Improving Alfalfa Seedling Establishment: Understanding and Managing the Components of Wet Soil Syndrome

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Abstract

Alfalfa stand health and persistence is dependent on rapid and uniform seedling emergence. Pathogens such as *Aphanomyces euteiches*, *Phytophthora medicaginis*, *Pythium* spp., *Fusarium* spp., and *Rhizoctonia solani* plague alfalfa seedlings leading to decreased stand health and an overall decrease in yield over an alfalfa stand's life. The objectives of this study were to test the efficacy of fungicides when used as seed treatments for control of seed rot and damping-off of alfalfa under field conditions, identify the microbial communities (bacteria, fungi, and oomycetes) associated with infected alfalfa seedlings and soil samples from sites with poor alfalfa establishment, and quantify the abundance of known seed rot and seedling root rot pathogens from sites with poor alfalfa establishment. Field trials were conducted in Wisconsin in 2020 and 2021 in fields with previous alfalfa stand establishment issues to test the efficacy of the five different fungicide seed treatments. Rhizosphere soil, bulk soil, and root samples were taken from each field trial site and DNA was extracted from the samples. The DNA samples were submitted to the University of Minnesota Genomics Center (UMGC) to identify the entire microbial community of the sites. qPCR assays were conducted using the rhizosphere soil and root DNA samples to identify the abundance of the pathogens present. Results from the field trial indicated seed treatments may aid in stand establishment and an increase in yield. Results from growth chamber assays indicated fungicide seed treatments do aid in protecting alfalfa from *Pythium* spp. and *Phytophthora medicaginis* but did not add any control to *Aphanomyces euteiches*. Seed treatments also added control in field soils when tested under controlled environments and results indicated that experimental fungicides not currently labelled for alfalfa offered more control as compared to fungicides currently used on alfalfa today. Oomycete sequencing revealed *A. euteiches* was the dominating pathogen at the plot locations in Wisconsin followed by *P. medicaginis*. *Pythium* diversity was high in the rhizosphere. *Phytophthora sansomeana* was identified in relatively high abundance. This pathogen could be contributing to seed rot and damping-off in alfalfa seedlings and may have gone previously unrecognized as contributing to wet soil syndrome in alfalfa. Fungal sequencing revealed pressure from potentially unrecognized pathogens, *Paraphoma radicina* and *Plectosphaerella cucumerina*. qPCR results confirmed high *A. euteiches* quantities at all plot locations and higher *A. euteiches* pressure in the race 2 susceptible cultivar, Ameristand, as compared to the race 2 resistant cultivar, MegaTron AA. *P. medicaginis* and *Pythium* spp. quantities were lower than *A. euteiches*, which is

consistent with sequencing relative abundance results. Sequencing of the microbial community identified pathogens that could be contributing to alfalfa seedling diseases and qPCR assays allowed for quantification of known pathogens. Results from this study will inform future alfalfa breeding efforts to ultimately lead to increase profitability for alfalfa producers that are plagued with alfalfa establishment failure due to wet soil syndrome.

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1.1. Importance of alfalfa

Alfalfa (*Medicago sativa*) is a perennial forage legume crop grown throughout the world (Fernandez, Sheaffer, Tautges, Putnam, & Hunter, 2019). Today, alfalfa is grown in most U.S. states. Top alfalfa producing states are Wisconsin and California which are also the top two dairy producing states. Because of its high protein, mineral content, and digestible fiber, alfalfa is an excellent feed source for lactating dairy cattle promoting high milk production (Fernandez et al., 2019). Although the number one use of alfalfa in the United States goes to feeding dairy cattle, alfalfa also provides nutritional benefits to beef cattle, horses, and other ruminant animals.

In addition to the benefits alfalfa offers to livestock, alfalfa also many environmental services. Since alfalfa is a legume crop, it can fix its own nitrogen, and provides sufficient nitrogen to the next one or two corn crops (Yost, Morris, Russelle, & Coulter, 2014). Additional rotation effects that benefit the next crop in the rotation, such as corn or wheat, can increase grain yields 5 to 20% (Bullied, Entz, Smith, & Bamford, 2002; Stanger & Lauer, 2008). Because alfalfa is a perennial crop, it builds the soil year over year by increasing organic matter content (Fernandez et al., 2019). The increased level of organic matter in the soils can lead to increased water and nutrient holding capacities. Alfalfa decreases the potential for soil erosion and prevents nutrient loss to surface and ground water.

Due to its deep taproot and ability to deposit organic matter deep into the soil, alfalfa also plays a role in carbon sequestration and can help combat climate change by removing carbon dioxide from the atmosphere. Alfalfa also offers a habitat for important beneficial insects such as honeybees and other insect pollinators. Of all the perennial forage crops, alfalfa has the highest yield potential (Lacefield, Henning, Rasnake, & Collins, 1997). Due to all the benefits listed above, alfalfa is often known as the “queen of forages.”

Medicago sativa, purple-flowered alfalfa, originated near modern day Iran (Fernandez et al., 2019). The exact date of origin is not known but is estimated to be around 10,000 BCE. The oldest written reference to alfalfa is from 1300 BC in Turkey. Alfalfa was an important crop in early Babylonian cultures as well as early Roman, Persian, and Greek societies.

Alfalfa was brought to the Americas by Spanish and Portuguese settlers during their conquest of Mexico, Peru, and Chile. Alfalfa was introduced to the western United States around the 1850s and to the midwestern United States by Wendelin Grimm, in the late 1800s. Grimm brought seed from his home in Germany to Minnesota and year over year selected from the plants that were able to survive harsh winters in Minnesota (Edwards, Russell, History, & Mar, 1938). Eventually, alfalfa with the ability to persist through Midwest winters was developed and was known as Grimm alfalfa.

Once established in the United States, alfalfa breeders began selecting for different traits such as improved winterhardiness as well as resistance to diseases and insect pests (Fernandez et al., 2019). Resistance is the main method of disease control in alfalfa. Most modern cultivars have resistance to six diseases as well as several insect and nematode pests. However, diseases continue to significantly reduce alfalfa herbage yield and stand persistence.

Alfalfa stand health and persistence is dependent on rapid and uniform seedling emergence (Samac, Dornbusch, & Ao, 2017). There are a number of pathogens that can infect alfalfa seedlings and cause seedling disease in cold, wet, poorly drained soils known as “wet soil syndrome.” Symptoms of seedling disease include seed rot, damping-off, root rot, and lack of nodule formation in alfalfa roots. “Wet soil syndrome” can also affect mature alfalfa stands, leading to the destruction of fine feeder roots which can lead to less nitrogen fixation, less water absorption, and less nutrient uptake. Past research has developed resistance to pathogens that cause seedling disease and has identified fungicides that provide some protection to these pathogens, but poor alfalfa seedling establishment is still an issue that face alfalfa producers. Overall, “wet soil syndrome” can cause decreased yields, poor winter survival, and a shorter stand life, leading to a lower return on investment.

Seedling diseases that commonly cause pre-emergence and post-emergence damping off in alfalfa are diseases caused by oomycete pathogens *Pythium* spp., and *Phytophthora medicaginis* and true fungal organisms *Rhizoctonia solani* and *Fusarium* spp. (Berg, Miller, Dornbusch, & Samac, 2017; Hancock, 1983). Pre-emergence damping off, or seed rot, and post-emergence damping-off can lead to reduced stand life and yields, and a decreased ability to survive harsh winter conditions. *Aphanomyces euteiches* can also cause root rotting in seedlings (Smith & Watson, 2014). Mature

alfalfa stands can also be affected by this complex of soilborne pathogens (Berg et al., 2017).

Pythium spp. are often considered the most damaging in inflicting damage on alfalfa seedlings (Berg at al., 2017). Alfalfa seedlings infected by *Pythium* spp. often result when planting into cool, wet soils in the early spring. *Pythium* infection can lead to seed rot, post-emergence damping off, and root rot. Infected seedlings may exhibit softened radicles and their cotyledons may have brown lesions. Often, the hypocotyls and roots of infected plants will appear water-soaked before eventual collapse and seedling death. In addition, infected roots lack root hairs. *Fusarium* spp. and *Rhizoctonia solani* are true fungal pathogens that can cause pre- and post-emergence damping off but have received much less attention by researchers.

Often, the infection from *Pythium* and *Phytophthora* leads to the root “forking” which is when adventitious roots form above the damaged primary taproot. Infection caused by these pathogens in mature stands during wet soils in the spring leads to the destruction of fine feeder roots. This can negatively impact the alfalfa plant’s ability to fix nitrogen and sequester needed nutrients and water from the soil. Overall, infection by these pathogens can lead to a reduction in yields, less ability to survive the winter, and shortened stand life.

Phytophthora root rot was the first seedling root rot identified in alfalfa (Erwin, 1954) and has been identified around the world where alfalfa is grown (Frosheiser & Barnes, 1973). Today, Phytophthora root rot is well controlled by the use of resistant cultivars but can still cause yield losses in areas with wet soil, long periods of standing water, or where soil drainage is poor (Alva, Lanyon, & Leath, 1985). The most dramatic losses from Phytophthora root rot occur in the seeding year (Lueschen, Barnes, Rabas, Frosheiser, & Smith, 1976).

Infection of *Phytophthora medicaginis* in the seedling year can lead to both pre- and post-emergence damping off. Symptoms of Phytophthora root rot in alfalfa seedlings include yellow or brown lesions along the root or root tip (Lueschen et al., 1976), that may develop into dark brown or black lesions (Frosheiser and Barnes, 1973). In addition, the taproot and lateral roots may be rotted, which prevents growth and causes adventitious roots to form above the rotted taproot. If the disease is severe enough and

prolonged wet conditions occur, yellow or reddish-brown coloring of leaves can occur aboveground. Overall, Phytophthora root rot can lead to stunting and thin stands.

Infection by Phytophthora root rot can also lead to weed invasion due to stand thinning. Because Phytophthora root rot can cause the taproot to rot, the infected alfalfa plant may not be able to survive droughts. In addition, a diseased plant may have a harder time surviving winter. This can all lead to a less productive and less persistent stand.

Aphanomyces root rot is a disease that can be found in both seedlings as well as mature stands (Samac, Yu, & Missaoui, 2021). Aphanomyces root rot is caused by the pathogen *Aphanomyces euteiches*. Unlike *Pythium*, *Fusarium*, *Rhizoctonia solani*, and Phytophthora root rot, Aphanomyces root rot does not cause seed rot or damping-off in alfalfa. In seedlings, Aphanomyces root rot typically causes stunted growth. *Aphanomyces euteiches* is also pathogenic on a number of other forage host crops such as red and white clover, common vetch, grain legumes, bean, faba bean, lentil, and chickpea.

In addition to stunting, cotyledons of seedlings infected by *A. euteiches* turn chlorotic and can turn necrotic if the disease is severe. Hypocotyls and roots of infected seedlings appear gray and water soaked at first and then may turn brown as the disease progresses. Typically, seedlings infected with *A. euteiches* can remain standing upright for a long period of time after infection. Because Aphanomyces root rot causes stunting in growth, it may hinder the alfalfa plant's ability to compete with weeds (Smith & Watson, 2014).

Mature plants infected with Aphanomyces root rot lack lateral and fibrous roots (Samac et al., 2021). Typically, mature plants become infected in the late summer during prolonged wet soil conditions (Samac, Rhodes, & Lamp, 2015). Currently, there are two races or pathotypes of Aphanomyces root rot, race 1 and race 2 with race 2 being more virulent (Smith & Watson, 2014). Overall, Aphanomyces root rot can lead to a reduction in nodulation, nitrogen fixation, root growth and nutrient and water uptake which can lead to a decrease in yield potential and stand persistence over time (Samac et al., 2021; Smith & Watson, 2014; Wiersma, Grau, & Undersander, 2013).

1.2. Pathogens involved in the wet soil syndrome

Phytophthora medicaginis, *Aphanomyces euteiches*, and *Pythium* spp. are oomycetes (Samac et al., 2021; Schroeder, Martin, Cock, Okubara, & Paulitz, 2013). One key difference in oomycetes and fungi is that their cell walls are comprised of different components. Oomycete cell walls contain cellulose whereas fungal cell walls are mainly comprised of chitin (Fawke, Doumane, & Schornack, 2015). In addition, fungal and oomycete cells differ morphologically. Oomycetes also have coenocytic hyphae and produce biflagellate zoospores (Schroeder et al., 2013). *Pythium* spp. and *Phytophthora medicaginis* belong to the Peronosporales, and *Aphanomyces euteiches* is in the Saprolegniales (Judelson, 2012).

Oomycete pathogens are commonly known as the “water molds,” and are often found in wet, poorly drained fields (Agrios, 2005). There are two spore stages in the *A. euteiches*, *Pythium* spp., and *Phytophthora medicaginis* life cycles: the oospore and zoospore stage (Samac et al., 2021; Schroeder et al., 2013). The oospore stage is the sexual stage, and the zoospore stage is the asexual stage. Oospores can overwinter and persist in soils for many years even in the absence of a host crop (Samac et al., 2021; Schroeder et al., 2013; Smith & Watson, 2014), making oomycete diseases particularly hard to control by use of crop rotation.

In prolonged saturated and wet soil conditions, oospores of *P. medicaginis* germinate (Frosheiser, 1980). A hypha is produced from the oospore that can infect an alfalfa root directly, or the oospore will produce sporangia in which asexual, motile zoospores are produced that can then infect the alfalfa root. Zoospores are motile in water by their flagella. Zoospores are attracted to all zones of an alfalfa root other than the root cap and can infect the root within two minutes of contact. Both susceptible and resistant cultivars are equally as attractive to the pathogen zoospore.

Once infection occurs, the zoospores encyst and attach to the root (Miller & Maxwell, 1984). After encysting, the *P. medicaginis* zoospores germinate and penetrate the alfalfa root. Two hours after zoospore penetration, growth of the pathogen is rapid. From epidermal cells the hyphae then penetrate into the outer cortex of the root. Typically, differences in resistant and susceptible plants can be noted 12 hours post-infection.

Plants resistant to *P. medicaginis* respond to infection with a hypersensitive response (Marks & Mitchell, 1971). Cells that were in contact with the hyphae become discolored and plasmolyze and growth of the pathogen ends in the epidermis and cortex of the root. On the other hand, in susceptible plants, complete colonization of the

pathogen takes place and hyphae grow into the stele. It should be noted that medicarpin, a phytoalexin, plays a key role in inhibiting the growth of *P. medicaginis* and is produced in resistant plants in response to the hypersensitive reaction (Vaziri, Keen, & Erwin, 1981).

There are currently two pathotypes of *A. euteiches*, race 1 and race 2. Both race 1 and race 2 are very widespread across the U.S. but race 2 was identified as being more prevalent in Minnesota and New York. However, in the bioassay used for race detection, the presence of race 2 strains masks the presence of race 1 strains, which may make race 2 strains appear more abundant (Samac et al., 2021). Currently, there is genetic resistance to both race 1 and race 2 in a number of cultivars (National Alfalfa and Forage Alliance, 2020). When using a soil baiting technique, surveys have found that *A. euteiches* is more prevalent in soils as compared to *P. medicaginis* (Munkvold & Carlton, 1995; Vincelli, Nesmith, & Eshenaur, 1994). When both *A. euteiches* and *P. medicaginis* are in the soil they can both infect an alfalfa plant; however, *A. euteiches* can inhibit colonization of *P. medicaginis* in an alfalfa plant (Vandemark, Ariss, & Hughes, 2010a).

