-0.37	0.0974	-0.38	0.0911	-0.57	0.0072
Degrees Longitude	-0.41	0.0653	-0.41	0.0623	-0.68	0.0007
Low Temperatures	-0.45	0.0389	-0.28	0.2150	-0.36	0.1106
Rainfall	-0.19	0.4009	-0.10	0.6596	-0.20	0.3850
Planting Date	-0.53	0.0135	-0.28	0.2237	-0.43	0.0538

A p-value below α=0.05 was considered to be significant.

varieties (Figure 2). At the Ingham, Lenawee, Saginaw, and Sanilac field sites, no seed treatments were predicted to result in break-even scenarios more than half the time. Though the correlation between observed partial returns and predicted probability of breaking even were significant ($p<0.0001$), the model only explained 25% of the variability between predictions and actual observations.

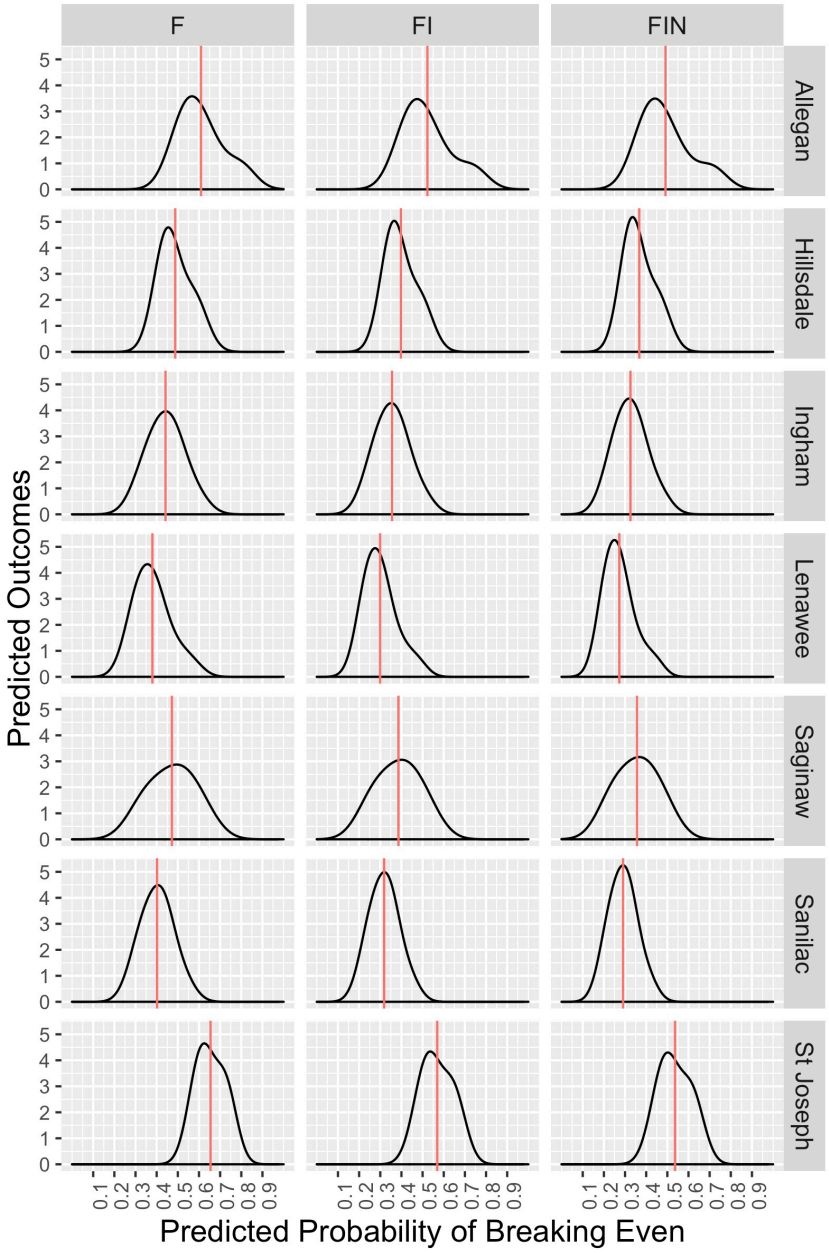
Varieties had significantly different net responses to seed treatment. Across sites and years, the net effect of F, FI, and FIN on Pioneer-2 plant stand was significantly higher than all other varieties α=0.05). Additionally, the net effects of seed treatments on Asgrow-2 were observed to be negative. For example, under field conditions in 2015, the root dry weight response of Asgrow-2 to FI was significantly lower than the response of Asgrow-1 and Pioneer

(p=0.0022 and p=0.0012, respectively). Moreover, across all sites in 2013, 2014, and 2015, the partial returns response of Asgrow-2 to FIN was significantly lower than Asgrow-1 (p=0.0222).

***Greenhouse study*.**

Across pathogen treatments, non-treated 'Sloan' had significantly lower emergence relative to

Figure 2. Density plots showing the distribution of predicted probabilities that seed treatment use will be profitable, by seed treatment and site. Red lines indicate the mean predicted probability that seed treatment will be profitable. Non-treated seed is the baseline scenario in which probability of being profitable is 50%



the NTC compared to the non-treated controls of Asgrow-1 and Asgrow-2 ($p<0.0008$, and $p<0.0001$, respectively).

When considering just Asgrow-1 and Asgrow-2, the main effect of seed treatment on emergence was non-significant ($p=0.2862$). However, the overall effect of FIN treatment on root dry weight was significantly lower than all other treatments ($p<0.0001$ for each comparison). F treatment was the only treatment that resulted in root dry weight significantly higher than the NTC ($p=0.0196$). The response of root dry weight to seed treatment was significantly higher for Asgrow-1 than for Asgrow-2 when averaged across pathogen and seed treatment.

Rhizoctonia solani AG 2-2 and *P. sojae* uniformly reduced seedling emergence across varieties and seed treatments ($p<0.0001$ and $p=0.0165$, respectively). Relative to the non-inoculated control, *R. solani* AG 2-2, *F. oxysporum*, *P. sojae*, and *Py. sylvaticum* significantly reduced root dry weight of non-treated seed across both commercial varieties tested ($p<0.05$). Compared to the non-inoculated controls, all three seed treatments prevented significant root dry weight reductions due to *P. sojae* ($p>0.05$), but the F and FIN treatments had significant root dry weight reductions due to *R. solani* AG 2-2 ($p=0.0003$ and $p<0.0001$, respectively); no treatment prevented significant root dry weight reductions due to *Py. sylvaticum* or *F. oxysporum* ($p<0.05$).

Discussion

This study was conducted to more clearly elucidate the effect of seed treatment on soybean productivity in the upper Midwest of Michigan. Results from this study indicate that seed treatment is unlikely to break even relative to no treatment across years, sites, and varieties, which differ from the findings of some previous studies in which use of fungicide seed treatments (Poag et al. 2005; Esker and Conley 2012) and seed treatments containing an insecticide (Esker and Conley 2012; Gaspar et al. 2015) were found to be profitable across sites and years. Several other studies have demonstrated similar results to the current study, showing that seed treatment use does not consistently improve yield and profits across growing conditions (Bradley 2008a; Cox et al. 2008; Cox and Cherney 2014; Dorrance et al. 2009a; Gaspar et al. 2014; Schulz and Thelen 2008). In the current study, overall negative yield effects of each seed treatment relative to the NTC were observed in 2014. Though this may be due to the later-than-normal planting dates across sites (Bradley 2008b), profitability of seed treatment in 2015, with normal planting dates across sites, remained low.

Seed treatment was not able to prevent significant root dry weight reductions relative to non-inoculated plants for all representative soilborne pathogens tested in the greenhouse study, and nematode reproductive factors and fecundity were not significantly impacted by seed treatment. Imidacloprid seemed to have efficacy in controlling low aphid populations, but efficacy of clothianidin for limiting aphid populations was not observed. Findings from this study indicate that the efficacy of some commercial seed treatments may be limited.