In wet soil conditions, oospores of *A. euteiches* germinate and form a sporangium that produces primary spores, which germinate to form zoospores (Gangneux et al., 2014). Zoospores of *A. euteiches* are attracted to root hair zone of the alfalfa seedlings and can germinate within ten minutes of contact (Samac et al., 2021). Like infection by *P. medicaginis*, there is no difference in attraction to root hair zones by zoospores of *A. euteiches* between susceptible and resistant cultivars. After infection of the pathogen, roots of susceptible cultivars are colonized rapidly with the pathogen growing in the cortical cells and intercellular spaces, leading to cell degradation and death.

In resistant cultivars, no signs of disease occur and hyphae of *A. euteiches* do not grow past the penetrated epidermal cell and a few neighboring cells. Like infection by *P. medicaginis*, varieties resistant to *Aphanomyces euteiches* display a hypersensitive response triggering immunity to the pathogen. In susceptible plants, oospores will be present in the roots and visible under a microscope. In resistant plants, no or very few oospores will be present in the plant roots.

Around the world, 15 species of the oomycete pathogen *Pythium* have been identified that can cause pre-emergence and post-emergence damping off in alfalfa (Berg et al., 2017). Of those 15 species, *P. irregulare*, *P. sylvaticum*, and *P. ultimum* var.

ultimum are the most aggressive and widespread alfalfa pathogens. *Pythium* pathogens have thousands of hosts and are present in almost all agricultural soils in the world.

Pythium spp. are necrotrophic pathogens, meaning they feed on dead plant tissue after killing the living tissue. Seed rot and post-emergence damping off occurs after the *Pythium* pathogens attack the embryo, hypocotyl, and emerging radicles of the alfalfa seeds and seedlings. *Pythium* pathogens also cause destruction of the root hairs and fine feeder roots on mature alfalfa plants (Berg et al., 2017). This can lead to a reduction in root biomass causing symptoms of nutrient deficiencies and reductions in yield.

Like other oomycetes, *Pythium* spp. can survive many years in the soil as thick-walled oospores even during extreme cold and extreme heat (Schroeder et al., 2013). Under cold, wet soil conditions, *Pythium* oospores germinate and form sporangia. The developed sporangia then form a discharge tube which forms a vesicle. Zoospores are then released from the vesicle and are chemotactically attracted to root and seed exudates.

Once in contact with the seed or roots, the zoospores will encyst and form cell walls, they germinate, and then infect the seed or root. The zoospores will kill living plant tissue and colonize inside the tissue. Once inside the plant tissue, oospores will then be produced by the pathogen, which produce sporangia or sexual reproductive structures, oogonia and antheridia. The antheridia then fertilize the oogonia to produce oospores. *Pythium* oospores can be spread throughout a field by irrigation or tillage.

Along with *Pythium*, true fungal species such as *Rhizoctonia solani* and various *Fusarium* spp. also infect alfalfa seedlings and can cause both pre- and post-emergence damping-off and can also feed on fine feeder roots of mature plants (Berg et al., 2017). Both *Fusarium* spp. and *Rhizoctonia solani* (Hancock, 1983) are recognized as pathogens contributing to pre-emergence and post-emergence damping-off, but additional information regarding individual species and strains of the pathogens causing disease is still needed. Berg et al. (2017) found that *Fusarium incarnatum-equiseti* and *F. oxysporum* were the two most aggressive *Fusarium* strains in causing both seed rot and seedling root rot on alfalfa. *F. oxysporum* is also the pathogen that causes Fusarium wilt in alfalfa (Samac et al., 2021).

1.3. Control of seedling diseases

Current control measures for combating seedling diseases in alfalfa are the combined use of fungicide seed treatments (Berg et al., 2017) and genetic resistance for Phytophthora root rot and Aphanomyces root rot (Samac et al., 2021). Currently, both Apron XL and Stamina are fungicides used to control seedling diseases in alfalfa (Berg et al., 2017). Apron XL is commonly used to control damping off caused by *Pythium* spp. and *Phytophthora medicaginis*. The active ingredient of Apron XL is mefenoxam; R, S-2[2,6-dimethylpthenyl)-methoxyacetylamino]-propionic acid methyl ester). Mefenoxam's enantiomer, metalaxyl, has also been used to manage damping off in alfalfa and a number of other legumes (Berg et al., 2017). Apron XL however does not control other pathogens causing seedling damping-off such as *Rhizoctonia solani*, *Fusarium* spp., and *A. euteiches* (Samac et al., 2017). In addition, Berg et al. (2017) identified *Pythium* strains in Minnesota that were not well-controlled by Apron XL. Extended use of mefenoxam and metalaxyl fungicides may have increased resistance; however, metalaxyl resistant strains of *Pythium* were observed before its widespread use.

Stamina fungicide seed treatment is labelled to control *A. euteiches, Phytophthora medicaginis*, *Fusarium* spp., and *Rhizoctonia solani* but Stamina does not offer adequate control of *Pythium* spp. Stamina is a quinone outside inhibitor (QoI) fungicide. The active ingredient of Stamina is pyraclostrobin; carbamic acid, [2-[[[1-(4-chlorophenyl)-1*H*-pyrazol-3-yl]oxy]methyl]phenyl]methoxy-,methyl ester). Another fungicide treatment in addition to Stamina that can offer control to the true fungal species of *Fusarium* spp. and *Rhizoctonia solani* is Maxim (Syngenta, 2022). Maxim's active ingredient is Fludioxonil and it is a group 12 fungicide. Fludioxonil (Maxim) is labelled for alfalfa but is not widely used currently among the alfalfa seed marketers.

Genetic resistance to both Phytophthora root rot and Aphanomyces root rot is currently available to alfalfa producers (National Alfalfa and Forage Alliance 2022; Samac, Yu, and Missaoui 2021). Phytophthora root rot genetic resistance was developed before Aphanomyces root rot resistance (Samac et al., 2021). Phytophthora root rot resistant varieties were available for use in the southwestern U.S. in the late 1960's (Lehman, Erwin, & Stanford, 1969). Selection for resistance to Phytophthora root rot in the Midwest U.S. began in the early 1970s (Frosheiser & Barnes, 1973), with the adoption of resistant cultivars actively used in the 1980s (Frosheiser, 1980). Initial selection in the Midwest was conducted in a field nursery setting by selection of healthy plants from pathogen infected soil (Frosheiser & Barnes, 1973).

Resistance to Phytophthora root rot in alfalfa led to improved yields and winterhardiness. Phenotypic selection using disease screens is still the most common way to select varieties with resistance and over time, resistance to Phytophthora root rot has remained stable (Samac et al., 2021). Failure of varieties with genetic resistance to Phytophthora root rot was caused by other pathogens such as *A. euteiches* and *Pythium* spp. and led to the development of Aphanomyces root rot resistant varieties.

Aphanomyces root rot was identified as a source of seedling root rot in alfalfa when varieties with resistance to Phytophthora root rot failed in the 1980s (Delwiche, Grau, Holub, & Perry, 1987). The release of a cultivar with genetic resistance to Aphanomyces root rot happened in the early 1990s (Grau, 1992). When varieties with resistance to both Phytophthora root rot and Aphanomyces root rot began to fail, the identification of a second pathotype, Aphanomyces root rot race 2, occurred (Malvick & Grau, 2001; Munkvold et al., 2001). Development of a race 2 resistant cultivars then took place. There are an increasing number of varieties with resistance to race 1 and race 2 each year (National Alfalfa and Forage Alliance, 2022).

To market varieties with resistance to both Phytophthora root rot and Aphanomyces root rot, seed companies must follow standard test protocols outlined by the North American Alfalfa Improvement Conference (Fitzpatrick, Brummer, Hudelson, Malvick, & Grau, 1998; Nygaard, Tofte, & Barnes, 1995). Once the test is conducted, plants are scored on a scale of 1-5: 1=no necrosis of roots or hypocotyl; 2=slight necrosis of roots or hypocotyl; 3=necrosis of roots and lower hypocotyl and moderate stunting of stems; 4=extensive necrosis of roots, hypocotyls, and cotyledons, and severe stunting of stem; 5=dead seeding (Fitzpatrick et al., 1998; Nygaard et al., 1995). Scores of 1 and 2 are resistant plants and scores of 3-5 indicate susceptible plants. Varying levels of resistance are classified based on how the plants score in the standard test. If a cultivar scores 50% or greater resistant plants, then that cultivar is classified as having high resistance. A score of 31-50% is resistant, 15-30% is moderate resistance, 6-14% is low resistance, and 0-5% is susceptible (National Alfalfa and Forage Alliance, 2022).

There is a standard test for Pythium seed rot and damping-off (Altier, Barnes, Thies, & Samac, 1995), but the test is not widely used for cultivar development. Because *Pythium* spp. can destroy lateral and fine feeder roots of mature plants, in addition to infecting seedlings, genetic resistance could protect alfalfa seedlings as well as protect mature plant roots (Berg et al., 2017). This genetic resistance to *Pythium* can lead to more productivity and stand persistence. Recent research has shown genetic resistance

to Pythium can be achieved after two cycles of selection (Samac et al., 2017). In addition, genetic resistance to one species of Pythium seems to offer adequate resistance to others.

In addition to the above chemical and genetic controls, cultural controls can also be deployed to mitigate losses from alfalfa seedling diseases but may be a challenge for farmers to adopt. To mitigate losses from *Pythium*, growers should seed into warmer soils to prevent oospore germination in cold, wet soils (Schroeder et al., 2013). In addition, farmers should avoid seeding alfalfa into wet, clay, poorly drained fields (Malvick, Grünwald, & Dyer, 2009; Undersander et al., 2011). However, this can be challenging if a grower only farms fields with a clay soil type. Overseeding to avoid potential losses is also widely adapted in the industry (Berg et al., 2017) but this increases grower expenses. Another cultural control method is to avoid continuous rotation back into alfalfa, as disease inoculum can build (Smith & Watson, 2014). Crop rotation, however, can be ineffective in controlling the oomycete diseases since the oospores of the oomycete pathogens (*Pythium*, *Phytophthora*, and *Aphanomyces*) can survive in the soil without the presence of a host crop for many years (Samac et al., 2015; Smith & Watson, 2014).

1.4. Results of similar studies-seed treatments

Recent research has identified fungicide seed treatments that are effective against *Pythium* spp*.*, *Phytophthora medicaginis*, *A. euteiches*, and *Fusarium* spp. in agar plate assays (Samac et al., 2017). The research tested the efficacy of different soybean fungicide seed treatments against alfalfa pathogens. The fungicide treatment combinations that showed the most activity against the pathogens were: Apron XL (mefenoxam), Intego Solo (ethaboxam), and EverGol Energy (metalaxyl, penflufen, and prothioconazole).

The research found that Apron XL had excellent activity on both *Pythium* spp. and *Phytophthora medicaginis* but was poor against *A. euteiches* and *Fusarium* spp. EverGol Energy showed good control on *A. euteiches* and *Fusarium* spp. and excellent control on both *Pythium* spp. and *Phytophthora medicaginis*. Intego Solo had excellent control on *Phytophthora*, and *Aphanomyces*, very good control on *Pythium*, but was poor against *Fusarium*.

In addition to the recent research conducted by Samac et al., 2017, other fungicide efficacy has been tested against both *Pythium* spp. and *Phytophthora* in corn

and soybean crops (Radmer et al., 2017) and against *A. euteiches* in field peas (Wu et al., 2018). In the study by Radmer et al. (2017), the sensitivity of 22 isolates of 10 pathogenic *Pythium* spp. to fungicides were tested in a lab setting using both seed and seedling assays. Fungicides used were Dynasty (azoxystrobin), Intego Solo (ethaboxam), Apron XL (mefenoxam), Stamina (pyraclostrobin), and Trilex (trifoxystrobin). Of the fungicides tested, Apron XL (mefenoxam) and Intego Solo (ethaboxam) showed the most control on the *Pythium* spp. tested, including *Pythium irregulare*, *P. ultimum* var. *ultimum* and *P. sylvaticum*, as opposed to the strobilurin fungicides used. Intego Solo (ethaboxam) was effective against all but one of the *Pythium* spp. at both medium and high concentration levels, which may be due, in part, to its multiple modes of action (Uchida, Roberson, Chun, & Kim, 2005).

Ethaboxam is specifically labelled for oomycetes (Kim, Chun, Jeon, Lee, & Joe, 2004), and the study by Samac et al. (2017) found that it offered control of *A. euteiches* in lab tests in addition to controlling *Pythium* and *Phytophthora*. In both a field setting and lab setting, Intego Solo was evaluated for effectiveness against *A. euteiches* infection of field peas (Wu et al., 2018). The study revealed that Intego Solo provided protection to seedling blight in field peas caused by *A. euteiches* in a greenhouse setting but not in a field setting. The limited control in the field setting may have been due to the presence of other soilborne pathogens, complex and varying soil conditions, or that the fungicide may have degraded.

In other fungicide efficacy trials, isolates of *Pythium* were identified that were not well controlled by Apron XL or Stamina seed treatments in Minnesota alfalfa fields. Berg et al. (2017) identified six out of 10 *Pythium* strains that were not well controlled by Apron XL. Only 75% or less of the plants were controlled when tested against six of the 10 strains meaning that some aggressive *Pythium* strains can potentially rapidly colonize inside the seed and bypass the control of the seed treatment. This would then negatively impact seed germination and therefore establishment by causing seed rot. The species of *Pythium* identified that were not well controlled by Apron XL (mefenoxam) fungicide seed treatment were *Pythium irregulare*, *P. sylvaticum*, *P. conidiophorum*, *and P. ultimum var. ultimum*. The study did find that Apron XL offered more control on a *P. sylvaticum* strain that can cause both seed rot and root rot versus the *P. sylvaticum* strain that caused seed rot. More aggressive isolates may also be present outside of Minnesota. In addition, Apron XL seed treatments are not effective on *Rhizoctonia*, *Fusarium*, or *Aphanomyces*.

In addition to identifying more aggressive Pythium strains that were not controlled by Apron XL or Stamina, Berg at al. (2017) also identified *Fusarium* spp. that caused significant seed rot and seedling root rot in alfalfa. *Fusarium* spp. were previously identified to cause seed rot and damping-off, but identification of pathogenicity of specific *Fusarium* strains was lacking. *F. oxysporum* and *F. incarnatum-equiseti* were both identified as *Fusarium* spp. causing aggressive seed rot in alfalfa that have been previously overlooked. Samac et al. (2017) confirmed that Apron XL was ineffective in controlling the *Fusarium* spp. identified. Similar to *Pythium*, genetic resistance to *Fusarium* may be warranted to help control the seed and root rot caused by the pathogen in alfalfa (Berg et al., 2017).

1.5. Results of similar studies-microbial community analysis

Accurate identification of pathogens present in soils with poor establishment is needed to correctly guide future breeding efforts. One means of community analysis is amplification and sequencing of target genes from soil or plant materials. A field trial in high disease environments in Ohio compared plant populations and yield of different soybean cultivars with varying resistance to soybean oomycete pathogens *Phytophthora*, *Pythium*, and *Phytopythium* (Navarro, Wijeratne, Culman, Benitez, & Dorrance, 2021). The research explored the oomycete community composition in cultivars with varying levels of resistance and also analyzed how the genotypes and environments affected diversity of *Phytophthora*, *Pythium*, and *Phytopythium* in rhizosphere soil. They found that the cultivar, Kottman, with moderate resistance to the pathogen *Phytophthora sojae*, which causes stem and root rot in soybeans, had a lower abundance of *P. sojae* as compared to the cultivar, Sloan, which had moderate susceptibility to *P. sojae*. The cultivar also affected the *Pythium pachycaule* abundance where species abundance was higher in Sloan as opposed to Kottman. No other *Pythium* spp. or *Phytopythium* spp. were affected by genotype/cultivar. The study also found that the moderately resistant cultivar Kottman had a statistically higher ($P<0.01$) early plant population as compared to the moderately susceptible cultivar Sloan in six out of 11 environments. In addition, Kottman had significantly higher yield than Sloan in five of 10 environments.

Using DNA based approaches to identify the abundance, composition, and diversity of pathogens in soils has been conducted in other crops such as field pea (Taheri, Chatterton, Gossen, & Mclaren, 2017b). Taheri et al. (2017b) aimed to measure

the abundance and frequency of oomycete species in the rhizosphere soil with asymptomatic and diseased field pea plants in 26 pea fields in Canadian prairies. The study also identified whether there were oomycete microbial community differences among the regions analyzed in Canada, which were, Alberta, Saskatchewan, and Manitoba.

Previously *Fusarium* spp. were identified as the main contributors to root disease in field pea; however, the methods used were often culture dependent methods that could have biases. Non-culture-based methods using DNA based approaches provide a greater picture of all pathogens that are present and that could cause root rot in field peas. This study focused on the role that oomycetes may play in causing pea root rot that may have been underestimated before. Sequencing of the ITS1 region revealed that *Pythium* was the most abundant oomycete genus and that *P. heterothallicum* was the most abundant species. It was also more abundant in sites with healthy plants. No plant inoculation was performed with *P. heterothallicum,* so the role of the pathogen in pea root disease remains unknown.

Aphanomyces was detected 57% of samples but in low abundance in Canadian pea fields (Taheri et al. 2017b). The authors hypothesized it was identified in such low abundance in the sequencing results because there are three nucleotide mismatches between the universal ITS7 primer and its binding site on the *A. euteiches* ITS region. The authors designed new oomycete primers, ITS6 and ITS7a.e. to improve *A. euteiches* detection (Taheri, Chatterton, Gossen, & Mclaren, 2017a) that replaced the mismatched nucleotides in the ITS7 and ITS6 primers with degenerate nucleotides. Using the new primers, the authors saw improved results in *Aphanomyces* detection at both a genus and species level. Abundance of *A. euteiches* increased from 0.2% of total reads when the ITS6 and ITS7 primers were used to 15% of total reads when the ITS6 and ITS7a.e. primers were used revealing that the abundance of *A. euteiches* was underestimated when using the ITS6 and ITS7 primers.

Other *Aphanomyces* species such as *A. caldogamus* were detected in higher abundance with the degenerate primer use. This species previously was not identified using the ITS6 and ITS7 primers. However, this species was only confirmed at a 97% identity meaning its identity is still ambiguous.

Using the newly designed degenerate primers, *Pythium heterothallicum* was still the most abundant oomycete species identified, but overall percent of relative abundance decreased from just over 40% using the ITS6 and ITS7 primers to a relative

abundance of around 35% using the newly designed degenerate primers. Similar to their findings with the ITS6 and ITS7 primers, *P. heterothallicum* was still more abundant in healthy versus diseased soils when using the ITS6 and ITS7a.e. primers. On the contrary, *A. euteiches* abundance was higher in diseased sites as compared to healthy sites, leading the authors to believe that *A. euteiches* could be a species significantly contributing to root rot in pea that has gone underestimated in the past because of use of soil and baiting pathogen isolation techniques for detection and quantification.

In addition to an increase in *A. euteiches* reads and relative abundance, using the newly designed ITS6 and ITS7a.e. primers resulted in an increase in total reads. The number of high-quality reads more than doubled with the use of the ITS6 and ITS7a.e. primers as compared to the ITS6 and ITS7 primers used in the previous study. At a genus level, *Pythium* abundance decreased and *Aphanomyces* abundance increased. The use and adoption of degenerate primers show that improper primer design can lead to an underestimation of some species that could be contributing to the pea root rot complex. In addition, the use of incorrect primers can result in an underestimation of total number of species in the soil. The new primers resulted in a more accurate estimation of the oomycete community in the Canadian fields used in the study.

Along with the above field pea study, oomycete and fungal community composition was identified in winter rye fields in Iowa (Bakker, Moorman, & Kaspar, 2017). In this study, community composition was evaluated on dying rye cover crop fields in Boone, Iowa in 2013 and 2014 using amplicon sequencing. Before planting the rye cover crop, corn and soybeans were sown in the cropping system. Rye was planted in the fall of 2013 and then received a glyphosate application the following spring on April 22.

Sampling then took place after the glyphosate application at three subsequent time periods: April 25, May 9, and May 23 to observe how the fungal and oomycete communities may change as the rye cover crop dies over the period of the month following herbicide application. DNA extraction of the samples took place, and the DNA was amplified by PCR using ITS7 and ITS6 primers for oomycete sequencing amplifying the ITS region. For fungal amplification the 18S rRNA gene was amplified. After PCR amplification the products were purified and submitted for pyrosequencing.

Results indicated that previous methods that relied on baiting for pathogen isolation may have overlooked oomycete pathogens responsible for corn root diseases. *Pythium volutum* and *Pythium* sp. F86 as well as *Lagena radicicola* were the topmost

abundant oomycete species identified from amplicon sequencing. Pathogenicity assays revealed that both of the *Pythium* spp. identified caused root disease in corn. The *Pythium* sp. F86 did not respond to the time frame of termination (community composition did not change based on sampling time). However, *L. radicicola* relative abundance did decrease with time following the glyphosate application. In addition, *P. volutum* relative abundance increased over sampling time.

Pythium volutum was not previously recognized in the Midwest as a species that was pathogenic and caused root disease on corn. However, media used previously for isolation may not have been conducive for growth of oomycetes and the species could have gone unrecognized for causing disease in corn in the past. Both the *Pythium* spp. and *L*. *radicicola* could have previously been missed in causing significant root disease when relying on the standard isolation techniques of the pathogens. The results demonstrated the advantages of using high throughput sequencing to evaluate community composition as compared to baiting and isolation methods alone, as the dominant oomycete species identified in this study are not easily cultured.

The fungal sequencing revealed that the community composition greatly shifted based on sampling date. In addition, five OTUs were identified in every plot sampled indicating that these fungal species were the most prevalent in the roots of rye cover crops at the Iowa sites sampled. It should be noted that the fungal diversity may have been underreported because there are many fungal taxa which share identical 18S rRNA gene sequences, and therefore were not correctly identified.

Overall, complete microbial community analysis allows for the accurate identification of potential pathogens that may be present in the soil and/or roots that could lead to pathogen invasion and subsequent infection and disease. Indexing soils using DNA based sequencing provides a more accurate assessment of all potential pathogens and the abundance of those pathogens in the soil microbial community, as compared to the standard baiting isolation methods.

1.6. Results of similar studies-PCR assays to quantify pathogens in plants and soil

Quantitative polymerase chain reaction (qPCR) assays have been developed to measure the abundance of certain pathogens known to cause seedling disease in alfalfa such as *Phytophthora medicaginis* (Vandemark & Barker, 2003), *A. euteiches* (Gangneux et al., 2014), *Pythium irregulare, P. sylvaticum*, and *P. ultimum* (Schroeder et

al., 2013). For the detection of *A. euteiches*, a qPCR assay was developed that can detect less than 10 oospores of the pathogen per gram of soil (Gangneux et al., 2014). The test was developed to allow the fast, specific, and sensitive detection of *A. euteiches* in pea fields in France. *Aphanomyces euteiches* is very pathogenic in peas, and as of late, there are only a few varieties with partial resistance. Therefore, avoiding soil infested with the pathogen is a solution that many producers rely on in combating the disease.

Previously, a bioassay has been used to evaluate the inoculum potential of *A. euteiches* in fields. However, this takes weeks to obtain results and is not quantitative. The adoption of a qPCR assay to quickly detect the pathogen at accurate levels offered a more efficient and accurate way to identify the pathogen in fields. The qPCR assay was designed by isolating DNA of pure pathogen cultures as well as infested soil. Primers were designed to amplify the ITS1 region of the rDNA operons and of all the primers designed and tested in the study, the primer pair Ae_ITS1_39F and Ae_ITS1_167R proved to be the most sensitive and specific in detecting the pathogen.

To ensure that the primer pair only amplified *A. euteiches* and no other *Aphanomyces* species, *A. cladogamus* and *A. cochliodies* DNA were tested in qPCR assays and results showed no amplification of other species besides *A. euteiches*. A total of 40 isolates of *A. euteiches* were used in the assay and were accurately amplified by the primers. Various inoculum levels were compared, and the assay proved effective in identifying pathogen levels as low as 10 oospores per gram of soil. Both SYBR Green and Taq-Man assays were tested and SYBR Green proved to be more sensitive. The authors concluded that the use of qPCR to detect *A. euteiches* in field soils is fast, accurate, and sensitive to low amounts of the pathogen. qPCR targeting the ITS1 region of *A. euteiches* can allow researchers, agronomists, and farmers to not only identify the pathogen load in the soils but can also lead to greater research in the future surrounding the effects of compounds, microorganisms, and soil properties and their role in either enhancing or suppressing the pathogen in the soil.

qPCR assays to detect *P. medicaginis* in alfalfa were developed using Taq-Man probes (Vandemark and Barker 2003). However, the amounts of pathogen DNA in the alfalfa roots were measured versus the amount of pathogen in the soil. Three different cultivars were used that represented high resistance, resistance, and susceptibility to

Phytophthora root rot in alfalfa. The plants were inoculated, and DNA extracted from the bulked samples of the three cultivars.

DNA was then run through the qPCR assay using primers specific to *P. medicaginis*: forward primer p990F, reverse primer p1050R, and probe p1010CT. DNA from pure pathogen cultures was used in 0.001, 0.01, 0.1, 1.0, 5, 25, 50, and 100 ng concentrations to generate a standard curve. To ensure that the primers only amplified DNA of *P. medicaginis*, DNA was isolated from pure cultures of other oomycete species and used in qPCR to see if other species besides *P. medicaginis* were amplified. The qPCR *P. medicaginis* specific primers did not amplify any other oomycete species besides *P. medicaginis*.

The results indicated that pathogen quantities were significantly different among the three varieties compared. The pathogen quantity was the lowest in the highly resistant cultivar and was the highest in the susceptible cultivar. In addition, the primer pair used was able to detect pathogen amounts at a very low quantity of 0.001 ng meaning it is highly sensitive and accurate. Using qPCR to detect the amounts of the pathogen in the plant can save time as this method is much faster than the typically used hyphal staining technique. Overall, using these primer pairs can allow the identification of the amount of *P. medicaginis* in a targeted sample and can also distinguish between resistant and susceptible cultivars.

Previous studies have revealed that *A. euteiches* can inhibit colonization of *P. medicaginis* in alfalfa roots (Vandemark, Ariss, & Hughes, 2010b). In addition, in previous soil surveys, *A. euteiches* was identified as being more prevalent in the soil than *P. medicaginis* (Munkvold & Carlton, 1995; Vincelli et al., 1994). However, the methods used in both studies relied on the soil baiting technique to identify the prevalence of the two-root rot causing pathogens. These results could potentially be inaccurate because of differences in plant colonization by *A. euteiches* and *P. medicaginis*. DNA based approaches using quantitative PCR may help clarify and confirm that *A. euteiches* is more prevalent than *P. medicaginis* since qPCR uses DNA from the soil and roots and does not rely on pathogen colonization (Gangneux et al., 2014; Vandemark & Barker, 2003).

Primers for PCR assays have been developed for many different *Pythium* spp. and are reviewed by Schroeder et al. (2013). The primers listed in the study include

primers used for end point PCR and qPCR. Among the qPCR list of primers, *Pythium* spp. such as *P. irregulare*, *P. sylvaticum*, and *P. ultimum var. ultimum* are listed. The region targeted by these primers is the ITS region.

In Australia, routine DNA-based testing services for soilborne diseases have been developed so farmers can know the pathogen levels in their soils before planting crops, thus avoiding stand failures or yield loss due to disease (Ophel-Keller, McKay, Hartley, Herdina, & Curran, 2008). The testing services offer DNA based assays to identify the amounts of fungi and nematodes in the soil. It is offered by the South Australian Research and Development Institute (SARDI). Using a quantitative polymerase chain reaction (qPCR) approach, they are able to identify the amounts of targeted pathogens in the soil. From there, the service has correlated the amount of pathogen identified to the amount where infection can occur, and farmers can therefore make management decisions based on the findings.

1.7. Research objectives

Past research has developed resistance to pathogens that cause seedling disease and has identified fungicides that provide some protection to these pathogens, but poor alfalfa seedling establishment is still an issue that face alfalfa producers. In addition, recent research has found more aggressive strains of *Pythium* in Minnesota not controlled by Apron XL or Stamina fungicide seed treatments (Berg et al., 2017). Current research has identified fungicide seed treatments that offer advanced protection to a number of seedling diseases (Samac et al., 2017), but field studies testing those effective fungicide seed treatments to control more aggressive strains of pathogens are needed to provide alfalfa producers with more tools to combat “wet soil syndrome.” Therefore, I plan to investigate the efficacy of EverGol Energy and Intego Solo fungicide seed treatments in a field setting where alfalfa stand establishment has been problematic, and more aggressive pathogen isolates may be present. Results from field trials testing the seed treatments can be used to guide future efforts of seed marketers to add new treatments to their seed to increase the protection against seedling pathogens and allow farmers to increase their yield per acre over the life of their alfalfa stand by decreasing their chances of seedling establishment issues, leading to a higher return on investment.

Current methods for identifying oomycete and fungal pathogens that infect alfalfa seedlings involve using a seedling baiting method (Berg et al., 2017), or isolation from

soil by plating soil dilutions on agar media (Jeffers & Martin, 1986). However, these methods are not highly quantitative and accurate identification of the abundance of pathogens present in the soil community is needed. DNA based sequencing can provide a more accurate inventory of the soil microbial community to identify which pathogens are present in soils of alfalfa fields with poor seedling establishment and identify the abundance of those pathogens present (Bakker et al., 2017). In this research, I plan to use DNA from soil samples of rhizosphere soil and bulk soil, as well as from alfalfa plant roots from locations where poor seedling establishment has occurred in the past to accurately identify the pathogens present in the soil community through oomycete, fungal, and bacterial sequencing, and community analysis. I will use the soil from the field locations where the fungicide seed treatments will be tested. Once pathogens are identified by sequencing, I will inoculate alfalfa plants with the identified pathogen to determine its role in potentially contributing to wet soil syndrome in alfalfa seedlings.

Taxonomists have proposed splitting the genus *Pythium* into four genera, based primarily on sporangium morphology, *Ovatisporangium, Globisporangium, Elongisporangium,* and *Pilasporangium* (Uzuhashi, Tojo, & Kakishima, 2010). For simplicity, I will continue to use the genus *Pythium*, which is most familiar to agronomists, when discussing results of community analysis. However, OTUs were classified using the SINTAX algorithm (Edgar, 2016b) using the reference database described in Bakker et al. (2017) with the proposed genus names, so the OTU species designations in my results will follow the taxonomy in the reference database.

Quantitative PCR assays have been developed to identify the quantity of oomycete pathogens known to cause seedling disease in alfalfa such as *Phytophthora medicaginis* (Vandemark & Barker, 2003), *A. euteiches* (Gangneux et al., 2014), *Pythium irregulare, P. sylvaticum*, and *P. ultimum* (Schroeder et al., 2013). In this research I plan to generate standard curves through qPCR for each of these pathogens using pure cultures of each pathogen. I will then compare the standard curve of the pathogen DNA to qPCR assays run on rhizosphere soil DNA, bulk soil DNA, and root DNA sampled from the field locations where the fungicide seed treatments will be tested that have had poor alfalfa establishment issues in the past, as well as request soils from fields with previous alfalfa stand establishment issues across the Upper Midwest and Great Lakes regions. This will help me to identify which pathogens are the most prevalent across a wide range of soils. I will then seed alfalfa into the soil to correlate the amount of the pathogen identified in qPCR assays to the level of disease in the

bioassays to correlate the level of risk associated with the amount of the pathogen, similar to what was done in the development of the routine qPCR test in Australia (Ophel-Keller et al., 2008).