Similar to the findings of previous studies, plant stand was improved at fewer than half of the field sites tested (Bradley 2008b; Bradley et al. 2001; Guy et al. 1989; Schulz and Thelen

2008). Even when present, significant plant stand differences did not consistently translate to significant yield differences at any site, possibly because losses in stand tended to be uniformly distributed within the row (data not shown). Soybean plants have been shown to compensate for moderate, evenly-distributed stand losses by yielding more seed on secondary branches (Stivers and Swearingin 1980). Growers may be less likely to replant moderate stand losses that are evenly dispersed due to replanting costs and the lower yield potential associated with later planting dates (Wilmot et al. 1989). Although plant stand losses may be due to pre-emergence damping-off caused by soilborne pathogens (Broders et al. 2007a; Dorrance et al. 2003b, 2009a), plant stand may be reduced by other factors. For example, stand-reducing seed corn maggot may proliferate when substantial amounts of organic material are added to the soil, such as when crop or weed residues are tilled under soon before planting (Hammond 1991). Although seedcorn maggot was observed at the St. Joseph and Sanilac field sites in 2015 where improvements in stand from the FIN treatment were observed, no seedcorn maggot was observed at the Allegan field site where yield benefits from FIN were observed (personal observation). The roles of seedcorn maggot and stand loss in determining profitability of seed treatments remain unclear.

Benefits from seed treatments may only be observed if inoculum density of soybean pathogens are sufficiently high in the rhizosphere of soybean seedlings (Raftoyannis and Dick 2002; Sippell and Hall 1981). *Pythium heterothallicum* and species of the *Py. ultimum* complex were recovered at a high rate, which corresponds well to findings of recent oomycete community surveys in soybean (Rojas et al. in press; Zitnick-Anderson and Nelson 2015). Species from the *Py. ultimum* complex have previously been shown to be highly aggressive on soybean (Coffua et al. in press; Kirkpatrick et al. 2006a; Wei et al. 2010). Different studies have shown that *Py. heterothallicum* causes significant disease in soybean (Zitnick-Anderson and Nelson 2015; Rojas

et al. in press), though another study found *Py. heterothallicum* to be non-pathogenic on soybean (Jiang et al. 2012), indicating that virulence may vary between isolates, as has been demonstrated in additional previous studies (Broders et al. 2007a; Olson et al. 2016; Zhang and Yang 2000).

A high incidence of *Fusarium* species isolated from soybean seedlings has been previously reported (Broders et al. 2007b; Cui et al. 2016; Rizvi and Yang 1996). Many *Fusarium oxysporum* isolates are pathogenic on soybean and are able to cause seedling mortality and consequent losses in plant stand (Arias et al. 2013; Datnoff and Sinclair 1988; Farias and Griffin 1990). Isolates from the *F. oxysporum* complex were isolated from the Allegan and Sanilac field sites more frequently than other sites, which may have contributed to the observed beneficial effect of seed treatment on plant stand at those sites. Though stand loss was not caused by the *F. oxysporum* isolate in the current greenhouse study, virulence of *F. oxysporum* species has been shown to be variable (Arias et al. 2013). Members of the *F. graminearum* and *F. equiseti* complexes have also previously been shown to cause disease on soybean seedlings at sufficiently high inoculum concentrations (Broders et al. 2007b; Ellis et al. 2010; Goswami et al. 2008), but these *Fusarium* species may not have caused differences in plant stand or yield between field sites because they were recovered at a somewhat even rate across field sites.

In the greenhouse study, limited seed treatment efficacy was observed for the control of *Fusarium oxysporum* and *Pythium sylvaticum*, species that were isolated from diseased soybean seedlings in 2015. Previous studies have indicated that certain *Fusarium* isolates may easily develop insensitivity to fungicides common in seed treatment formulations (Broders et al. 2007b; Ellis et al. 2010), though the extent of fungicide resistance in *Fusarium* species remains unclear. Because the observed lack of seed treatment control could be caused by numerous factors, the efficacy of seed treatment in managing seedling pathogens may need more evaluation.

At the four sites where plant stand benefits from seed treatment were not observed, *Trichoderma* species represented more than 20% of isolates collected. The potential for *Trichoderma harzianum* to be used as a seed-applied bio-control agent in field crop production has been demonstrated in previous studies (Carvalho et al. 2014; El-Katatny et al. 2006; Paulitz et al. 1990; Pugliese et al. 2011), and other *Trichoderma* species may also have the ability to suppress pathogen activity (González et al. 2012; Harman et al. 2004). Although *Trichoderma* species considered for biocontrol have been shown to be sensitive to multiple seed-applied fungicides (McLean et al. 2001; Sarkar et al. 2010), oomycete-targeting seed treatments are compatible with seed-applied *Trichoderma* species (Howell et al. 1997). The Allegan County site in 2015 was the only field site with a significant FIN yield benefit and the only site where no *Trichoderma* species were recovered. Seedlings in fields with comparatively higher populations of certain *Trichoderma* species may have benefited from the antagonism of *Trichoderma* species to seedling pathogens, reducing the benefits that would otherwise be observed from seed treatment use.

Efficacy and profitability of seed treatments may remain difficult to assess because seed treatments have been shown to have effects beyond their intended use. Previous studies have indicated that high rates of metalaxyl may result in reduced yield in the absence of pathogens (Guy et al. 1989; Schmitthenner 1985) and that other seed-applied fungicides can cause phytotoxicity and plant stand reductions (Bradley 2008a). Imidacloprid has been previously reported to cause phytotoxicity in tomato, cucumber, and other crops (Ebel et al. 2000; Taylor and Salanenka 2012), corresponding to some phytotoxicity symptoms that were observed on seedlings in the current greenhouse study. In the current study, Asgrow-2 root weight was significantly reduced by FIN use relative to the NTC, indicating that the FIN seed treatment may

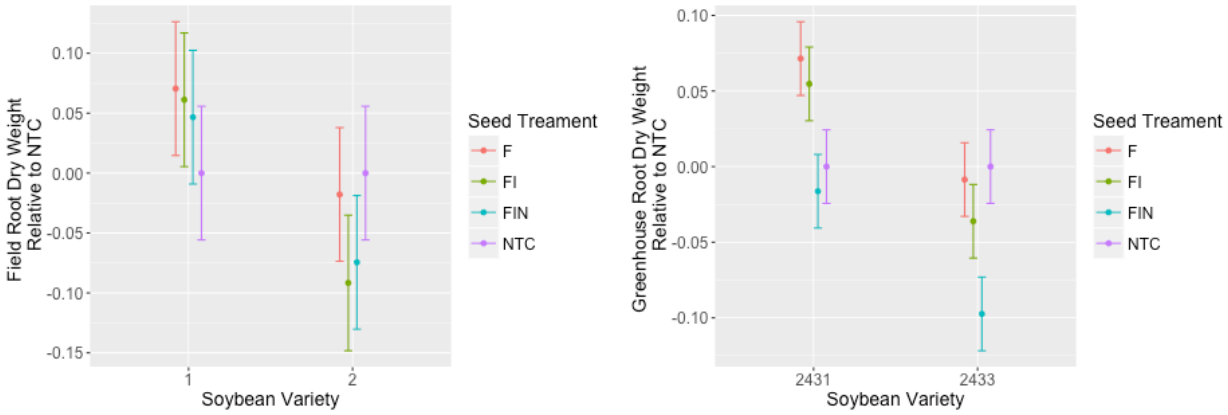
adversely affect growth of certain soybean varieties. Conversely, neonicotinoid insecticides have been shown to induce systemic acquired resistance (Ford et al. 2010). For example, imidacloprid was shown to reduce drought stress and stimulate plant defense in barley (Elbert et al. 2008). However, direct effects of seed treatment components on soybean seedling health remain unclear and may warrant further study to improve seed treatment recommendations.

Seed treatment effects were influenced by field site. Soybeans planted at the Allegan field site exhibited high responsiveness in yield to FIN across varieties in 2013 and 2015 (Table 9) and had higher likelihood of breaking even than soybeans planted at the Lenawee, Saginaw, and Sanilac sites (Figure 2) where partial returns from the use of FIN were found to be significantly lower the NTC. Site-specific trends may be related to regional climate effects of Lake Michigan, local soils, historical management of individual field sites, or other factors. Though longitude was significant in the MLE model for predicting the probability of positive economic returns, it is unclear if a real geographic factor impacted seed treatment efficacy or if longitudinal separation of field sites acted as a proxy for other site-specific factors that were unaccounted for in our model. Site-specific trends in seed treatment profitability may be related to edaphic factors, as was suggested by the significant negative correlations observed between site characteristics and partial returns (Table 10). Edaphic and climatic factors may determine the abundance of pathogens at particular sites, as previous studies have shown that these factors impact the variability of soil microbial communities in the rhizosphere of soybean seedlings (Rojas et al. 2013; Broders et al. 2009; Saremi et al. 1999). It is worth noting that the Allegan county site had the lowest pH, clay content, and soil organic matter in 2013 when yield of FI and FIN treatments were highest (Table 2), likely impacting the correlation of these variables with seed treatment efficacy. Because these edaphic factors have been known to influence nutrient

availability and microbial communities (Lumsden et al. 1976; Rousk et al. 2010, 2009), more investigation into the influence of edaphic factors on seed treatment efficacy is warranted.

Seed treatment benefits also were influenced by soybean variety, as has been previously reported (Lueschen et al. 1991; Esker and Conley 2012). Across field sites in 2015, root dry weight and yield of Asgrow-2 had a significantly lower net response to seed treatment than Asgrow 1. The same significant pattern of root growth response to seed treatment for Asgrow-1 and Asgrow-2 also was observed under greenhouse conditions (Figure 3), indicating that the compatibility of seed treatments with specific seed lots or soybean varieties may remain fairly consistent across environments. As new soybean varieties come onto the market, field tests to evaluate their susceptibility to seedling disease and compatibility with commercial seed treatments may help to describe the effect that a given soybean variety may have on seed treatment efficacy.

Figure 3. Comparison of root dry weight responses of soybean to seed treatment by soybean variety, comparing greenhouse and field results. Seed treatments – F: fungicide, FI: fungicide & insecticide, FIN: fungicide, insecticide, & nematode biocontrol.



Seed treatments evaluated in this study were not shown to be profitable across growing conditions in Michigan, although positive responses were observed for somemsites and years. Given the variability in seed treatment efficacy reported across soybean varieties, environments,

and planting conditions observed in the current study and several previous studies (Gaspar et al. 2014; Esker and Conley 2012; Bradley 2008a; Cox and Cherney 2014), regional studies are needed to determine which factors may drive seed treatment profitability. Results from the current study indicate that seed treatment may be more economical in early-planted fields with low clay content and low pH. However, the ability of these factors to predict profitability of seed treatment is incomplete. By determining which factors related to field site and soybean variety can be used to predict seed treatment profitability, a model could be developed for making seed treatment recommendations based on pre-season risk factors. Soybean producers in Michigan should take measures to determine the responsiveness of their fields and soybean varieties to seed treatment and whether seed treatment will provide economic benefit to their cropping systems.

CHAPTER 3. PATHOGENICITY AND VIRULENCE OF OOMYCETES ON COMMON BEAN AT TWO TEMPERATURES

Introduction

The most important food legume is common bean (*Phaseolus vulgaris*), accounting for more than half of all food legume production internationally (Miklas et al. 2006). Although common bean is planted on substantial land area in Michigan and other parts of temperate North America (National Agricultural Statistics Service 2015), farmers in Latin America and East Africa account for the majority of global production (Blair 2013; Broughton et al. 2003; Miklas et al. 2006). Bean yields throughout the world's bean production regions are frequently compromised by seedling disease and root rot, often caused by *Pythium* species (Blair et al. 2010; Broughton et al. 2003; Hoch and Hagedorn 1974; Navarro et al. 2008).

Pythium is a ubiquitous genus in the oomycetes that contains more than 150 species (Senda et al. 2009), many of which are phytopathogenic. *Pythium* species may reduce yield by causing pre and post-emergence damping-off of seedlings or root rot in mature plants (Hendrix and Campbell 1973a). *Pythium* species have been reported to cause serious losses in a wide variety of important crops (Bala et al. 2010), including common bean (Li et al. 2014; McCarter and Littrell 1970; Nzungize et al. 2011). Severity of damping-off and root rot caused by *Pythium* species often varies by isolate and can also depend on the characteristics of environment, host, and *Pythium* species composition (Broders et al. 2009; Matthiesen et al. 2016; Roncadori and McCarter 1972; Wong et al. 1984).

Seed-applied fungicides and root rot-resistant germplasm are commonly utilized in bean production for control of pathogenic *Pythium* species (Abawi and Corrales 1990; Keinath et al. 2000). Though fungicide seed treatments have been successful in reducing stand and yield losses in common bean (Locke et al. 1983; Keinath et al. 2000), certification regulations may limit the use of fungicides in organic production (Koch et al. 2010). Moreover, fungicide insensitivity has been identified in multiple oomycete species and remains a consideration for future fungicide use (Broders et al. 2007a; Falloon et al. 2000; Matthiesen et al. 2016; Mazzola et al. 2002; Munera and Hausbeck 2016; Taylor et al. 2002). Chemical disease control may be best utilized in conjunction with resistant germplasm (Abawi and Corrales 1990). Breeding for *Pythium* resistance consequently remains important for maintaining sustainable disease control. Characterization of relationships between *Pythium* species and common bean may help breeders to utilize key pathogenic *Pythium* species in selecting for resistant bean varieties, particularly since bean resistance to *Pythium splendens* did not translate to resistance to *Pythium aphanidermatum* in a recent study (Binagwa et al. 2016).

Common bean domestication occurred historically in Central America and the Andes mountains; varieties originating from these regions have been classified into two gene pools – the Middle American and the Andean (Benchimol et al. 2007; Gepts 1988). Andean varieties have been considered to be more susceptible to root rot pathogens than Middle American varieties (Blair et al. 2010; Conner et al. 2014), with several studies indicating that genes conferring root rot resistance are present in Middle American varieties more frequently than in Andean varieties (Nicoli et al. 2011; Román-Avilés and Kelly 2005; Schneider et al. 2001a). To our knowledge, no study has been conducted to compare *Pythium*-induced seedling disease and root rot resistance between gene pools.

Though 34 *Pythium* species have been associated with bean plants (USDA-ARS 2016), the pathogenicity and virulence of these and other *Pythium* species remains largely uncharacterized. Studies in Australia and East Africa have evaluated the pathogenicity of multiple regionally-important *Pythium* species (Gichuru et al. 2014; Li et al. 2014; Nzungize et al. 2011). As far as we know, however, a study evaluating pathogenicity of regionally important *Pythium* species has not been conducted in North America. Because temperatures and edaphic conditions have previously been shown to impact *Pythium* growth, aggressiveness, and species composition (Broders et al. 2009, 20; Cantrell and Dowler 1971; Pieczarka and Abawi 1978b; Wei et al. 2010), more descriptive evaluations of *Pythium* pathogenicity and virulence may be attained by using multiple temperatures and assays. By determining *Pythium* species that are most problematic for bean production, bean breeders will be equipped to identify and develop resistance to the most aggressive *Pythium* species. Additionally, pathologists may be enabled to improve recommendations for the management of *Pythium*-induced damping-off and root rot.

To promote improved management of Pythium root rot, a study was conducted to 1) determine the pathogenicity and comparative virulence of select North American and East African oomycete species on common bean, 2) contrast the *Pythium* disease severity on varieties representing the Andean and Middle American gene pools, and 3) examine the effect of two temperatures on virulence of oomycete species on bean.

Methods

Bean seeds were obtained from the Michigan State University Dry Bean Breeding Program. 'Red Hawk' kidney bean of the Andean gene pool and 'Zorro' black bean of the Middle American gene pool are common varieties planted in Michigan and were used in this study to represent their respective gene pools when evaluating *Pythium*-induced seed rot and root rot.

A total of 85 isolates of 28 oomycete species were tested for pathogenicity on common bean, including two *Phytopythium* species (previously *Pythium* clade K) (de Cock et al. 2015) and two varieties of *Pythium ultimum* that were regarded as separate *Pythium* species in this study based on differences in zoospore production and morphology (Barr et al. 1996). Three arbitrarily-selected isolates of each species were evaluated in the study unless otherwise noted (Table 11).

Pythium species used in the study came from one of three sources. First, many oomycetes were isolated in 2011 and 2012 from soybean plants in Arkansas, Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Nebraska, North Dakota, and Wisconsin as part of the Oomycete-Soybean Coordinated Agricultural Project (OSCAP) (Rojas et al. 2013). Second, *Pythium* species were isolated in 2014 and 2015 from bean as part of a *Pythium* survey of dry bean fields in Michigan (Jacobs and Chilvers, unpublished). Finally, four additional oomycete isolates were collected from bean by Mukankusi Clare Mugisha in East Africa and were also included in the study. *Pythium* species from the OSCAP and bean isolations were included in the study if they met one of the following requirements: species were recovered at a rate above 2.5% in the OSCAP survey (Rojas et al. in press), were recovered at a rate above 2.5% in the *Pythium*

survey from bean (Jacobs, unpublished data), or were known pathogens of bean (USDA-ARS 2016).

Table 11. Isolates used to determine pathogenicity and virulence of *Pythium* species on common bean, including number of isolates, phylogenetic clade, original host of isolate, and location of isolate collection.