The objectives of this research are: 1) Test the efficacy of fungicides when used as seed treatments for control of seed rot and damping-off of alfalfa under field and growth chamber conditions; 2) Identify the entire microbial communities (bacteria, fungi, and oomycetes) associated with infected alfalfa seedlings and soil samples from sites with poor alfalfa establishment; and 3) Quantify the abundance of known seed rot and seedling root rot pathogens from sites with poor alfalfa establishment. Overall, the goal of this project is to identify and develop new tools to help alfalfa producers and alfalfa breeders identify the components of poor seedling establishment, manage seedling diseases, and increase yields per acre as a result of the tools.

Chapter 2: Materials and Methods

2.1. Objective #1: Test the efficacy of fungicides when used as seed treatments for control of seed rot and damping-off of alfalfa under field conditions.

2020 Field Trial

Field Trial Design and Locations

Field trials were conducted in Wisconsin from May to September in 2020 and 2021. In 2020, five locations were chosen where stand establishment problems had occurred in the past. Locations were in Beaver, Wisconsin; Emerald, Wisconsin; Marshfield, Wisconsin; Unity, Wisconsin; and West Salem, Wisconsin (Table 2.1). The soil type in each location was a silt loam. In 2020, two cultivars and six different fungicide seed treatments were tested in the listed locations. Cultivars used were Roundup Ready Stratica and HarvXtra MegaTron provided by CROPLAN by WinField United. Stratica has high resistance to Phytophthora root rot and Aphanomyces root rot race 1. MegaTron has high resistance to Phytophthora root rot, Aphanomyces root rot race 1, and Aphanomyces root rot race 2. Both are Roundup Ready and fall dormancy 4 cultivars.

Six fungicide seed treatments applied to cultivars Stratica and MegaTron were tested for efficacy under field conditions at the five above locations. Treatments were as shown in Table 2.2.

Stand Counts and Dry Matter Yield

Stand counts were conducted at the first trifoliate stage and again at the 4 to 6 trifoliate stage to obtain plant counts per linear foot for each cultivar and treatment. Forage harvest was conducted at 60 days after planting and again at first flowering in September. Pesticides were applied throughout the season to manage weeds and insects. After concluding the 2020 field trial it was discovered that the fungicide seed treatments had been incorrectly applied, therefore the seed treatments were invalid and stand count and yield data obtained from the 2020 will not be presented.

2021 Field Trial

Field Trial Locations and Study Design

In 2021, three locations were chosen where stand establishment problems had occurred in the past. Locations were in Frankfort, Wisconsin; Spencer, Wisconsin; and West Salem, Wisconsin. In 2021, two cultivars and five different fungicide seed treatments were tested. Cultivars used were Roundup Ready AmeriStand 415NT RR and HarvXtra MegaTron AA provided by CROPLAN by WinField United and Forage Genetics International. AmeriStand 415NT has high resistance to Phytophthora root rot and Aphanomyces root rot race 1. MegaTron AA has high resistance to Phytophthora root rot, Aphanomyces root rot race 1, and Aphanomyces root rot race 2. Both are Roundup Ready and are fall dormancy 4 cultivars.

The five different seed treatment combinations are shown in Table 2.2. Treatments were applied at the labelled rates. WinField United treated and coated all naked seed in small batch treatments at their River Falls, Wisconsin facility. A total of 500 grams of each cultivar with each seed treatment was provided. Treatment 1 received only rhizobia and a plant growth regulator (Ascend) and was defined as the "base" treatment or application; Treatment 2 received base application and Evergol Energy at 3.0 fl oz/cwt; Treatment 3 received base application, Apron XL at a rate of 0.64 fl oz/cwt, and Stamina at a rate of 1.5 fl oz/cwt; Treatment 4 received the base treatment, Apron XL at a rate of 0.64 fl oz/cwt, Stamina at a rate of 1.5 fl oz/cwt, and Intego Solo at a rate of 0.2 fl oz/cwt; Treatment 5 received the base treatment, Apron XL at a rate of 0.64 fl oz/cwt, Stamina at a rate of 1.5 fl oz/cwt, Intego Solo at a rate of 0.2 fl oz/cwt, and Maxim at a rate of 0.16 fl oz/cwt.

Each of the five seed treatments of each cultivar were replicated seven times at each of the three locations. Each location was planted in a randomized complete block design. The individual plot sizes were 3' x 16' with five rows per plot with a total area size of 54' x 73' at each location. All three trial sites were planted on May 5, 2021. Planting rates were 12 grams of seed per plot. Border rows and alleys were planted with Roundup Ready seed. The Spencer and Frankfort locations were planted using a Wintersteiger planter by the USDA ARS team. The West Salem location was planted using a Carter planter by Forage Genetics International.

Pesticide Applications

Plots were treated with pesticides and fertilizer throughout the growing season. Pesticides were applied at all locations to manage for weeds and insects throughout the

season. The first herbicide application at all locations was made before the second stand count. Applications were done as shown in Table 2.3.

Weather Data

Weather was tracked using the WinField United R7 tool. Each plot was mapped to the exact location in R7 so that weather data was accurate for each location. The West Salem plot had the capacity to be irrigated and was irrigated on 5/13/21 with ¾ inches of water and 6/08/21 with 1 inch of water. The Horst and Frankfort locations did not have the capacity to be irrigated and depended on rainfall only. Total rainfall in inches for each location are shown in Table 2.4.

Stand Counts and Yield

Stand plant counts were conducted twice during the season: once at the first trifoliate stage and again at the 4 to 6 trifoliate stage. In each plot, plants were counted in three random 12-inch sections within the three center rows to obtain plant counts per linear foot for each cultivar and treatment.

First harvest took place at 67 days after planting at all locations. The second harvest took place 35 days after first harvest at the Spencer and Frankfort locations. At the West Salem location, the second harvest took place 37 days after first harvest. Cutting height was 3-4 inches from ground level.

Fresh weight was measured for each plot. A subsample was taken for each plot and placed into a 140 °F forced air drying oven for 7 days. Subsamples were weighed and dry matter conversions were calculated and used to obtain dry matter yields for each plot.

Growth Chamber Standard Tests and Soil Bioassays

The protection provided by the 2021 seed treatments was tested with standard methods for evaluating resistance to *Aphanomyces euteiches, Phytophthora medicaginis,* and *Pythium* spp. (Altier et al., 1995; Fitzpatrick et al., 1998; Nygaard et al., 1995). Three replications of each cultivar x treatment were planted in randomized complete block design: Ameristand treatment 1 to treatment 5 and MegaTron AA treatment 1 to treatment 5, and three to four replications of check cultivars Agate, Saranac, WAPH-1, and WAPH-5. Saranac is the susceptible check for both the Aphanomyces root rot and Phytophthora root rot tests. Agate is resistant to

Phytophthora root rot. WAPH-1 is highly resistant to Phytophthora root rot and Aphanomyces root rot race 1. WAPH-5 is highly resistant to Phytophthora root rot and Aphanomyces root rot races 1 and 2. None of the check cultivars used have genetic resistance to *Pythium*. All check varieties were untreated seed.

To plant the standard tests, 48-cell plastic flat inserts were placed into plastic 1020 trays (10.94" W x 21.44" L x 2.44" D) and each cell was filled with 50 mL of medium grade vermiculite. The vermiculite was leveled and 25 seeds were added by scattering evenly on top of the vermiculite. Then, 10 mL of germination mix (Sungrow) was added on top of the seed. The flats were watered lightly immediately following planting and placed in a growth chamber where temperatures were kept at 25 °C/18 °C (day/night) with a 16 h photoperiod.

One week following planting, trays were inoculated with *A. euteiches* or *P. medicaginis*, depending on the test conducted. For *A. euteiches*, the standard test protocol was followed (Fitzpatrick et al., 1998). The race 1 test inoculation rate was 400 zoospores per plant and the MF-1 isolate was used. For the race 2 test, inoculation varied depending on isolate used. For the MER4 race 2 isolate, the inoculation rate was 200 zoospores per plant. For the NC1 race 2 isolate, the inoculation rate was 400 zoospores per plant. Trays were then flooded for five days following inoculation to encourage zoospore infection. Plants were rated for disease symptoms 14 days after inoculation on a 1 to 5 scale.

For the *P. medicaginis* pathogen standard test, seedlings were inoculated 7-10 days following planting. The standard test protocol was followed on inoculation rate, flooding, and scoring (Nygaard et al., 1995). Briefly, a 7-day-old agar plate culture of *P. medicaginis* was homogenized in 1 L of water, then 10 ml of homogenate was added to each cell with 25 seedlings. Soil was flooded for 5 days then plants rated 14 days after inoculation on a 1 to 5 scale.

To evaluate the effectiveness of the seed treatments against *Pythium* spp., the agar plate standard test of Pythium seed rot and damping-off resistance was conducted (Altier et al., 1995). *Pythium* species used were *P. irregulare* isolate Bec56, *P. ultimum* var*. ultimum* isolate Was53, and *P. paroecandrum* isolate L3. Agar plugs of each strain were placed in the center of a 1.5% water agar plate and cultured at 25°C. After 3 days, 25 seeds were then placed on the surface of the mycelium and plates incubated in a lighted incubator with a 16 h photoperiod at 18°C. Seedlings were rated for disease

symptoms after 5 days on a 1 to 5 scale: 1 (resistant) = healthy seedling, root free of necrosis; 2 (resistant) = infected seedling, primary root tip necrotic but firm; 3 (moderately susceptible) = infected seedling, primary root tip soft and rotted; 4 (susceptible) = dead seedling, germinated seed with emerged radicle rotted; and 5 (susceptible) = dead seed, ungerminated and rotted.

To further test the seed treatments, all treatments on the two cultivars and the checks were tested in soil bioassays in growth chambers with field soil. To plant the bioassays, 48-cell inserts were placed into 1020 trays and each cell was filled with 50 mL of each bulk soil from the 2020 and 2021 field locations. Soil was leveled and 25 seeds were added by scattering evenly on top of the soil in a randomized complete block design with three replications of each cultivar x treatment. Then, 10 mL of germination mix was added on top of the seed. The flats were then watered lightly immediately following planting and placed in a growth chamber where temperatures were kept at 25 °C/18 °C (day/night) with a 16 h photoperiod. One week following planting, trays were flooded to encourage oospore germination and infection from oomycete pathogens. Trays were flooded for a total of three days. Plants were scored for disease symptoms 21-days after planting.

Soils were obtained from alfalfa fields in Illinois, Indiana, Michigan, Minnesota, New York, Ohio, South Dakota, and additional sites from Wisconsin. Seed used in these bioassays were check cultivars and MegaTron AA treatments #1-5. Bioassays were conducted as detailed above. Information on previous crop and crop rotation history, county, alfalfa establishment history, years of established alfalfa, herbicides used (if applicable), were requested. All bioassays grown had no chance of herbicide carryover in the soil.

The plants were scored on a 1 to 5 scale: 1=no necrosis of roots or hypocotyl; 2=slight necrosis of roots or hypocotyl; 3=necrosis of roots and lower hypocotyl and moderate stunting of stems; 4=extensive necrosis of roots, hypocotyls, and cotyledons, and severe stunting of stem; 5=dead seedling (Fitzpatrick et al., 1998; Nygaard et al., 1995). Scores of 1 and 2 are healthy plants and scores of 3 to 5 indicate diseased plants. Attention was given to the standard check cultivars to determine which root rot was most prevalent. For example, if Saranac had 0-5% resistant plants and Agate had 25-40% resistant plants, this indicated the presence of Phytophthora root rot. If both Saranac and Agate had 0-5% resistant plants but WAPH-1 and WAPH-5 had 50-60% resistant plants, then this would indicate the presence of Aphanomyces root rot race 1. If

WAPH-1 had 0-5% resistant plants along with Agate and Saranac but WAPH-5 had 50-60% resistant plants, this would indicate the presence of Aphanomyces root rot race 2. If all of the four check cultivars are susceptible, this would indicate the presence of *Pythium, Rhizoctonia solani, Fusarium,* or another pathogen that had previously gone unrecognized in causing damage to alfalfa seedlings.

Phytophthora sansomeana **Pathogenicity Tests**

Phytophthora sansomeana was analyzed for pathogenicity on alfalfa using two assays. First, the standard test protocol that is used for *P. medicaginis* was used to determine if *P. sansomeana* caused either damping-off or root rot in alfalfa seedlings. Isolates used were isolated from corn plants in Illinois and obtained from Dr. Dean Malvick, University of Minnesota. Three replications of all standard check cultivars were used in the assay along with MegaTron AA treatments 3 and 5 and Ameristand with treatment 1. At 7 days after planting, half of a culture plate of each isolate was homogenized in 1 L of water and 10 mL added to each cell of 25 seedlings. The flats were then flooded for 5 days and were kept at 25 °C/18 °C (day/night) with a 16 h photoperiod. Plants were scored for disease symptoms 21-days after planting.

The second assay to determine the pathogenicity of *P. sansomeana* was conducted using soil infested with the two *P. sansomeana* isolates. To infest soil, 2,000 grams of synthetic soil was mixed with ¼ of a culture plate of each isolate that was ground with 250 mL ddH2O. Three replications of all standard check cultivars were used in the assay along with MegaTron AA treatments 3 and 5 and Ameristand with treatment 1. To plant the bioassays, 48-cell inserts were placed into 1020 trays and each cell was filled with 50 mL of the *P. sansomeana* infested soil mixture. Soil was leveled and 25 seeds were added by scattering evenly on top of the soil in a randomized complete block design. Then, 10 mL of non-inoculated germination mix was added on top of the seed. The flats were then watered lightly immediately following planting and placed in the growth chamber where temperatures were kept at 25 °C/18 °C (day/night) with a 16 h photoperiod. At the first trifoliate stage, trays were flooded to encourage zoospore formation and infection. Trays were flooded for a total of five days. Plants were scored for disease symptoms 26-days after planting.

Statistical Analyses: Field Trial, Bioassays, and Standard Pathogen Assays

Statistical analyses were conducted to examine the differences in cultivar, treatment, location, and cultivar by treatment for stand counts, dry matter tons per acre yield, and percent resistant plants in the bioassays and standard pathogen tests using R version 4.1.2 (R Core Team, 2021). Packages used were: tidyverse, shiny, agricolae, plotly, shinyWidgets, leaflet, broom, and kableExtra. All three 2021 locations were statistically analyzed for significant differences in stand counts and yield. Both 2020 and 2021 field trial location bioassay results were statistically analyzed, as well as the standard pathogen test results for *A. euteiches* race 1 and race 2, *P. medicaginis*, and *Pythium irregulare*, *P. ultimum* var*. ultimum,* and *P. sylvaticum*.

Mean analyses were compared for four different categories for stand count, yield, and bioassay results: 1) all locations, cultivars, and treatments; 2) split by location; 3) split by location and pooled across cultivars; 4) split by location and cultivar. For the standard pathogen tests, mean analyses were compared for the following categories: 1) across all isolates and cultivars; 2) by isolate, across all cultivars; 3) by cultivar, across all isolates; and 4) by cultivar and isolate. Among each of these analyses, one-way ANOVAs were conducted, and significant statistical differences were determined between each of the five different treatments using least significant difference test for each analysis. Significance was determined at $p \leq 0.05$ or $p \leq 0.10$.