Species	N	Clade	Host	Location
Phy. cucurbitacearum	1	K	CB	U
Phy. aff. vexans	2	K	CB, SB	MI, KS
Py. CAL_2011f	3	B	CB	MI, MI, MI
Py. acanthicum	2	D	SB	KS, MI
Py. aff. *diclinum*	3	B	SB	IA, ND, ND
Py. aff. *dissotocum*	3	B	SB	AR, MI, IA
Py. aff. *torulosum*	3	B	CB, SB	MI, MI, IA
Py. aphanidermatum	3	A	CB, SB	IL, NE, MI
Py. attrantheridium	3	F	CB, SB	MI, MI, IA
Py. coloratum	3	B	CB, SB	MI, MI, MN
Py. conidiophorum	3	B	SB	IA, IL, NE
Py. deliense	1	A	CB	U
Py. heterothallicum	3	I	CB, SB	MI, MI, IA
Py. inflatum	3	B	CB	MI, MI, MI
Py. irregulare	3	F	CB, SB	MI, KS, WI
Py. lutarium	2	B	SB	IN, MI
Py. myriotylum	3	B	CB	MI, MI, MI
Py. oopapillum	3	B	SB	IL, IN, MI
Py. pachycaule	3	B	SB	MN, NE, IA
Py. paroecandrum	3	F	SB	AR, IN, AR
Py. perplexum	3	J	SB	ND, NE, MI
Py. rostratifingens	3	E	SB	IA, MI, NE
Py. spinosum	4	F	SB	AR, AR, IN, IN
Py. sylvaticum	8	F	CB, SB	IN, ND, NE, MI,
Py. torulosum	3	B	SB	MN, MI, MI
Py. ultimum	5	I	CB, SB	U, U, IL, KS, MN
Py. ultimum var. *sporangiiferum*	3	I	SB	IL, IN, KS
Py. ultimum var. *ultimum*	3	I	SB	MI, IL, WI

Host from which *Pythium* isolates were originally collected include CB: common bean or SB: soybean. Isolates collected from the NIFA oomycete soybean coordinated agricultural project (OSCAP) (n=57) came from the following locations: AR, Arkansas; IA, Iowa; IL, Illinois; IN, Indiana; KS, Kansas; MI, Michigan; MN, Minnesota; ND, North Dakota; NE, Nebraska; and WI, Wisconsin. Additional isolates (n=24) were collected by Chilvers lab from dry bean in MI (Michigan). Isolates from Uganda (U) were collected by Mukankusi Clare Mugisha (n=4).

Pathogenicity assays.

A growth chamber seedling assay and petri dish seed assay were both conducted to evaluate the pathogenicity and virulence of *Pythium* species on bean. For both assays, the 85

Pythium isolates were arbitrarily divided into seven experiments; each experiment included twelve *Pythium* isolate treatments, at least one non-inoculated control (NIC) treatment (NIC treatments with and without inoculum substrate were used for the seedling assay) and an inoculated control (IC) treatment using an isolate of *Pythium ultimum* var. *ultimum* (MISO_8-10) known to be highly virulent. The presence of the common control treatments in each experiment allowed for comparisons among species across experiments. Each experiment was repeated three times, constituting three experimental trials. These trials included three replications of each treatment, resulting in a total of nine treatment replications per experiment.

In the seedling assay, *Pythium*-colonized rice was used as inoculum. To prepare inoculum, 20 mL of dH_2O were mixed with 30 g of parboiled white rice in a 125 mL Erlenmeyer flask. Flasks were covered with aluminum foil and autoclave sterilized. Autoclaved rice was agitated aseptically by carefully pounding flasks against rolls of paper towel and autoclave sterilized a second time. Isolates of *Pythium* spp. were grown on corm meal agar amended with pimarcin, ampicillin, rifampicin, and pentochloronitrobenzene (Jeffers and Martin 1986) amended with benomyl (10μg/mL) (CMA-PARPB). Using a flame-sterilized cork borer, rice was inoculated with five, 0.6 cm diameter plugs from the leading edge of 2 to 3-day-old mycelial growth. Inoculated rice was incubated at room temperature for 10-14 days.

Insulated 354-mL paper cups (Solo Cup Company, Lake Forest, IL) were filled with the following layers: 200 mL of medium vermiculite, 7 grams of inoculum (unless a non-inoculated control), 70 mL medium vermiculite, six bean seeds, and 70 mL medium vermiculite. Cups were placed into a growth chamber and watered with the MSU Growth Chamber nutrient water – water fertilized with half-strength Hoagland solution (Hoagland and Arnon 1938) – until water dripped from the bottom. Beans were grown at 20°C and 85% relative humidity with 10 hours of

darkness and 14 hours of light with light intensity of 500-590 mE. After 12 days, bean emergence was recorded, roots were washed with tap water, and all roots recovered from the cup were scanned at 300 dpi resolution on a flatbed scanner (Epson Perfection V600, Epson, Suwa, Nagano, Japan). All roots and shoots were separated and dried in a drying oven at 38°C until dry weight stabilized. Root dry weight was measured and recorded. Total root area and root length of each plant was determined from the scanned root images using Assess 2.0 software (American Phytopathological Society, St. Paul, MN).

Koch's postulates were fulfilled for the seedling assay by re-isolating the *Pythium* species from bean roots. Within both bean varieties, a representative plant was selected from each isolate treatment. From this plant, approximately 1 cm of darkened root tissue was plated onto CMA-PARPB to re-isolate the *Pythium* isolate from each bean variety. Re-isolations were performed three times for each isolate treatment – once from each trial. Mycelia from the original cultures used for inoculation and from the cultures isolated from root samples were transferred and grown out on CMA-PARPB. A crude DNA extraction was performed for original cultures and re-isolations by collecting pinhead sized mycelia pieces with a sterile wooden toothpick, placing mycelia into a 1.5 mL centrifuge tube containing 100 µL of sterile, filtered water, and holding the tube contents at 94°C in a heat block for 10 minutes. Samples were cooled on ice for at least 5 minutes and were either used immediately for DNA amplification or were transferred to a -20°C freezer for later use. PCR reactions for 25 µL samples contained 19.1 µL sterile, filtered water, 2.5 µL 10x DreamTaq Buffer, 0.2 µL of 25 mM dNTP, 0.5 µL of 10 µM primers ITS6 and ITS7, 0.2 µL of 5U/µL DreamTaq Polymerase, and 2 µL crude DNA. PCR parameters to amplify DNA samples included the following: 94°C for 3 min; 35 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 1 min; 72°C for 7 min; and hold at 4°C. The PCR products were utilized for single-

strand confirmation polymorphism (SSCP) as described by Kong *et al* (2004, 2005). DNA from the original *Pythium* cultures and the cultures isolated from bean roots in the study were run side by side in a polyacrylamide gel to compare banding patterns. If banding patterns of the re-isolated culture matched the banding pattern of the original culture in at least two of three instances, the oomycete isolate was considered to be associated with roots of that bean variety.

In the seed assay, the same *Pythium* isolates used in the seedling assay were used on the same two bean varieties. The seed assay was conducted at 20°C and 26°C, corresponding approximately to the normal maximum daytime air temperatures in Michigan in May and June (Prism Climate Group, Oregon State University 2016). *Pythium* species were grown out on water agar (2%) for two days. One, 0.6 cm^2 plug from the leading edge of mycelial growth of each isolate was transferred onto the center of a fresh water agar plate. Cultures were incubated in the dark at one of the two treatment temperatures. After two days of incubation, bean seeds were surface disinfected by soaking them in a 0.495% sodium hypochlorite solution. Based on preliminary trials (data not shown), 'Red Hawk' and 'Zorro' beans were soaked for 20 minutes and 10 minutes, respectively. Ten bean seeds were placed in a circle 5 mm away from the edge of each petri dish. Cultures with 'Red Hawk' and 'Zorro' seed were then returned to the respective temperature conditions and incubated for an additional 7 days. At the end of incubation, the disease severity rating for each seed was recorded using the following scale modified from Rojas et al. (in press) : 0 = seed germinated, 1 = seed germinated with reduced growth (no visible lesions), 2 = seed germinated with reduced growth and lesions, 3 = seed germinated with coalesced lesions and/or surface partially colonized by visible mycelial growth and 4 = no germination/completely colonized.

Statistical analysis.

All data was analyzed using proc mixed from SAS statistical software, version 9.3 (SAS Institute Inc., Cary, NC, USA). Pearson correlations were conducted using the Hmisc package (Harrell 2016) in R version 3.2.3 (R Core Team 2015).

In the seedling assay, the presence or absence of inoculum substrate resulted in no significant differences between the NIC with rice and the NIC without rice. For comparisons between the two bean varieties, data from the seedling assay was thus normalized within each trial to a percentage of the "NIC with rice" data means. Oomycete species, bean variety, and their interaction were treated as fixed effects while random effects included isolate nested within the interaction among *Pythium* species, experiment, and trial.

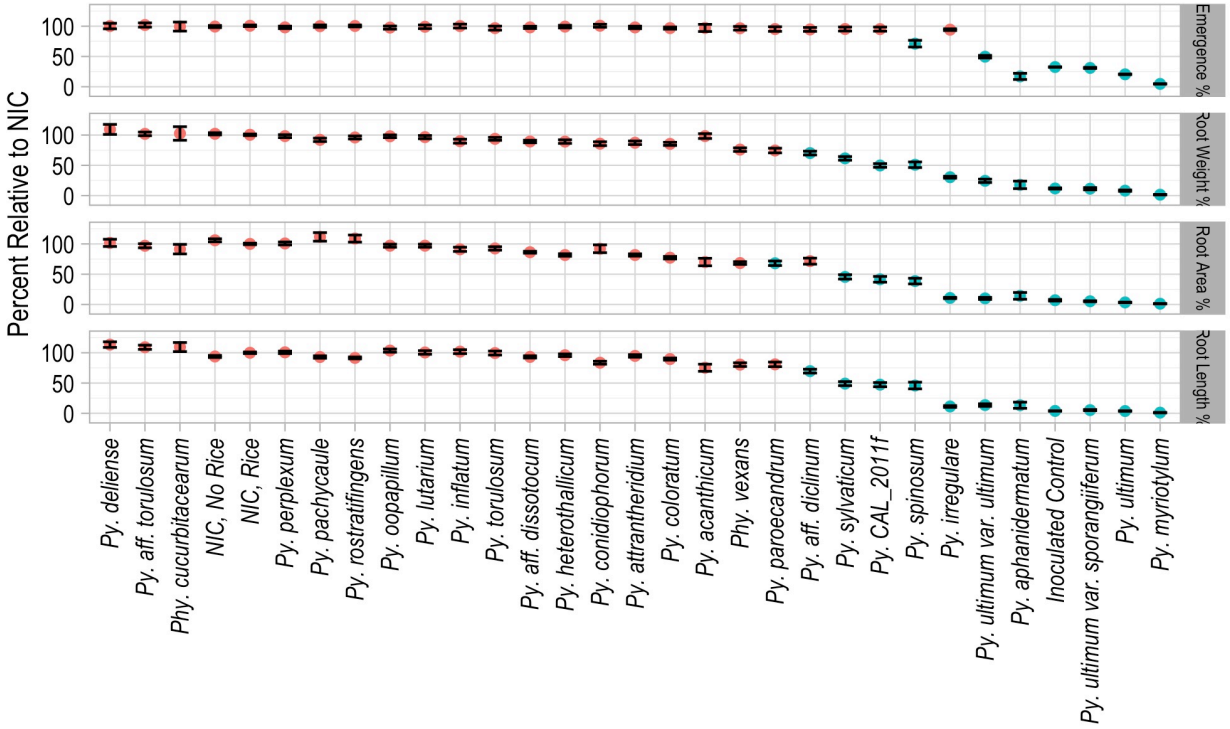
Clustering analysis was performed for each bean variety to group *Pythium* species together based on similarities in virulence. The least square means of the root dry weight, root length, root measurement, and seedling emergence data from the seedling assay were scaled and utilized as proxies for virulence. Cophenetic distances were determined using the Cord Distance (CRD) method (Foster and Bills 2011), which returned a cophenetic correlation and a root mean square error above 0.95. The CRD method was specified within the pheatmap R package to build a dendogram and heat map (Raivo Kolde 2015).

In the seed assay, disease severity ratings were converted to a continuous disease severity index (DSI), as has been performed by Li et al. (2014). Oomycete species, bean variety, temperature, and all interaction effects were treated as fixed effects while random effects included isolate nested within the interaction among *Pythium* species, experiment, and trial**.**

Results

When averaged across both bean varieties in the seedling assay, 11 *Pythium* species significantly reduced emergence, root dry weight, root area, or root length relative to the NIC (Figure 4). Six *Pythium* species (*Py. aphanidermatum*, *Py. myriotylum*, *Py.spinosum*, *Py. ultimum*, *Py. ultimum* var. *sporangiiferum*, and *Py. ultimum* var. *ultimum*) significantly reduced

Figure 4. Emergence, root dry weight, root length, and root area across bean varieties inoculated with oomycete species in the seedling assay, expressed as a percentage relative to the non-inoculated control (NIC), with [non-inoculated] rice. The inoculated control was an isolate of *Py. ultimum* var. *ultimum* known to be virulent on bean. Blue points indicate species that caused significant reductions in emergence or root growth relative to the NIC by Tukey's HSD, α=0.05. Red points indicate no significant difference.



emergence and all root growth parameters across both varieties; the effect of bean variety on emergence was non-significant (p=0.5737). Three additional *Pythium* species (*Py. irregulare, Py. sylvaticum*, and *Py. CAL_2011f*) caused significant reductions in root dry weight, root area, and root length relative to the NIC without significantly reducing emergence.

Table 12. Seedling assay emergence, root dry weight, root area, and root length of 'Red Hawk' and 'Zorro' dry bean inoculated with oomycete species, expressed as a percentage relative to the non-inoculated control with non-inoculated rice. "RH" and "Z" Refer to 'Red Hawk' kidney bean (Andean) and 'Zorro' black bean (Middle American), respectively. "Inoculated control" refers to a *Pythium ultimum* var. *ultimum* isolate known to be virulent.

		Emergence %		Root Weight %		Root Area %		Root Length %	
Treatment	**N**	**RH**	**Z**	**RH**	**Z**	**RH**	**Z**	**RH**	**Z**
Non-inoculated control	63	96.8	96.3	0.69	0.42	15.6	12.2	155.9	113.0
Inoculated control	63	22.2*	38.6*	0.07*	0.06*	1.4*	0.6*	5.3*	5.0*
Phy. cucurbitacearum	9	90.7	90.7	0.56	0.40	10.5	9.8	124.2	108.7
Phy. vexans	18	95.4	96.3	0.46*	0.46	9.5*	11.5	124.3	141.6
Py. CAL_2011f	27	92.0	85.8	0.38*	0.15*	6.4*	3.8*	76.4*	34.4*
Py. acanthicum	18	96.3	98.2	0.67	0.42	12.2	10.4	137.4	99.2
Py. aff. *diclinum*	27	89.5	93.8	0.45*	0.31	10.3*	9.2	120.8*	99.8
Py. aff. *dissotocum*	27	96.3	96.3	0.63	0.47	14.1	12.7	174.7	139.3
Py. aff. *torulosum*	27	97.5	91.4	0.56	0.21*	11.6*	6.2*	125.4	64.9*
Py. aphanidermatum	27	14.2*	17.9*	0.10*	0.06*	1.4*	1.5*	12.4*	15.4*
Py. attrantheridium	27	95.1	99.4	0.56	0.39	14.0	12.4	161.9	134.3
Py. coloratum	27	96.3	95.7	0.55*	0.39	13.2	11.8	155.1	125.1
Py. conidiophorum	27	95.7	100.0	0.54*	0.38	13.2	11.4	147.0	118.0
Py. deliense	9	96.3	92.6	0.66	0.41	13.4	9.9	146.1	103.6
Py. heterothallicum	27	97.5	99.4	0.56	0.41	13.3	12.9	155.3	142.0
Py. inflatum	27	97.5	88.3	0.50*	0.18*	11.6*	5.1*	123.5	54.9*
Py. irregulare	27	90.1	95.1	0.21*	0.16*	1.8*	1.4*	22.3*	15.3*
Py. lutarium	27	97.2	97.2	0.64	0.47	13.9	13.1	136.0	124.3
Py. myriotylum	27	8.6*	0.0*	0.02*	0.00*	0.4*	0.0*	5.1*	0.2*
Py. oopapillum	27	95.7	94.4	0.68	0.46	14.2	12.7	146.4	122.5
Py. pachycaule	27	94.4	98.8	0.59	0.40	15.9	15.1	157.3	133.7
Py. paroecandrum	27	93.8	84.0	0.50*	0.26*	9.6*	6.4*	109.5*	70.9*
Py. perplexum	27	97.5	93.2	0.69	0.46	15.0	12.9	151.3	113.4
Py. rostratifingens	27	95.7	98.8	0.61	0.43	15.6	14.0	155.7	133.7
Py. spinosum	36	75.5*	57.4*	0.29*	0.13*	4.3*	2.9*	50.9*	30.1*
Py. sylvaticum	72	96.1	86.8	0.37*	0.21*	6.4*	4.3*	70.5*	42.0*
Py. torulosum	27	95.1	95.1	0.72	0.52	14.6	13.2	134.9	107.7
Py. ultimum	45	27.8*	12.2*	0.09*	0.02*	0.6*	0.5*	7.7*	2.2*
Py. ultimum var. *sporangiiferum*	27	24.1*	36.1*	0.08*	0.06*	0.8*	0.8*	6.4*	6.2*
Py. ultimum var. *ultimum*	27	39.0*	59.8*	0.17*	0.14*	1.1*	1.6*	16.5*	14.3*

Within each column, values marked with an asterisk are significantly different from the non-inoculated control by Tukey's HSD, α=0.05.