In the field trial and bioassay statistical analyses, for all locations, cultivars, and treatments mean analysis, all means were analyzed across all replicates that contained both cultivars and all five treatments. Split by location analysis only included locations that included two or more complete replicates. In the split by location and pooled across cultivars analysis, only locations that included two or more complete replicates were analyzed. In the split by location and pooled across cultivar analysis, the cultivar effects are pooled (i.e., cultivar is not a source of variation in the ANOVA model). In the split by location and cultivar analysis, only locations that included two or more complete replicates were included. In these analyses, the ANOVA was run separately for each cultivar.

2.2. Objective #2: Identify the microbial communities (oomycetes, fungi, and bacteria) associated with alfalfa seedlings and soil samples from sites with poor alfalfa establishment.

The entire microbial community (oomycetes, fungi, and bacteria) associated with alfalfa seedlings were identified from root and soil samples from each of the untreated

control plot at each location. In 2020, soil samples and root samples were taken from each of the five sites after the first cut. Samples were dug from the control plots (treatment 1 as described in Table 2.1) in the outer two rows for Stratica and MegaTron replicates. Three “core” samples at 6-8 inches deep and about 5 x 5 inches wide from each cultivar were gently taken using a tiling shovel. Total sample number was 24 samples per each location.

Each core sample was enclosed in a plastic bag and placed on ice for transport to the lab. The bulk soil of each core was shaken off onto clean bench paper. Bulk soil clumps were broken up, and 50 mL of bulk soil was placed into a sterile 50 mL Nalgene tube and frozen at -80 °C. The remaining bulk soil was placed into a ziplock bag and stored at 4 °C.

For rhizosphere soil collection, the alfalfa roots of each core were tapped onto a fine screen over the clean bench paper to remove the soil closest to the roots. The rhizosphere soil was placed into a sterile 15 mL Nalgene tube and stored at -80 °C. Both the bulk soil and rhizosphere soil collected in 2020 was used for DNA extractions.

Plant roots were washed and blotted dry. Roots were placed into 15 mL sterile Nalgene tubes and stored at -80 °C.

In 2021, soil samples and root samples were taken from each of the three sites after the first cut. Samples were dug from the control plots (treatment 1) in the outer two rows for both Ameristand and MegaTron AA replicates. Three “core” samples per each control plot for each variety were gently taken using a tiling shovel as described above. For each variety and treatment there are seven replications with a total of 42 samples per location. Each core was processed to obtain bulk and rhizosphere soil and washed roots as described above.

DNA Extractions

From each plot, 0.33 g of rhizosphere sample from each of the three cores was mixed together to form one bulk rhizosphere sample per plot. From this bulk sample, a 0.25-gram subsample of soil was added to the PowerBead Tube of the Qiagen DNAeasy PowerSoil kit (Qiagen Sciences, Germantown, MD). For root DNA extractions, two roots from each core sample were homogenized into a fine powder using liquid nitrogen and a mortar and pestle and combined to form a bulk sample. DNA was extracted from 100 mg of the bulk sample using the Qiagen DNeasy Plant Pro kit.

For bulk soil DNA extractions from 2020 plot samples, bulk soil samples were pooled together representatively from each core (3.333 grams from each core) so that 10 grams total was mixed and used for extractions. In 2021, since bulk soil cores were combined into one tube on the day of processing, 10 grams was weighed from each core sample tube for each plot. Total DNA was extracted from alfalfa field soil samples using 10 g of soil. For bulk soil DNA extractions, the Qiagen DNeasy PowerMax Soil kit was used according to the manufacturer's protocol (Qiagen Sciences, Germantown, MD). DNA was quantified, and quality assessed using a Nanodrop2000 spectrophotometer.

Amplicon Sequencing

After DNA extractions, rhizosphere soil DNA and root DNA were used for amplicon sequencing to identify the oomycete, fungal, and bacterial communities in the endosphere (root) and rhizosphere at each plot location for both 2020 and 2021. For oomycete sequencing, DNA was first amplified using primers ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS7a.e. (5'-WGYGKTCTTCATCGATGTGC-3') (Taheri et al., 2017a). These primers amplify a 350 bp fragment of the internal transcribed spacer (ITS) region between the 18S and 5.8S ribosomal DNA subunits. Each reaction consisted of 12.5 ul 2xGoTaq Master Mix (Promega, Madison, WI), 0.25 ul forward primer, 0.25 ul reverse primer, 7 ul purified water, and 5 ul of rhizosphere soil or root DNA. The cycling protocol was based on that of Hossain et al. (2021) with denaturation at 94 °C for 3 minutes followed by 3 cycles of 94 °C for 1 minute, 64 °C for 30 seconds, and 70 °C for 40 seconds. These cycles were then followed by 34 cycles of 94 °C for 1 minute, 52 °C for 30 seconds, and 70 °C for 40 seconds. Finally, the run was completed with a 10-minute final extension at 72 °C.

PCR products were purified using the Qiagen QIAquick PCR Purification Kit/QIAquick PCR & Gel Cleanup Kit following the manufacturer's protocol. Purified products were quantified using a Nanodrop2000. If necessary, samples were diluted with sterile water so that all samples were 1-100 ng DNA/ul.

The purified PCR amplicons for each root and rhizosphere sample were submitted to the University of Minnesota Genomics Center (UMGC) for amplicon sequencing using the Illumina MiSeq platform. Run/read length was MiSeq 2x300 bp

1/8th lane-stowaway. Two sequencing runs took place. This process was identical for both 2020 and 2021 submissions.

Total DNA rhizosphere and roots were submitted for fungal and bacterial amplicon sequencing. For both root and rhizosphere soil DNA, 20 ul of pure DNA was submitted. Concentrations ranged from 1 ng/ul to 100 ng/ul. Any sample over 100 ng/ul was diluted 1:1 by using 15 ul of DNA and 15 ul water. For fungal sequencing, ITS1 primers were used. For bacterial sequencing, 16S V3-V4 primers were used. Run/read lengths for both fungal and bacterial sequencing were MiSeq 2x300 full run. Two sequencing runs took place. Total sample number for both runs were 80 in 2020 and 84 in 2021.

Data Processing of Oomycete Amplicons

Amplicon sequencing results were processed using the UPARSE pipeline with the usearch software (version 11; Edgar, 2013). Briefly, sequences corresponding to forward and reverse primers and low-quality ends (20 and 50 bases from forward and reverse reads, respectively) were trimmed prior to pairing reads (maxdiffs=5). High-quality reads (maximum expected error=1, minimum length=230) were generated and denoised using the unoise3 algorithm (Edgar, 2016a) to produce zero-sum operational taxonomic units (OTUs). Paired reads were mapped to OTU representatives at 97% similarity to produce an OTU table. OTUs were classified using the SINTAX algorithm (Edgar, 2016b) using the oomycete reference database described in Bakker et al. (2017) supplemented with a *Medicago sativa* ITS sequence (NCBI accession KY968953.1) to capture plant-derived sequences.

Statistical Analyses: Oomycetes, Fungi, and Bacteria

Data were imported to R version 4.1.2 (R Core Team, 2021). Packages used in analyses were: vegan, ggplot2, plyr, grid, Hmisc, Rmisc, pheatmap, compositions, ecodist, labdsv, cowplot, ggpubr, gridExtra, reshape2, and RColorBrewer. Packages were also used from Bioconductor: BiocManager, DESeq2, phyloseq, ANCOMBC, and microbiome. Only OTUs identified as Stramenopiles were used for oomycete analyses. All samples were rarefied to a depth of 8,000 sequences. After rarefication, three samples were discarded. For fungi, only OTUs identified to a fungal phylum were used for analyses. All samples were rarefied to a depth of 10,000 sequences.

After rarefication, alpha diversity measures were calculated. Measures calculated were: Shannon diversity, inverse Simpsons diversity, species richness, and Pielou's evenness. Non-metric multidimensional scaling (NMDS) (k=2) was conducted using the Bray-Curtis dissimilarity calculation. The significance of location and compartment in structuring communities was calculated using permutational multivariate analysis of variance (PERMANOVA). Beta dispersion among compartments was then evaluated by ANOVA for fungi.

Following these analyses, individual NMDS plots were compiled for both types of samples: endosphere and rhizosphere following the Bray-Curtis dissimilarity calculation. The significance of location in structuring communities was also conducted using PERMANOVA. After these analyses, species-level bar plots were compiled for the endosphere and rhizosphere to represent the most abundant OTUs (>0.3%) for each sample type at each of the eight locations. Abundances were converted to proportions and the ggplot2 package in R was used to create the bar plots. Heat maps were also created which showed the top 30 most abundant taxa for each sample type and location. This was created using the pheatmap package in R.

For oomycetes, using the DESeq2 package from BioConductor, differential abundance was performed. First, low-abundance reads (< 20 counts or found in < 3 samples) were removed from analysis prior to running the DESeq2 algorithm with a local dispersion fit using the model ~Location + Variety. Wald tests were used to contrast varieties with different resistances to *Aphanomyces* races. After that, a table of differentially abundant OTUs was created and significant OTUs were displayed. Normalized counts for *Aphanomyces euteiches* OTU480 was plotted. Differential abundance using ANCOMBC method was then performed to confirm DESeq2 results.

Phytophthora sansomeana **DNA Extraction and Sequencing**

The ITS1 rDNA sequence of the two isolates used in the *P. sansomeana* pathogenicity assays was determined to verify identification of the isolates. DNA was extracted from mycelium grown on a potato dextrose agar plate using the MP Bio FAST DNA extraction kit following the manufacturer's instructions. DNA was amplified using the oomycete community primers with the same protocol used for root and rhizosphere oomycete amplification. The PCR products were purified using the Qiagen QIAquick PCR Purification Kit/QIAquick PCR & Gel Cleanup Kit following the manufacturer's

protocol. Purified products were sequenced in both directions by the University of Minnesota Genomics Center with the forward (ITS6) primer and reverse (ITS7ae) primers.

2.3. Objective #3: Quantify the abundance of known seed rot and seedling root rot pathogens from sites with poor alfalfa establishment.

Pure Culture DNA Extractions for Standard Curve Generation

To generate a standard curve for the qPCR assays for the known pathogens, DNA was extracted from pure cultures of *A. euteiches, P. medicaginis, Pythium irregulare, P. ultimum* var*. ultimum*, and *P. sylvaticum* using the MP-Bio FAST DNA kit according to the manufacturer's protocol. DNA samples were quantified using a Nanodrop2000. Serial 10-fold dilutions were made from each pure pathogen DNA sample for standard curve generation from 10 ng/ul to 0.0001 ng/ul.

Quantitative Polymerase Chain Reactions (qPCR)

DNA in soil and root samples of *A. euteiches*, *P. medicaginis, Pythium irregulare, P. sylvaticum* and *P. ultimum* var. *ultimum* was measured by quantitative PCR assays adapted from Vandemark et al. (2002) using SYBR Green detection and species-specific primers (Table 2.5). The target region for all primers is the internal transcribed spacer (ITS). Each 25 ul PCR reaction consisted of SYBR Green Supermix with ROX (BioRad Laboratories, Hercules, CA), 0.25 ng/ul of species-specific forward and reverse primers, and 5 ul of bulk soil DNA or 2 ul root or rhizosphere DNA. All reactions were run in triplicate using an ABI 7500/7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min. A melting curve analysis was conducted according to the ABI software. A linear relationship existed between Ct values and DNA concentrations of the standard curve. Concentrations of pathogen DNA in soil or root samples were determined by comparing the Ct values to that of the standard curves.

Table 2.1. Locations of field trials in 2020 and 2021 and soil types.

Year	Location	Soil Type	Percent of AOI		Soil Type	Percent of AOI	Soil Type	Percent of AOI
2020	Beaver, WI	Loyal silt loam	43.4		Marshfield silt loam	1.2	Withee silt loam	55.4
2020	Emerald, WI	Amery silt loam	4.1		Freeon silt loam	71.5	Magnor silt loam	24.3
2020	Marshfield, WI	Withee silt loam	100					
2020	Unity, WI	Loyal silt loam	21.1		Marshfield silt loam	7.3	Withee silt loam	71.5
2020	West Salem, WI	Seaton silt loam	73.3		Toddsville silt loam	26.7		
2021	Frankfort, WI	Loyal silt loam	83.3		Marshfield silt loam	2.6	Withee silt loam	14.1
2021	Spencer, WI	Loyal silt loam	80.3		Marshfield silt loam	0.2	Withee silt loam	19.6
2021	West Salem, WI	Seaton silt loam	73.3		Toddsville silt loam	26.7		

Soil information from USDA Natural Resources Conservation Services: SSURGO Database

Table 2.2. A) Seed treatments used in 2020 field trials. **B)** Seed treatments used in 2021 field trials.

A

Treatment	Components
1	Untreated Control (Nitragen rhizobia, Zn, Mn)
2	Penflufen + Prothioconazole + Metalaxyl (EverGol Energy)
3	Mefenoxam + Pyraclostrobin (Apron XL + Stamina)
4	Treatment 3 + Ethaboxam (Treatment 3 + Intego Solo)
5	Treatment 3 + Ethaboxam + Fludioxonil (Treatment 3 + Intego Solo + Maxim)
6	Treatment 3 + WinField United proprietary experimental biological treatment

B

Treatment	Components
1	Untreated Control (Nitragen rhizobia, Zn, Mn)
2	Penflufen + Prothioconazole + Metalaxyl (EverGol Energy)
3	Mefenoxam + Pyraclostrobin (Apron XL + Stamina)
4	Treatment 3 + Ethaboxam (Treatment 3 + Intego Solo)
5	Treatment 3 + Ethaboxam + Fludioxonil (Treatment 3 + Intego Solo + Maxim)

Table 2.3. Timing and rates of herbicides, insecticides, and fertilizers in the 2021 field trial.

Location	Glyphosate Application	Rate	Insecticide Application	Rate	Fertilizer Application	Rate
Frankfort, WI						
1st application	6/8/2021	44 oz/acre	6/8/2021	2 oz/acre	6/8/2021	20 oz/acre
2nd application	7/18/2021	44 oz/acre	7/18/2021	2 oz/acre	7/18/2021	20 oz/acre
3rd application			7/31/2021	1.9 oz/acre		
Spencer, WI						
1st application	6/8/2021	44 oz/acre	6/8/2021	2 oz/acre	6/8/2021	20 oz/acre
2nd application	7/18/2021	44 oz/acre	7/18/2021	2 oz/acre	7/18/2021	20 oz/acre
3rd application			7/31/2021	1.9 oz/acre		
West Salem, WI						
1st application	5/30/2021	44 oz/acre	6/12/2021	2 oz/acre	5/30/2021	20 oz/acre
2nd application			7/28/2021	1.9 oz/acre	7/14/2021	214 lbs/acre
3rd application						
***Note:** all pesticide applications were tank mixed with an adjuvant to reduce drift and increase canopy penetration.*						
Adjuvants used: InterLock and MasterLock						
Fertilizers used: Ammonium sulfate on all plots for 1st application and on Frankfort and Spencer for 2nd application; 5-0-34-15S-0.5B used at West Salem for 2nd application						
Insecticides used: Baythroid XL (first application for all plots; second application for Frankfort and Spencer), Warrior II (second and third application for West Salem; third application for Frankfort and Spencer)						
1st applications took place before 1st harvest						
2nd applications took place after 1st harvest and before 2nd harvest						

Table 2.4. Rainfall totals throughout the 2021 field trial at each location.