Figure 5. 'Red Hawk' dry bean clustering analysis for pathogenicity and virulence of *Pythium* species. Red, yellow, and blue coloration represents low, moderate, and high disease pressure. Group 1: "Seed-Rot" pathogens, Group 2: "Root-Rot" pathogens, Group 3: Minor pathogens, Group 4: Non-pathogenic. "NIC, Rice" and "NIC, No Rice" refer to the non-inoculated controls with and without non-inoculated rice substrate, respectively.

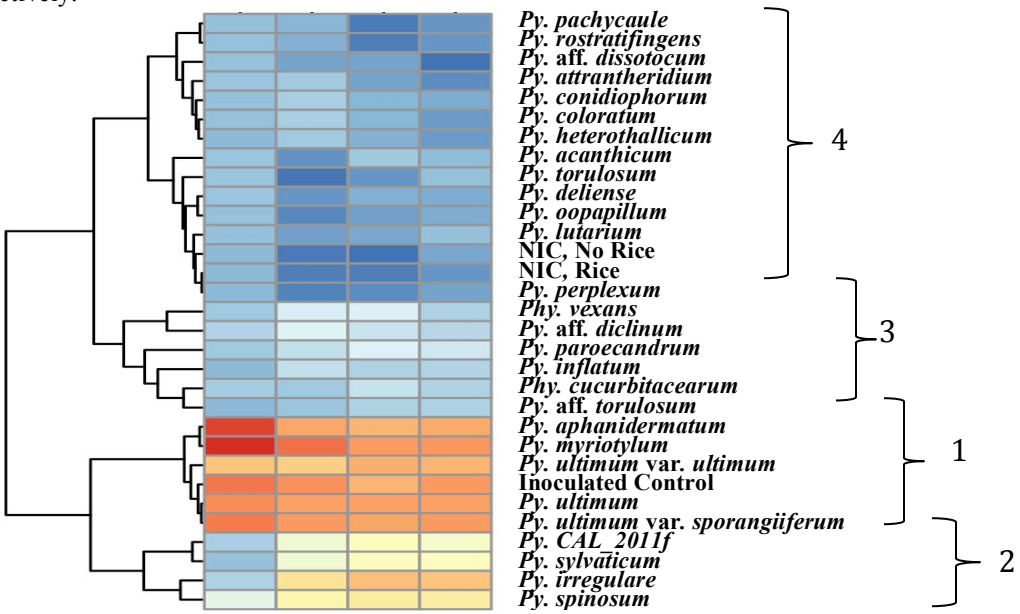
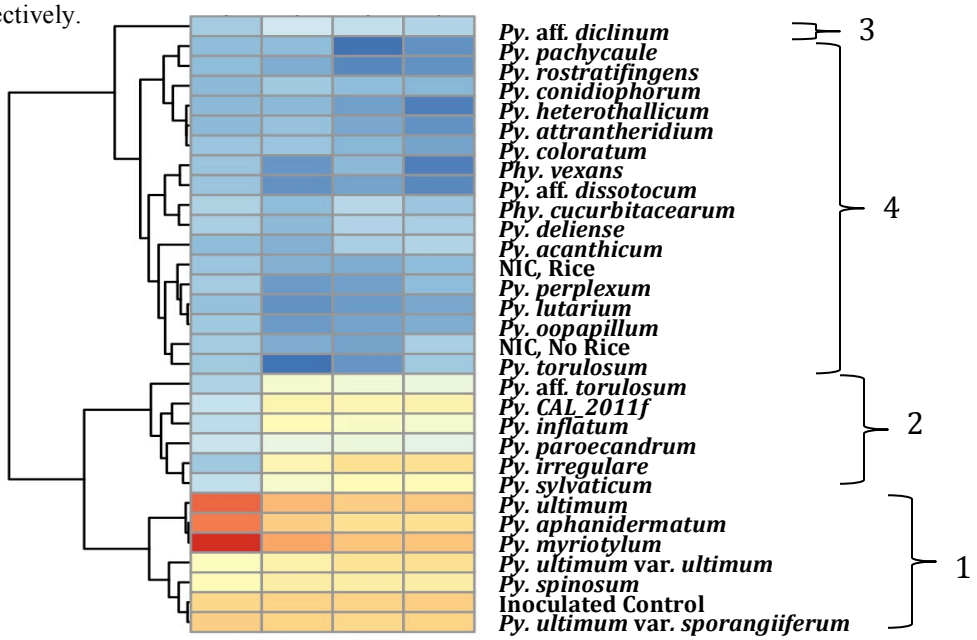


Figure 6. 'Zorro' dry bean clustering analysis for pathogenicity and virulence of *Pythium* species. Red, yellow, and blue coloration represents low, moderate, and high disease pressure. Group 1: "Seed-Rot" pathogens, Group 2: "Root-Rot" pathogens, Group 3: Minor pathogens, Group 4: Non-pathogenic. "NIC, Rice" and "NIC, No Rice" refer to the non-inoculated controls with and without non-inoculated rice substrate, respectively.



Bean variety significantly impacted the effects of *Pythium* species on root growth ($p<0.0001$). At least one root growth parameter was significantly reduced by 16 and 12 of the

Pythium species evaluated for 'Red Hawk' and 'Zorro,' respectively (Table 12). Relative to their respective NIC, 'Zorro' challenged with *Pythium* species yielded significantly greater root dry weight, area, and length than 'Red Hawk' ($p<0.0001$, $p=0.0022$, and $p<0.0001$, respectively).

The cluster analysis across seedling assay parameters was conducted separately for each

Figure 7. Seed assay disease severity index (DSI) across 'Zorro' and 'Red Hawk' dry beans, by temperature. Red points are significantly different from the non-inoculated control (Tukey, α=0.05). Points are marked with standard errors. Within a species, '*' indicates significantly different DSI between temperatures by Tukey's HSD, α=0.05. NIC refers to the non-inoculated control.