LOCATION	Total Rainfall (in.): Planting to Final Harvest	Total Rainfall (in.): Before 1st Stand Count	Total Rainfall (in.): Before 2nd Stand Count	Total Rainfall (in.): Before 1st Harvest	Total Rainfall (in.): After 1st Harvest
Frankfort, WI	21.757	2.708	5.728	11.655	10.102
Spencer, WI	17.702	2.291	4.503	10.38	7.322
West Salem, WI	23.433	1.876	3.275	11.956	11.487
	*Calculated by taking sum of rainfall from planting to final harvest	*Calculated by taking sum of rainfall total for all days before 1st count	*Calculated by taking sum of rainfall total for all days before 2nd count	*Calculated by taking sum of rainfall total for all days before 1st cut	*Calculated by taking sum of total rainfall after 1st cut and before 2nd cut

Table 2.5. Primers used in qPCR reactions.

Target Species	Target DNA	Primers (5' to 3')	Reference
Aphanomyces euteiches	ITS1	Ae_ITS1_39F [5′- TGAGGCTTGTGCTCTTTTCA-3′]	(Gangneux et al., 2014)
		Ae_ITS1_167R [5′-GA AAGTTGTATAGAATTGACAAGCT-3′]	
Phytophthora medicaginis	ITS	p990F [5′- d-GGTGGGTGGAACGAAGGA-3′]	(Vandemark & Barker, 2003)
		p1050R [5′-d-TGGCAGCGGAGATCCAA-3′]	
Pythium irregulare grps I and II	ITS	PiF [5′-GTAGCATGCGTGTTTGCTTA-3′	(Kernaghan, Reeleder, & Hoke, 2008)
		PiR [5′-GCAAGCTGTGCATTCATTGC-3′]	
Pythium sylvaticum	ITS	SYL1F [5′-GTGTCTCGCTGTGGTTGGTATATTTG-3′]	(Schroeder, Okubara, Tambong, Lévesque, & Paulitz, 2006)
		SYL2R [5′-CTTCTGCCAATTGCACAAGTGC-3′]	
Pythium ultimum var. *ultimum*	ITS	ULT1F [5′-GACACTGGAACGGGAGTCAGC-3′]	(Schroeder et al., 2006)
		ULT4R [5′-AAAGGACTCGACAGATTCTCGATC-3′]	

Chapter 3: Results

3.1. Objective #1: Test the efficacy of fungicides when used as seed treatments for control of seed rot and damping-off of alfalfa under field conditions.

Results of 2021 Field Trial

Two alfalfa cultivars, treated with four different fungicide combinations and a control without treatment were established in three locations in Wisconsin. Rainfall was below average in May, therefore, there was likely limited pathogen infection early in the season (Table 3.1). First stand counts were conducted at the first trifoliate stage and second stand counts were conducted at the 4-6 trifoliate stage. First harvest was conducted 60 days after planting and second harvest was conducted at first flowering in September. Differences in stand counts and forage yield were found for some fungicide treatments at some locations.

Stand Counts

Treatments had no effect on the first stand counts ($p>0.10$) at the first trifoliate stage, but effects were observed in the second stand counts, at the 4 to 6 trifoliate stages over all locations and cultivars (Fig. 3.1). Differences in the second stand counts were between treatment 1, the untreated check, and treatment 5, the Apron XL, Stamina, Intego Solo, and Maxim combination ($p=0.067$) (Fig. 3.1A). The second stand count mean for treatment 1 combined across all locations and cultivars was 31 plants per linear foot. The second stand count mean for treatment 5 combined across all locations and varieties was 36 plants per linear foot. No significant differences were observed between the control and treatments 2, 3, and 4 ($p>0.10$).

When separated by location, no significant differences in plant numbers were observed for first or second stand count at the Frankfort, WI location ($p>0.10$) over both cultivars (Table 3.2). The largest differences observed were between treatment 1 and treatment 2. The treatment 1 stand count was 35 plants per linear foot and treatment 2 stand count was 31 plants per linear foot. Like the first stand count, no significant differences were found among treatments in the second stand count over both cultivars at the Frankfort location ($p>0.10$). Treatments 1 and 2 stand counts were both 30 plants per linear foot and treatments 3, 4, and 5 stand counts were all 31 plants per linear foot, respectively.

When separated by location and cultivar, no significant differences in plant numbers were observed for first or second stand count at the Frankfort location ($p>0.10$). In the first stand count, largest differences were between Ameristand treatment 1 and Ameristand treatment 2 and 3. Ameristand treatment 1 stand count was 38 plants per linear foot and treatments 2 and 3 were 32 plants per linear foot. Largest differences in MegaTron AA were between treatments 3 and 2, 4, and 5. Treatment 3 stand count was 33 plants per linear foot and treatments 2, 4, and 5 stand counts were 30 plants per linear foot. In the second stand count at the Frankfort location, the largest differences for Ameristand were between treatments 4 and 5 and treatment 2. Treatment 4 and 5 plants per linear foot were 32. Treatment 2 stand count was 28 plants per linear foot. In MegaTron AA the largest differences were observed between treatment 2 and treatments 1 and 3. Treatment 2 stand count was 33 plants per linear foot. Treatment 1 and 3 stand count values were 29 plants per linear foot.

At the West Salem location when pooled over both cultivars, no significant differences in plant numbers were observed in first or second stand count among the treatments ($p>0.10$). The largest differences in the first stand count were between treatment 4 and treatment 2. Treatment 4 stand count was 43 plants per linear foot. Treatment 2 stand count was 40 plants per linear foot. For second stand count, the largest differences were observed between treatment 5 and treatments 1 and 3. Treatment 5 stand count was 45 plants per linear foot. Treatments 1 and 3 stand counts were 39 plants per square foot.

When separated by location and cultivar, there were no significant differences in plant numbers in first and second stand counts at the West Salem location ($p>0.10$). In the first stand count, the largest differences in Ameristand were between treatment 2 and treatments 3 and 4. Treatment 2 stand count was 46 plants per linear foot. Treatments 3 and 4 stand counts were 44 plants per linear foot. In the MegaTron AA cultivar, the largest differences were observed between treatment 4 and treatment 2. Treatment 4 plants per linear foot were 42. Treatment 2 plants per linear foot were 35. In the second stand count, the largest differences in Ameristand were between treatment 5 and treatment 3. Treatment 5 stand count was 45 plants per linear foot. Treatment 3 stand count was 38 plants per linear foot. In the MegaTron AA cultivar, largest differences were observed between treatment 5 and treatments 1 and 2. Treatment 5

stand count was 44 plants per linear foot. Treatment 1 and 2 stand counts were 38 plants per linear foot.

When separated by location, no significant differences in plant numbers were observed for the first stand count at the Spencer, WI location ($p>0.10$). The largest differences were observed between treatment 1 and treatments 2 and 4. The treatment 1 stand count was 26 plants per linear foot. Treatment 2 count was 30 plants per linear foot and treatment 4 stand count was 30 plants per linear foot. Significant differences were not observed for the second stand count at the Spencer, WI location but largest differences were between treatment 1 and treatments 3 and 5 ($p=0.112$). Treatment 1 count was 25 plants per square foot. Treatment 3 count was 30 plants per square foot and treatment 5 count was 32 plants per square foot.

When separated by cultivar and location, statistical differences were not observed for the first stand count ($p=0.182$) or second stand count ($p =0.154$) at the Spencer, WI location for Ameristand. In the first stand count, the largest differences were observed between treatment 1 and treatments 2 and 5. In the second stand count, differences were observed between treatments 1 and treatments 3 and 5.

Overall, there were few statistical differences in treatments observed in stand counts. The results show that seed treatments resulted in small but non-significant changes in stand establishment due to differences in stand count densities in the treated plots as opposed to the untreated plots. There was a trend for the increased stand with seed treatments with Ameristand, the cultivar lacking resistance to Aphanomyces race 2.

Dry Matter Yield

Significant statistical differences were not observed in the first harvest yield or second harvest yield across all locations and cultivars combined ($p>0.10$). The largest differences in first harvest yield across all locations and cultivars were between treatment 5 and treatment 2. Treatment 5 mean yield was 1.512 dry matter tons per acre and treatment 2 mean yield was 1.441 dry matter tons per acre. The largest differences in the second harvest yield were between treatment 1, untreated check, and treatment 5, the Apron XL, Stamina, Intego Solo, and Maxim combination. Second harvest mean yield for treatment 1 combined across all locations and varieties was 1.268 dry matter tons per acre. Second harvest mean yield for treatment 5 combined across all locations

and varieties was 1.347 dry matter tons per acre. No differences were observed between treatments 2, 3, and 4.

When split by location and pooled over both cultivars, no statistical differences were observed for first and second harvest yield at the Frankfort, WI location ($p>0.10$). The largest differences in yield for first harvest were observed between treatment 3 and treatment 4. Treatment 3 yield was 1.326 dry matter tons per acre and treatment 4 yield was 1.283 dry matter tons per acre. The largest differences in yield for second harvest were observed between treatment 1 and treatments 3 and 5. Treatment 1 yield was 1.141 dry matter tons per acre. Treatment 3 yield was 1.285 dry matter tons per acre and treatment 5 yield was 1.262 dry matter tons per acre.

When split by location and cultivar, significant differences were not observed among treatments for either the first or second harvests at the Frankfort, WI location ($p>0.10$). At first harvest, the largest differences in Ameristand were observed between treatment 5 and treatment 2. Treatment 5 yield was 1.353 dry matter tons per acre and treatment 2 yield was 1.229 dry matter tons per acre. In the MegaTron AA cultivar, largest differences in first cutting yield were observed between treatment 1 and treatment 5. Treatment 1 yield was 1.383 dry matter tons per acre and treatment 5 yield was 1.294 dry matter tons per acre. Second harvest largest yield differences at the Frankfort, WI location in Ameristand were observed between treatments 3 and 1. Treatment 3 yield was 1.306 dry matter tons per acre and treatment 1 yield was 1.126 dry matter tons per acre. In MegaTron AA, the largest differences were observed between treatment 1 and treatment 3. Treatment 1 yield was 1.156 dry matter tons per acre and treatment 3 yield was 1.264 dry matter tons per acre.

At the West Salem, WI location and pooled across both cultivars, statistical differences were not observed for the first or second harvest yield ($p>0.10$). The largest differences in first harvest yield were between treatment 5 and treatment 2. Treatment 5 yield was 2.120 dry matter tons per acre and treatment 2 yield was 1.991. Largest differences in second harvest yield were between treatment 1 and 3 and treatment 5. Treatment 1 yield was 1.423 dry matter tons per acre. Treatment 3 yield was 1.438 dry matter tons per acre and treatment 5 yield was 1.538 dry matter tons per acre.

When cultivars were not pooled, and treatments were analyzed by location and by cultivar, statistical differences were not observed for first harvest in both cultivars at

the West Salem, WI location ($p>0.10$). For second harvest, differences were not observed in Ameristand but were observed for MegaTron AA at the West Salem, WI location ($p=0.043$). The largest differences in first harvest yield at West Salem, WI were observed between treatments 5 and 2 in the Ameristand cultivar. Treatment 5 yielded 2.124 dry matter tons per acre and treatment 2 yielded 1.90 dry matter tons per acre. Largest yield differences were observed between treatments 5 and 3 in the MegaTron AA cultivar. In the MegaTron AA cultivar, treatment 5 yielded 2.116 dry matter tons per acre and treatment 3 yielded 1.950 dry matter tons per acre. Significant differences were observed at the West Salem location in second harvest yield among the MegaTron AA cultivar but not among the Ameristand cultivar. Second harvest largest yield differences in the Ameristand cultivar at West Salem were observed between treatments 5 and 2. Treatment 5 yielded 2.547 dry matter tons per acre and treatment 2 yielded 1.40 dry matter tons per acre. Significant yield differences were observed between treatments 1 and 5 as well as between treatment 3 and all other treatments in the MegaTron AA cultivar ($p=0.043$) (Fig. 3.1). In the MegaTron AA cultivar, treatment 1 yielded 1.400 dry matter tons per acre and treatment 5 yielded significantly higher at 1.527 dry matter tons per acre. In addition, treatment 3 yielded significantly lower than all other treatments at 1.380 dry matter tons per acre.

At the Spencer, WI location when pooled over both cultivars, no significant differences were observed between the treatments for both first and second harvest yield ($p>0.10$). The largest difference in first harvest was between treatment 4 and treatment 2. Treatment 4 yield was 1.111 dry matter tons per acre. Treatment 2 yield was 1.035 dry matter tons per acre. For the second harvest, the largest difference was observed between treatments 1, 2, and 5 and treatment 4. Treatments 1, 2, and 5 all yielded 1.240 dry matter tons per acre. Treatment 4 yielded 1.222 dry matter tons per acre.

When separated by location and cultivar, no significant differences were among treatments for both first and second harvest at the Spencer, WI location ($p>0.10$). For first harvest, the largest differences in Ameristand were observed between treatments 3 and 2. Treatment 3 yield was 1.075 dry matter tons per acre. Treatment 2 yield was 0.868 dry matter tons per acre. In MegaTron AA, the largest differences were observed between treatment 2 and treatment 3. Treatment 2 yielded 1.202 dry matter tons per acre and treatment 3 yielded 1.125 dry matter tons per acre. For the second harvest, the

largest differences in Ameristand were observed between treatments 3 and 2. Treatment 3 yielded 1.235 dry matter tons per acre. Treatment 2 yielded 1.082 dry matter tons per acre. For MegaTron AA, the largest differences in second harvest yield were observed between treatment 2 and treatment 3. Treatment 2 yielded 1.377 dry matter tons per acre and treatment 3 yielded 1.235 dry matter tons per acre.

Overall, there were few significant statistical differences in treatments observed in dry matter yield. The results show that seed treatments did aid in improving stand establishment as well as improved yield in first harvest at certain locations as well second harvest at certain locations and all locations pooled together. This could be due to more plants establishing in those plots with fungicide seed treatments compared to the untreated control leading to an increase in overall dry matter tons per acre for the treated plots.

Growth Chamber Assays

Standard Pathogen Test Results

Seed treatments were tested using both cultivars and check cultivars in growth chamber assays with single pathogens utilizing the standard test protocols to evaluate the protection of treatments against Aphanomyces root rot race 1 and race 2, Phytophthora root rot, and Pythium seed rot and damping-off. Seed treatments coupled with genetic resistance significantly improved resistance to Phytophthora root rot and to *Pythium* spp. but seed treatments did not provide added control in addition to the genetic resistance to Aphanomyces root rot. Cultivars with genetic resistance to both Aphanomyces root rot and Phytophthora root rot provided significantly more resistance to the pathogens as opposed to the susceptible cultivars.

Aphanomyces euteiches-Race 1

The susceptible checks, Saranac and Agate showed low percent resistant plants 10%, and 0%, respectively, which is within range of the standard test (Fig. 3.2). WAPH-1 and WAPH-5 are resistant checks and were above the expected 35-60% resistant plants: WAPH-1 showed 86% resistant plants and WAPH-5 showed 96% resistant plants. Both Ameristand and Megatron AA are HR for race 1. In the control treatment (treatment 1), Ameristand had 59% resistant plants and Megatron AA had 85% resistant plants, as expected for HR cultivars. None of the seed treatments significantly increased

the percent resistant plants; however, the resistant checks and MegaTron AA treatments 1 through 5, offered significantly more control as compared to Ameristand treatments 1, 2, and 3 and the susceptible checks (p=0.0001). This indicates that while the Ameristand cultivar still offers high resistance to Aphanomyces root rot race 1, the MegaTron AA cultivar offers more advanced control.