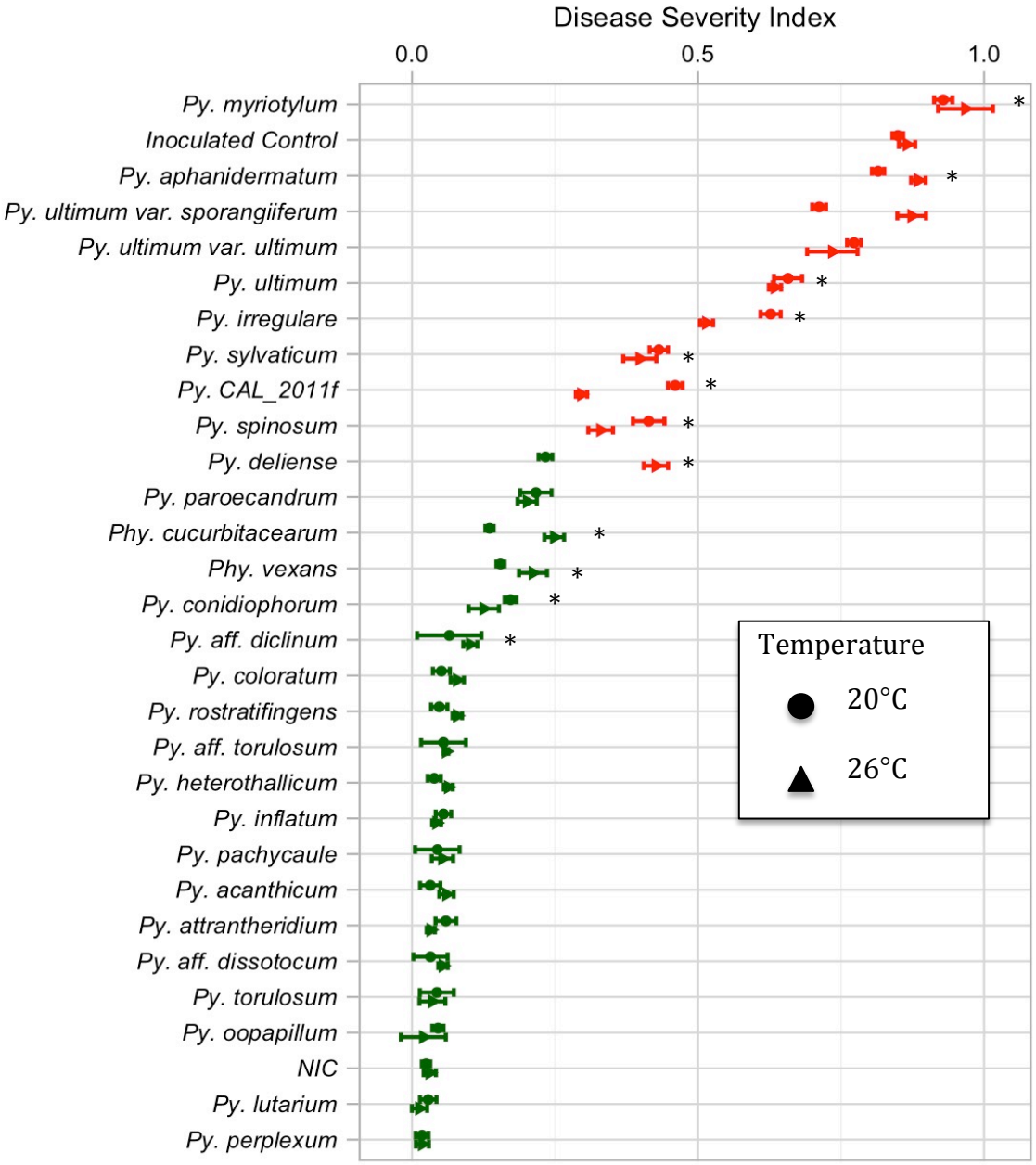


Table 13. Seed assay disease severity index (DSI) of 'Red Hawk' (RH) kidney bean and 'Zorro' (Z) black bean.

Treatment	RH		Z	
Non-inoculated control	0.04	a	0.07	a
Inoculated control	0.84	g	0.93	h
Phy. cucurbitacearum	0.24	a-e	0.19	a-e
Phy. vexans	0.28	b-e	0.30	c-e
Py. CAL_2011f	0.34	c-e	0.45	d-f
Py. acanthicum	0.03	ab	0.08	a-c
Py. aff. diclinum	0.05	a	0.14	a-c
Py. aff. dissotocum	0.02	a	0.10	a-c
Py. aff. torulosum	0.02	a	0.13	a-c
Py. aphanidermatum	0.81	g	0.92	h
Py. attrantheridium	0.02	a	0.10	a-c
Py. coloratum	0.03	a	0.13	a-c
Py. conidiophorum	0.11	a-c	0.22	a-d
Py. deliense	0.31	a-e	0.40	c-f
Py. heterothallicum	0.03	a	0.11	a-c
Py. inflatum	0.02	a	0.11	a-c
Py. irregulare	0.49	ef	0.68	fg
Py. lutarium	0.03	ab	0.05	a-c
Py. myriotylum	0.87	g	0.90	gh
Py. oopapillum	0.04	a	0.06	ab
Py. pachycaule	0.03	a	0.09	a-c
Py. paroecandrum	0.18	a-d	0.27	b
Py. perplexum	0.03	a	0.04	ab
Py. rostratifingens	0.03	a	0.13	a-c
Py. spinosum	0.40	de	0.50	ef
Py. sylvaticum	0.34	de	0.41	e
Py. torulosum	0.06	ab	0.05	ab
Py. ultimum	0.71	g	0.80	gh
Py. ultimum var. sporangiiferum	0.80	g	0.83	gh
Py. ultimum var. ultimum	0.69	fg	0.76	gh

Within each variety, values marked with the same letter are not significantly different; Tukey's HSD, α=0.05.

bean variety. *Pythium* species clustered into four main virulence groups for 'Red Hawk' seedlings (Figure 5) and four main virulence groups for 'Zorro' seedlings (Figure 6).

In the seed assay, DSI were highly correlated with each of the parameters measured from the seedling assay ($p<0.0001$, Pearson's $r>0.85$). Across *Pythium* species and bean varieties,

significantly more disease was caused on bean at 26°C than at 20°C (p=0.0210). However, the significant species x temperature interaction indicated that the extent of disease caused by individual *Pythium* species may depend on changes in temperature (p<0.0001). At 20°C and 26°C, nine and ten *Pythium* species were observed to cause a significantly higher disease severity index score than the NIC, respectively (Figure 7). At 26°C, the severity of disease caused by *Pythium deliense* significantly increased relative to 20°C (p<0.0001) and became significantly different from the NIC (p=0.0012). Thirteen *Pythium* species had significantly different mean DSI at 20°C than 26°C (Figure 7, p<0.05).

Seed assay DSI of *Pythium* species were significantly higher on 'Zorro' than 'Red Hawk' (p<0.0001). Although 10 of the same *Pythium* species caused a significant DSI on 'Zorro' and 'Red Hawk,' two additional *Pythium* species caused a significant DSI on 'Zorro' (Table 13).

Table 14. Seed assay disease severity index (DSI) by *Pythium* clades and temperature

	DSI	
Clade	20°C	26°C
A	0.69 Ba	0.79 Aa
B	0.18 Ac	0.17 Ac
D	0.22 Abc	0.23 Abc
E	0.06 Ac	0.09 Abc
F	0.38 Ab	0.33 Bb
I	0.69 Aa	0.71 Aa
J	0.03 Ac	0.03 Ac
K	0.17 Bbc	0.25 Abc

Within each clade, values marked with the same capital letter are not significantly different by Tukey's HSD, α=0.05. Within each temperature, values marked with the same lowercase letter are not significantly different by Tukey's HSD, α=0.05.

Within species, isolate virulence was observed to be quite variable. Within *Py. sylvaticum*, significant variability among isolates (n=8) was observed in DSI (p<0.0001) and root growth parameters (p<0.0001 for all parameters). Species virulence, as measured by DSI, was

similarly variable within clade ($p<0.0001$). Clades A (n=4 isolates) and K (n=3) had significantly higher DSI at 26°C than 20°C, whereas Clade F (n=21) exhibited the opposite trend (Table 14).

Discussion

This study was conducted to describe the relationship between select *Pythium* species and seedling growth of two bean varieties from different gene pool backgrounds at two temperatures. Findings from the current study indicate that many *Pythium* species can cause seedling disease in bean with bean variety and temperature influencing the disease severity observed. Nine *Pythium* species caused significant disease across bean varieties, temperatures, and assays. However, bean variety susceptibility to seven additional *Pythium* species evaluated in the current study varied depending on temperature and assay. *Pythium* species caused higher disease severity at 26°C than 20°C overall, but virulence response to temperature varied by *Pythium* species. Due to the observed variation in pathogenicity and virulence between bean varieties and temperatures observed in the current study, evaluation of *Pythium* species virulence on bean should be conducted across diverse bean germplasm and environmental conditions.

Ten *Pythium* species that exhibited pathogenicity in this study have been previously reported as pathogens of common bean, including *Phytopythium vexans*, *Py. aphanidermatum*, *Py. irregulare*, *Py. myriotylum*, *Py. paroecandrum*, *Py. spinosum*, *Py. sylvaticum*, *Py. ultimum*, *Py. ultiumum var. sporangiiferum*, and *Py. ultimum var. ultimum* (Kobriger and Hagedorn 1984; Li et al. 2014; Nzungize et al. 2011; Olson et al. 2016; Papias et al. 2016). This study constitutes the first report of *Py. inflatum*, *Py. deliense*, and *Py.* CAL_2011f as seedling pathogens of common bean. *Pythium* species that reduced root growth parameters did not always produce visible lesions, similar to the results of previous studies (Favrin et al. 1988; Stanghellini and Kronland 1986), indicating that DSI may not always be sufficient for evaluating the pathogenicity and virulence of root rot pathogens.

Previous studies in Rwanda and Australia have found that *Phytopythium cucurbitacearum*, *Py. conidiophorum*, *Py. lutarium*, *Py. pachycaule*, and *Py. rostratifingens* were able to cause disease in bean (Li et al. 2014; Nzungize et al. 2011). However, isolates from the same *Pythium* species used in the current study did not cause significant DSI or reduce root growth or emergence relative to the NIC on either bean variety screened. *Pythium* species found to be non-pathogenic in our experimental conditions may have been prevented from exhibiting the virulence observed in previous studies by pH or other factors (Rojas et al. in press; Martin and Loper 1999). Though some *Pythium* species have exhibited differential zoospore accumulation or virulence across certain hosts (Augspurger and Wilkinson 2007; Mitchell and Deacon 1986; Ingram and Cook 1990), the original host of the *Pythium* isolate had no significant effect on emergence and root growth parameters measured in the current study ($p>0.10$).

In the current study, *Pythium* species were found to form groups with similar patterns of disease aggressiveness on common bean (Figures 5 and 6). *Pythium* species evaluated on each dry bean variety clustered into four main groups. Although nine of the *Pythium* species were consistently clustered into the two most virulent groups for both bean varieties, clustering variability was observed between bean varieties. In both bean varieties, "Group 1" contained pathogenic species that were highly aggressive in reducing root growth and able to significantly impact emergence, including *Py. aphanidermatum*, *Py. myriotylum*, and all species from the *Pythium ultimum* complex. "Group 2" contained pathogenic species that aggressively reduced root growth, but had no significant effect on emergence, such as *Py.* CAL_2011f, *Py. irregulare*, and *Py. sylvaticum*. "Group 3" contained likely pathogens that were weakly aggressive, usually causing significant differences from the NIC for at least two root growth parameters, such as *Py.* aff. *diclinum*. Finally, "Group 4" contained species that exhibited little or no negative impacts on

seedling growth or seed germination under growth chamber conditions, such as *Py. acanthicum* and *Py. perplexum*. Group 1, Group 2, and Group 4 correspond approximately to the general categories of "seed-rot pathogens", "root-rot pathogens", and non-pathogenic *Pythium* species described in recent oomycete pathogenicity studies (Rojas et al. 2013; Matthiesen et al. 2016; Wei et al. 2010). The weakly virulent Group 3, however, may be opportunistic or "subclinical" pathogens that can cause reductions in root growth without obvious damage to root tissue (Stanghellini and Kronland 1986; Favrin et al. 1988).

Intraspecific variability in virulence has been reported in multiple previous studies (Broders et al. 2007a; Olson et al. 2016; Zhang and Yang 2000) and also was observed in this study. Within *Py. sylvaticum* (n=8), significant differences among isolates were observed for all root growth parameters measured. Virulence differences may be caused by varied selection pressures placed on isolates from different crop rotations (Zhang and Yang 2000), intraspecific genetic differences (Broders et al. 2007a), or other factors. Significant differences among isolates within a species may also be related to confusion between *Pythium* species *sensu stricto* and affinity groups. For example, *Py. torulosum* isolates were found to cause disease on bean and soybean in previous studies (Matthiesen et al. 2016; Nzungize et al. 2011). Although *Py. torulosum* did not significantly reduce root growth relative to the NIC in the current study study, *Py. aff. torulosum* did. Similar results have been previously observed on soybean (Rojas et al. in press).

For 13 *Pythium* species tested in the seed assay, virulence was significantly different between 20°C and 26°C. Previous studies have demonstrated that *Pythium* species may lose virulence as temperature increases (Cantrell and Dowler 1971; Kobriger and Hagedorn 1984; Matthiesen et al. 2016). This is similar to the current study in which *Py.* CAL_2011f, *Py.*

conidiophorum, *Py. irregulare, Py. spinosum*, *Py. sylvaticum*, and *Py. ultimum var. ultimum*, caused significantly higher DSI at 20°C than 26°C. However, the virulence of seven *Pythium* species significantly increased as temperature increased from 20°C to 26°C, including *Py. aphanidermatum*, which has previously been reported to favor high temperatures (Wei et al. 2010; Gold and Stanghellini 1985; Thomson et al. 1971). *Py. deliense* was also found to favor higher temperatures, as it caused significantly higher DSI than the NIC at 26°C, but not at 20°C. The previously described effects of temperature on virulence indicate that temperature may impact whether a *Pythium* species is determined to cause disease. A study on soybean reported that *Py. torulosum* caused serious disease at 13°C, but caused substantially less disease at higher temperatures (Matthiesen et al. 2016). In the seedling assay, *Py. torulosum* was not found to cause disease at 20°C, but its virulence at lower temperatures on bean was not evaluated. Seed assay temperatures may have resulted in some *Pythium* isolates appearing non-pathogenic that are capable of causing significant disease at other temperatures. Previous studies have described the effect of temperature on *Pythium* virulence as a uniform trait of isolates within each *Pythium* species (Abad et al. 1994; Gold and Stanghellini 1985). Though some *Pythium* species may have uniform virulence responses to temperature, intraspecific variation in the effect of temperature on virulence was observed in the current study for *Py. paroecandrum* and *Pythium ultimum*. Each species included at least one isolate with significantly greater virulence at 20°C than 26° ($p<0.0001$ and $p=0.0004$, respectively) and at least one isolate with significantly greater virulence at 26°C than 20°C ($p<0.0001$ for each isolate). The variable effect of temperature on virulence within some species indicates that different isolates within a species may have different optimal temperatures at which severe disease is caused.

Bean variety significantly affected the perceived pathogenicity and virulence of *Pythium* species in the current study. In the seedling assay, 'Zorro' bean root growth as a percentage of the NIC was reduced less than 'Red Hawk' across parameters (Table 12). Based on the cluster analysis, 13 *Pythium* species were grouped into virulent clusters of 'Zorro' compared to 15 *Pythium* species on 'Red Hawk,' While root growth as a percentage of the NICs was lower in 'Red Hawk' than 'Zorro' in the seedling assay, disease severity of *Pythium* species on 'Zorro' was consistently higher than on 'Red Hawk' in the seed assay (Table 13). Across temperatures, fewer *Pythium* species caused significantly higher DSI relative to the NIC for 'Red Hawk' than for 'Zorro.' The effect of bean variety on *Pythium* virulence seemed to be dependent on assay selection.

Several factors may account for the different results observed from the two assays. The differences in seed size, rates of imbibition and germination, assay duration, nutrition of growing media, exposure to light, and availability of moisture may have affected disease development of *Pythium* species on the two bean varieties. Using the two bean varieties tested, results from this study did not show any consistent effect of common bean germplasm across assays on resistance to *Pythium*-induced seedling disease and root rot. Selection of assay is an important consideration when aiming to consistently describe disease severity of oomycetes on bean since accurate assessments of root rot resistance in bean varieties depends substantially on multiple assay conditions (Walker 1965).

Characterizing bean-*Pythium* interactions of representative isolates from *Pythium* species has multiple benefits for production of common bean. The most virulent *Pythium* species can be used for evaluation of *Pythium* resistance. Breeders for *Pythium* disease resistance may need to account for *Pythium* species causing both pre-emergent and post-emergent damping-off, as little

is known about the diversity of genetic factors driving interactions between plant host and each *Pythium* species (Okubara et al. 2014). Non-pathogenic *Pythium* species that are mycoparasitic or are beneficial for host growth, such as *Py. oligandrum*, (Mazzola et al. 2002; Zhu et al. 2015), could be utilized for promoting plant growth (Le Floch et al. 2003) or biocontrol of soilborne pathogens (Lutchmeah and Cooke 1985).

Determining the most aggressive *Pythium* species in common bean from among *Pythium* species abundant in Midwestern agricultural fields may also help the development of improved disease management practices. *Pythium* species that are abundant and highly virulent on common bean may be among the most important for monitoring changes in fungicide sensitivity in common bean production. Determining the effects of temperature on *Pythium* species virulence may also allow for improved disease management by adjusting cultural practices that impact soil temperature around the seed, such as planting date (Naseri and Mousavi 2013), planting depth (O'Brien et al. 1991), or tillage practices.

From the findings of the current study and many previous studies (Li et al. 2014; Nzungize et al. 2011; Robertson 1976), Pythium damping-off and Pythium root rot can be caused by members of numerous *Pythium* species. Clearer characterization of the pathogenesis of virulent *Pythium* species in common bean may enable breeders to develop *Pythium*-resistant varieties more effectively. Although distinctions have been reported between root rot resistance traits for oomycete species and fungal species (Hagerty et al. 2015), common bean germplasm has been identified that confers resistance to root rots caused by both oomycetes and true fungi, such as *Fusarium* and *Rhizoctonia* species (Tu and Park 1993; Porch et al. 2014; Hagedorn and Rand 1978). Bean varieties may exhibit improved resistant to multiple root rot pathogens due to having modified root exudates (Keeling 1974; Okubara et al. 2014; Schroth and Hildebrand

1964), differences in cell wall tannins (Islam et al. 2003), or other physiological and molecular differences.

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