***Aphanomyces euteiches*-Race 2**

Two race 2 isolates were used in assays to test the seed treatments. Isolate NC-1 was isolated from North Carolina soil in the 1980s and has been used widely in standard tests while isolate MER4 was isolated from NY soil in 2013 and is a highly aggressive isolate.

In the MER4 test the susceptible checks, Saranac, Agate and WAPH-1 showed low percent resistant plants with 3%, 12%, and 0% resistance respectively, which is within range of the standard test (Fig. 3.3). WAPH-5 is the race 2 resistant check and showed greater than the expected 35-60% resistant plants with 67% resistance. Ameristand is susceptible to Aphanomyces root rot race 2 and MegaTron AA is highly resistant to race 2.

In the control treatment (treatment 1) MegaTron AA had 55% resistant plants which the expected range in cultivars that offer high resistance to the pathogen (>50%). Ameristand treatment 1 had 4% resistant plants indicating that the cultivar is very susceptible to Aphanomyces root rot race 2. When Ameristand was treated with the fungicide seed treatments, the largest improvement in protection was seen with treatment 5. Ameristand treatment 5 had 23% resistant plants which is still only considered moderate resistance.

Similar to the Ameristand treatment 5 increase in percent resistant plants, MegaTron AA treated with treatment 5 also saw the largest increase in percent resistant plants at 69% resistance. However, no significant differences (p>0.10) were observed between any of the treatments as compared to the control treatment (treatment 1). MegaTron AA treatments 1 through 5 and WAPH-5 offered significantly more control as compared to Ameristand treatments 1, 2, 3, 4, and 5, and the susceptible checks (Saranac, Agate, and WAPH-1) (p=0.0001; Fig. 3.3). Ameristand treatment 5 offered significantly more control as compared to Ameristand treatment 1 and the susceptible checks.

In the NC1 test, the susceptible checks, Saranac, Agate and WAPH-1 showed low percent resistant plants with 6%, 8%, and 0% resistance respectively, which is within range of the standard test (Fig. 3.4). WAPH-5 is the race 2 resistant check and showed above the expected 35-60% resistant plants with 93% resistance. Ameristand is susceptible to Aphanomyces root rot race 2 and MegaTron AA is highly resistant to race 2.

In the control treatment (treatment 1), MegaTron AA had 53% resistant plants which is considering high resistance. Ameristand treatment 1 had 20% resistant plants. In the Ameristand cultivar, none of the fungicide seed treatments offered any added control. In MegaTron AA, no treatments offered significantly more control ($p>0.05$) as compared to the control (treatment 1); however, the largest increase in percent resistant plants was observed in treatment 5 which had 67% resistant plants. WAPH-5 offered significantly more control than Ameristand treatments 1 through 5 and the susceptible checks. WAPH-5 also offered significantly more control than MegaTron AA treatments 1 and 2. MegaTron AA treatments 1-5 and Ameristand treatment 1 offered significantly more control than the susceptible checks. Overall, the *A. euteiches* standard pathogen test results indicate that the rated resistance to race 1 and race 2 for Ameristand and MegaTron AA are accurate, and the treatments did not add a benefit in protecting alfalfa seedings for either resistant or susceptible cultivars.

Phytophthora medicaginis

Both Ameristand and MegaTron AA are rated as HR for Phytophthora root rot and without seed treatment should have at least 50% resistant plants. The Phytophthora root rot standard test assay revealed significant differences among treatments. When pooled across the two cultivars, differences were observed between treatments 3 (78% resistant), 4 (83% resistant), and 5 (84% resistant) compared to treatments 2 (53% resistant) and 1 (36% resistant) at a p-value < 0.0001. Treatment 2 also increased protection compared to the control treatment 1. The results indicate that treatments increase protection to Phytophthora root rot in addition to the genetic resistance. Seed treated with Apron XL and Stamina would offer the same amount of protection as those treated with the combination of Apron XL, Stamina, and Intego Solo (treatment 4) or the Apron XL, Stamina, Intego Solo, and Maxim combination (treatment 5).

When separated by cultivar, significant differences were observed between MegaTron AA treatments 4 (89% resistant) and 5 (86% resistant) compared to treatments 2 (50% resistant) and 1 (49% resistant) at a p=0.03 (Fig. 3.5). For Phytophthora root rot, Evergol Energy (treatment 2) did not protect seedlings. The results also indicate that the Apron XL and Stamina combination offer enhanced control of *P. medicaginis* and adding Intego Solo or Maxim did not provide any added control as compared to the Apron XL and Stamina combination in treatment 3.

When separated by cultivar, significant differences ($p < 0.0001$) were observed in Ameristand treatments 3 (84% resistant), 4 (77% resistant), and 5 (82% resistant) as compared to treatments 2 (56% resistant) and 1 (24% resistant) (Fig. 3.5). Statistical differences were also observed between treatment 2 and treatment 1 where treatment 2 increased protected plants over the control treatment 1. These results indicate that the disease resistance genetics to *P. medicaginis* in Ameristand did not provide adequate control of the isolate used in the test and that fungicide seed treatments provided added control of the pathogen. In addition, EverGol Energy did increase protection from the pathogen but did not offer as much control as the other seed treatments. These results show that the Apron XL and Stamina combination offers the most protection and that adding Intego Solo or Maxim to the Apron XL and Stamina combination would not provide any added control of the pathogen.

The susceptible check, Saranac showed low percent resistant plants 4%, which is within range of the standard test. Agate is rated as resistant to Phytophthora root rot (expected range of 25-40% resistant plants) and showed 13% resistance. WAPH-1 and WAPH-5 both are highly resistant to Phytophthora root rot and showed 77% and 75% resistant plants, respectively. WAPH-5 and WAPH-1 were considered statistically the same as both MegaTron AA and Ameristand with treatments 3, 4, and 5 while the susceptible check, Saranac was considered significantly lower than all cultivars and treatments.

Pythium Seed Rot and Damping-off

Seed treatments on the two cultivars were tested in standard tests using three isolates of *Pythium*, known to cause seed rot and damping-off of alfalfa. Results indicate that treatments 3, 4, and 5 on both cultivars offer control to all three of the *Pythium* species and that treatment 2 would not always offer good control to the species used. In

addition, untreated seed offered no control to the *Pythium* species used which indicates there is very little genetic resistance to *Pythium* and that dependence on fungicide seed treatments is necessary to obtain control of the pathogen.

Pythium irregulare **test**

The *Pythium irregulare* standard plate test assay using the Bec56 isolate revealed statistical differences among treatments. When pooled across the two varieties, statistical difference was observed between treatments 3 (75.7% resistance), 4 (68.5% resistance), and 5 (78% resistance) which was statistically more resistant than treatments 2 (54.9% resistance) and 1 (0.7% resistance) at a p-value < 0.0001. Treatment 2 was also statistically more resistant than treatment 1. The results indicate that treated seed offers adequate control of *Pythium irregulare* and that untreated seed does not offer any protection to the pathogen. These results also indicate that seed treated with Apron XL and Stamina offers just as adequate of control as treated with an Apron XL, Stamina, and Intego Solo combination or an Apron XL, Stamina, Intego Solo, or Maxim combination and that any of the combinations would offer more control to *P. irregulare* than EverGol Energy treatment.

When separated by cultivar, significant differences ($p<0.0001$) were observed between MegaTron AA treatment 5 (84% resistant) compared to treatments 2 (64% resistant), and 4 (66% resistant) (Fig. 3.6). Significant differences ($p<0.0001$) were observed between Ameristand treatments 3 (73% resistant), 4 (71% resistant), and 5 (72% resistant) compared to treatment 2 (46% resistant) (Fig. 3.6). Treatment 2 significantly increased protection over the control treatment 1. The results indicate that treated seed offers adequate control of *Pythium irregulare* and that untreated seed has no resistance to the pathogen. These results also indicate that seed treated with Apron XL and Stamina offers similar control to that treated with an Apron XL, Stamina, and Intego Solo combination or an Apron XL, Stamina, Intego Solo, or Maxim combination and that any of those combinations would offer more control of *P. irregulare* than the EverGol Energy treatment for some cultivars such as Ameristand.

Pythium ultimum **test**

The *Pythium ultimum* standard plate test assay using the Was53 isolate revealed statistical differences among treatments. When pooled across the two cultivars, a significant difference ($p<0.0001$) was observed between treatments 3 (85% resistant), 4

(80% resistant), and 5 (81% resistant) compared to treatments 2 (66% resistant) and 1 (0% resistance). Treatment 2 significantly increased protection over the control treatment 1. The results indicate that seed treated with the fungicide combinations enhances seed germination in the presence of *P. ultimum* compared to untreated seed which lacks resistance to the pathogen. These results also indicate that seed treated with Apron XL and Stamina offers similar control as treatment with the Apron XL, Stamina, and Intego Solo combination or an Apron XL, Stamina, Intego Solo, or Maxim combination. Similar to the *P. irregulare* test, the EverGol Energy treatment was less effective than the other treatments.

When separated by cultivar, significant differences ($p<0.0001$) were observed between MegaTron AA treatments 3 (80% resistant), 4 (86% resistance), and 5 (90% resistance) compared to treatments 2 (68% resistant) and the control (0% resistant) (Fig. 3.6). Treatment 2 significantly increased protection over the control treatment 1. Significant differences ($p<0.0001$) were observed between Ameristand treatment 3 (90% resistance) compared to treatments 2 (64% resistant) and 1 (0% resistance) (Fig. 3.6). Treatment 2 significantly increased protection over the control treatment 1. The results indicate that seed treated with the fungicide combinations enhances seed germination in the presence of *P. ultimum* compared to untreated seed which lacks resistance to the pathogen. These results also indicate that seed treated with Apron XL and Stamina offers similar control as treatment with the Apron XL, Stamina, and Intego Solo combination or an Apron XL, Stamina, Intego Solo, or Maxim combination. Similar to the *P. irregulare* test, the EverGol Energy treatment was less effective than the other treatments.

***Pythium paroecandrum* test**

The *Pythium paroecandrum* standard plate assay using the L3 isolate revealed significant differences ($p<0.0001$) among treatments when pooled across cultivars. Results showed significant differences between treatments 3 (72% resistant), 4 (84% resistant), and 5 (78% resistant) as compared to treatment 2 (36% resistant) and treatment 1 (6% resistant). Treatment 2 significantly increased protection over the control treatment 1. These results indicate that even when a less aggressive isolate is used, fungicide seed treatments increased seed germination compared to seed that was not treated with a fungicide. These results show that Treatments 3 through 5 would all offer adequate control to this *Pythium* species and that adding Intego Solo or Maxim to

the Apron XL and Stamina combination would not result in an improvement over the Apron XL and Stamina combination. The results also show that while treatment 2 provides some protection, the EverGol Energy treatment is less effective than the other treatments.

When separated by cultivar, results were consistent with results when cultivars were combined. In MegaTron AA, treatments 3 (77% resistant), 4 (84% resistant), and 5 (84% resistant) resulted in more control of *P. paroecandrum* (Fig. 3.6; $p<0.0001$) as compared to treatments 2 (45% resistant). Treatment 2 significantly increased protection over the control treatment 1. For Ameristand, treatments 3 (67% resistant), 4 (83% resistant), and 5 (73% resistant) provided more control ($p<0.001$) as compared to treatments 2 (28% resistant). The results show that treatment 2 (EverGol Energy) did not significantly increase protection from this pathogen and that treatments 3 through 5 would provide significant control. Treatments 3 through 5 had similar levels of control so adding Intego Solo and Maxim to the Apron XL and Stamina combination would not provide any additional control.

Phytophthora sansomeana pathogenicity test

The pathogenicity tests with *Phytophthora sansomeana* inoculated onto seedlings or incorporated into soil revealed potential infection from the pathogen. In the test using the standard test methods inoculating seedlings, a moderate number of plants showed disease symptoms (Fig. 3.7). Symptoms of the *P. sansomeana* infection was plant stunting and necrosis on the hypocotyl and upper root resulting in some plants with girdling lesions and cincture of the tap root. These symptoms are similar to those caused by *P. medicaginis*. In the *P. sansomeana* infested soil assay, fewer plants showed symptoms. Further testing is needed to evaluate whether *P. sansomeana* causes seedling disease in alfalfa including re-isolation of the pathogen from symptomatic plants.

Phytophthora sansomeana sequencing results

Sanger sequencing results of the pure *Phytophthora sansomeana* isolate were trimmed to cover the same ITS region used in amplicon sequencing of oomycetes in Illumina MiSeq. A comparison of the sequences revealed that Zotu3 (*Phytophthora sansomeana* OTU) from oomycete amplicon sequencing and sequences of the pure isolates, LJL 34 and LJL 12, were identical (268 / 268 bp). The *P. sansomeana* identified

in sequencing is the same as the pure pathogen isolate, validating that the isolates used in the pathogenicity assay were the same organism that would be found in the soil and roots.

Bioassay Results

Soils from each of the seven field locations, Beaver, Emerald, Frankfort, Spencer, Marshfield, Unity and West Salem (2020 and 2021) were used in a controlled environment to test protection of seeds of Ameristand and Megatron AA from soilborne disease with the fungicide seed treatments. Plants without symptoms of root rot or only mild root discoloration (rated 1 or 2) were scored as resistant. None of the seed treatments were highly effective in protecting seeds from seed rot, damping-off, and root rot pathogens in the field soils.

When pooled across all seven soils and cultivars, a significant difference ($p<0.0001$) was observed between treatments 4 (35% resistant) and 5 (35% resistant) and treatments 2 (25% resistant) and the control (23% resistant) (Fig. 3.8). These results could indicate there are *Pythium* spp. present at these locations that are resistant to, or not well controlled by Apron XL, which is a component of treatment 2, EverGol Energy, and is in treatment 3. In addition, these soils could have aggressive *A. euteiches* isolates not well controlled by EverGol Energy or Stamina, or *P. medicaginis* isolates also not well controlled by treatments 2 and 3. Addition of Intego Solo in treatment 4 and 5 and Maxim in treatment 5 provide a significantly greater amount of control, although the percent resistant plants was still low (35%).

Bioassay results revealed cultivars with genetic resistance to both Aphanomyces root rot and Phytophthora root rot provided more control as opposed to susceptible cultivars (Table 3.3). At most locations, the percent resistant plants for MegaTron AA with high resistance to both races of Aphanomyces root rot and Phytophthora root rot was similar to WAPH-5, the check with high resistance to both races of Aphanomyces root rot and Phytophthora root rot. In soils from the Unity and Spencer locations, the percent resistant plants for WAPH-5 and MegaTron AA were lower than or at the low end of the range of expected resistant plants for Aphanomyces or Phytophthora root rot (35-60%) suggesting the presence of other root rot pathogens in these soils.

For the Beaver soil , when separated by cultivar, the MegaTron AA control treatment (treatment 1) had the highest percent resistant plants (Fig. 3.9). This was

significantly more control than treatment 2 ($p<0.0001$), which had 36% resistant plants. However, this was not significantly different from the results of Megatron AA treatments 3 through 5 in soil from this location. In the Ameristand cultivar, very little protection to the pathogens in the soil was observed with the highest percent resistant plants being at 6% for Ameristand treatments 3 and 4. The standard check cultivars, Saranac, Agate, and WAPH-1 all showed 0% resistant plants to the pathogens present in the soil at this location. WAPH-5, which is highly resistant to Aphanomyces root rot race 1 and 2 and highly resistant to Phytophthora root rot, had 77% resistance to the pathogens present in this soil indicating the presence of Aphanomyces race 2 in this soil.

For the Emerald soil, no significant differences were found between any of the five fungicide seed treatments when pooled over both cultivars and when separated by cultivars. However, significant differences were found between cultivars (Fig. 3.10). WAPH-5 and MegaTron AA treatments 1 through 5 were considered significantly more resistant than Ameristand treatments 1 through 5 and Saranac, Agate, and WAPH-1 ($p=0.0001$). In the MegaTron AA cultivar, the largest differences were observed between treatment 1 (untreated control) and treatment 4. Treatment 1 had 42% resistant plants and treatment 4 had 61% resistant plants. In the Ameristand cultivar, very little protection to the pathogens present in the soil was observed. The largest percent resistant plants was in treatment 5 which had 13% resistant plants. In the check cultivars, Saranac and Agate both had 0% resistant plants and WAPH-1 had 4% resistant plants. WAPH-5 had 78% resistant plants. This indicates that *A. euteiches* race 2 may be the most prevalent pathogen contributing to disease in this soil as the highly resistant cultivars, MegaTron AA and WAPH-5 offered added control to the pathogen as compared to the race 2 susceptible cultivars.

For the Frankfort soil, significant differences were observed among the Ameristand cultivar in treatment 2 and treatment 4 (Fig. 3.11; $p=0.001$). Treatment 4 had 53% resistant plants and treatment 2 had 11% resistant plants. In MegaTron AA, the largest differences were observed between treatment 1 and treatment 5. Treatment 1 had 65% resistant plants to the pathogens found in this soil and treatment 5 had 76% resistant plants. In the standard check cultivars, both Saranac and Agate displayed 0% resistance to the pathogens found in the soil at this location. WAPH-1 showed 19% resistance and WAPH-5 showed 62% resistance.

For the Spencer soil, no significant differences were observed among the fungicide seed treatments when pooled over both cultivars and when separated by cultivar. However, significant differences were found between cultivars; MegaTron AA treatments 3 and 5 were considered significantly more resistant than Ameristand treatments 1 through 5 and Saranac, Agate, and WAPH-1 (Fig. 3.12; p=0.001). In MegaTron AA, the largest differences were observed between treatment 1 (28% resistant plants) and treatment 5 (54% resistant plants). In Ameristand the largest differences were between treatments 1 and 2 and treatment 3. Both treatment 1 and 2 had 0% resistant plants and treatment 3 had 17% resistant plants. In the standard checks, Saranac, Agate, and WAPH-1 all had 0% resistance to the pathogens present in this soil. In the WAPH-5 cultivar, 38% of the plants were resistant to the pathogens found in the soil. These results indicate that the fungicide seed treatments in treatment 5 in combination with the MegaTron AA cultivar would offer the largest control of the pathogens present in the soil. Because WAPH-5 was below 50% resistance there may be other pathogens present other than just *A. euteiches* race 2 such as true fungal pathogens or *Pythium* spp. or *P. sansomeana* that WAPH-5 does not have genetic resistance to.

For the Marshfield soil, significant differences were found among treatments when separated by cultivar (Fig. 3.13). In MegaTron AA, significant differences were found between treatment 5 (68% resistant) and treatments 2 (47% resistant) and 3 (45% resistant) (p=0.001). In Ameristand, greatest differences were seen between treatment 1 and treatment 5. Treatment 1 had 2% resistant plants and treatment 5 had 14% resistant plants. In the standard check cultivars, Saranac, Agate, and WAPH-1 all showed 0% resistance to the pathogens present in this soil while WAPH-5 had 69% resistance to the pathogens in this soil. The high resistant score in WAPH-5 indicates that Aphanomyces root rot race 2 is present in this soil.

For the Unity soil, no significant differences were observed among treatments across both cultivars. However, there were significant differences in cultivars with MegaTron AA treatment 4 and 5 offering significantly more control as compared to Ameristand treatments 1 through 5 and all four check cultivars (Fig. 3.14; p=0.0001). When cultivars were pooled, greatest differences were between treatment 1 with the least resistant plants (18% resistant) and treatment 5 with the most resistant plants (38% resistant) across both cultivars. The greater number of protected plants from treatment 5

suggests that fungal pathogens may be playing a role in seed rot and damping off in this soil. When separated by cultivar, in MegaTron AA, largest differences were observed between treatment 2 and treatment 5. Treatment 2 had 35% resistant plants and treatment 5 had 60% resistant plants.

In Ameristand, largest differences were between treatment 1 and treatment 5. Treatment 1 had 0% resistant plants and treatment 5 had 17% resistant plants to the pathogens in this soil. In the standard check cultivars, Saranac, Agate, and WAPH-1 all showed 0% resistant plants. WAPH-5 showed 15% resistant plants. Since WAPH-5 is HR to both Phytophthora root rot and Aphanomyces root rot, it's low percent resistance to the pathogens present in the Unity soil indicate that *Pythium* spp., *Phytophthora sansomeana*, or true fungal species may be the main causal pathogens in contributing to seedling disease. In addition, there could be aggressive *A. euteiches* or *P. medicaginis* isolates present that can overcome the high genetic resistance that WAPH-5 has to the two root rot pathogens.

For the West Salem soil, significant differences were not observed when cultivars were combined but differences were observed when separated by cultivar. In MegaTron AA, significant differences were observed between treatment 1 and treatments 2 ($p=0.0001$; Fig. 3.15). Treatment 1 had 19% resistant plants and treatment 2 had 48% resistant plants. In the standard check cultivars, Saranac, Agate, and WAPH-1 all showed 0% resistant plants while WAPH-5 had 66% resistant plants indicating the presence of race 2 Aphanomyces in this soil.

3.2. Objective #2: Identify the microbial communities (bacteria, fungi, and oomycetes) associated with alfalfa seedlings and soil samples from sites with poor alfalfa establishment.

Oomycete Sequencing Results

Oomycete community composition in the alfalfa endosphere and rhizosphere

Amplicon sequencing was conducted in 2020 and 2021 using Illumina MiSeq. Amplicons of the ITS region were submitted for both rhizosphere and root DNA samples from 2020 and 2021 plot locations. A total of 164 samples were submitted for amplicon sequencing from the eight plot locations in 2020 and 2021 plots combined. A total of 6,201,720 sequences were mapped to 735 unique oomycete Amplicon Sequence

Variants (ASVs)/Operational Taxonomic Units (OTUs). After rarefying to 8,000 sequences/samples and dropping three low-abundance samples, there were 1,288,000 sequences among 719 ASVs/OTUs. Two endosphere samples from Beaver and one rhizosphere sample from West Salem were the low-abundance samples that were dropped.

The most abundant OTU was identified as *A. euteiches* at 56% abundance across all endosphere samples and 39% abundance across all rhizosphere soil samples (Table 3.4 and 3.5). Across all endosphere samples *P. medicaginis* was identified at 11% abundance and 11% abundance across all rhizosphere soil samples. Interestingly, *P. sansomeana,* a pathogen not previously recognized to infect alfalfa, was identified with 6% abundance across all endosphere samples for all plots and 5% abundance across all rhizosphere soil samples for all plots. *Pythium/Globisporangium inflatum* and *P. sylvaticum* were also identified in high abundance; however, *A. euteiches* and *P. medicaginis* dominated the total endosphere community composition.

Oomycete populations in the endosphere varied by location (Fig. 3.16, Table 3.4). At the Beaver, Emerald, Marshfield, and West Salem locations, *A. euteiches* was the most highly abundant: 60% at Beaver, 76% at Emerald, 47% at Marshfield, 79% at West Salem in 2020, and 61% at West Salem in 2021. At the Spencer and Unity locations, *P. medicaginis* was more abundant at 31% and 44%, respectively. At the Beaver and Marshfield locations, *P. sansomeana* was more abundant than *P. medicaginis*.

Pythium/Globisporangium spp. abundances varied by location and by species. In the endosphere, *Pythium inflatum* and *Pythium/Globisporangium sylvaticum* (Zotu6) were the most abundant across all locations and samples (Table 3.4). As the heat map in Fig. 3.17 highlights, *P. sylvaticum* was higher in abundance in the 2021 locations and West Salem 2020 samples as compared to the other 2020 locations. Endosphere *P. sylvaticum* abundance was highest at the West Salem 2020 location at 9% abundance followed by the Spencer location at 7% abundance, West Salem 2021 location at 6% abundance, and the Frankfort location at 4% abundance. It was identified at 3% abundance at the Emerald site. *Pythium sylvaticum* is widespread and highly virulent on alfalfa seedlings (Berg et al., 2017).

Pythium inflatum (Zotu4, Zotu12) was also identified in relatively high abundance in the endosphere, as compared to the other *Pythium/Globisporangium* spp. (Fig. 3.16, 3.17; Table 3.4). Highest *P. inflatum* abundances were identified at the Clark County and Marathon County plot locations (Beaver, Marshfield, Unity, Spencer; Fig.3.16) as opposed to the West Salem and Emerald plot locations. Berg et al. (2017) studied the pathogenicity of *P. inflatum* and found that this species does not cause severe disease to alfalfa.

Pythium aritosporum was identified in relatively high abundance at the Emerald location at 12% in the endosphere. Pathogenicity of this *Pythium* sp. toward alfalfa is not well known. Of the other two known *Pythium* spp. that cause severe disease on alfalfa, *P. ultimum* and *P. irregulare*, only *P. ultimum* was identified in abundance at the plot locations and abundance was overall low across all locations (Table 3.4). *P. ultimum* abundance was highest at the West Salem 2020 and 2021 locations. *P. irregulare* was not identified in the top 50 most abundant oomycete species in the endosphere.

In the rhizosphere soil amplicon sequencing results, more diversity was observed among locations with more abundance across OTUs (Fig. 3.18, Fig. 3.19; Table 3.5). However, like the endosphere samples, the most abundant oomycete was *A. euteiches* at 39%. The second most abundant species in the rhizosphere was *P. medicaginis* at 11% abundance and *P. sansomeana* was the third most abundant across all locations at 5%. At the Unity and West Salem 2020 locations, *P. sansomeana* was more abundant than *P. medicaginis*, which slightly differs from the endosphere abundance of this species, as it was not highly abundant in the endosphere at the West Salem 2020 location.

Pythium/Globisporangium spp. abundance varied by location and abundances for *Pythium/Globisporangium* spp. were higher in the rhizosphere as opposed to the endosphere (Fig. 3.18). Like the endosphere composition, *P. inflatum*, *G. sylvaticum,* and *P. aritosporum* were relatively highly abundant across the plot locations and rhizosphere soil samples (Table 3.5). *G. perplexum* (Zotu11), *G. heterothallicum* (Zotu7), and *G. attrantheridium* (Zotu12) all increased in abundance in the rhizosphere soil sample composition as compared to the endosphere sample composition. *G. ultimum* abundance was still relatively low. Highest *P. ultimum* abundances were at the West Salem 2021 location and Emerald location.

Currently identified pathogens that cause seed rot, damping-off, and root rot in alfalfa that were identified in high abundance in sequencing results were *A. euteiches*, *P. medicaginis*, *Pythium ultimum*, and *P. sylvaticum*. Of the other *Pythium* spp. identified, there were a few species where pathogenicity toward alfalfa is unknown. Those species are *P. aritosporum, P. selbyi, P. monospermum,* and *P. radicola. P. aritosporum* has been reported to cause corn stalk rot in China (Gao, Zhang, & Li, 2016) and has also been reported to cause damping-off in both field pea (Alcala et al., 2016) and soybean (Zitnick-Anderson & Nelson, 2015).

Berg et al. (2017) studied the pathogenicity of *P. irregulare*, *P. sylvaticum*, *P. ultimum* var*. ultimum, P. attrantheridium*, *P. heterothallicum*, *P. pleroticum*, *P. perplexum*, *and P. inflatum* toward alfalfa, which were all species identified in relatively high abundance in amplicon sequencing. Of those, *P. irregulare, P. sylvaticum, and P. ultimum* var*. ultimum* were the most pathogenic toward alfalfa seedlings and strains of these species were identified that were not well controlled by metalaxyl/mefenoxam fungicides.

Phytophthora sansomeana was identified in relatively high abundance at the Beaver, Spencer, Marshfield, and Frankfort locations in endosphere samples and was identified in relatively high abundance at the Unity, West Salem 2020, and Frankfort sites in rhizosphere soil samples (Table 3.4 and 3.5; Fig. 3.16 and 3.18). *P. sansomeana* is closely related to *P. medicaginis*, which is the causal pathogen of Phytophthora root rot in alfalfa (Hansen et al., 2017). Previously, *P. sansomeana* had been identified in alfalfa fields in New York but was recovered from weed species. *P. sansomeana* is most known as a pathogen of soybeans.

Alpha diversity analyses for all locations

Alpha-diversity indices were estimated for each of the eight plot locations and both endosphere and rhizosphere sample types (Fig. 3.20). Non-metric multidimensional scaling (NMDS) and principal component analysis (PCA) plots were compiled for exploratory analyses to reveal certain patterns of similarity across the plots and sample types. First, the NMDS plot showed that sample types were strongly clustered together at each location and by each compartment, meaning rhizosphere soil samples at each location were similar and endosphere sample community composition at each location were similar (Fig. 3.21). There were differences however at each location between the

two sample types as the sample types at each location (rhizosphere soil vs. endosphere) were not clustered as closely together.

Second, the NMDS showed that locations Emerald and West Salem endosphere samples were quite similar in oomycete species composition (Fig. 3.21). Additionally, the NMDS plot revealed that the Beaver, Unity, Spencer, Frankfort, and Marshfield plots were more closely clustered together indicating more similarity in the species at those locations. The differences in the clustering between the locations could be because the Unity, Spencer, Beaver, Frankfort, and Marshfield locations are all located in Marathon and Clark counties whereas Emerald and West Salem are in St. Croix and La Crosse counties, respectively. Next, the PERMANOVA results showed the significance of location and compartment in structuring communities. The results indicated significant ($p<0.001$) correlation of location, compartment, and location: compartment in structuring the oomycete communities.

Differential Abundances

When comparing the four cultivars used across the two years of samples, *A. euteiches* (Zotu 480) was the only pathogen where abundance varied significantly based on cultivar (Fig. 3.22). This is consistent with cultivar disease ratings. All cultivars have high resistance to *P. medicaginis* and *A. euteiches* race 1, but only MegaTron and MegaTron AA have high resistance to *A. euteiches* race 2. The abundance of *A. euteiches* was lowest in 2021 in MegaTron AA (Fig. 3.22). Because MegaTron AA and MegaTron have genetic resistance to race 1 and race 2, the increased abundance of *A. euteiches* in Stratica and Ameristand, which only have resistance to race 1, may be due to infection by race 2 pathogens. The variation in counts in each cultivar is likely due to differences in *A. euteiches* abundance at each location. In addition, less *A. euteiches* pressure may have been observed in the 2021 compared to 2020 due to less rainfall before sampling in 2021 and therefore less pathogen pressure.

Fungal Sequencing Results

Fungal community composition in the alfalfa endosphere and rhizosphere

Amplicon sequencing was conducted in 2020 and 2021 using Illumina MiSeq. Amplicons of the ITS region were sequenced for both rhizosphere and root DNA samples from 2020 and 2021 plot locations. A total of 164 samples were submitted for

amplicon sequencing from between the eight plot locations in 2020 and 2021 plots combined. In total 3,101 Amplicon Sequence Variants (ASVs) were obtained for fungal community analyses for 2020 and 2021 combined. The most abundant species in the endosphere was *Fusarium waltergamsii* at 28% abundance (Table 3.6). This fungus is a member of the *F. solani* species complex, one of the most abundant in soil and often associated with plant roots. Other frequently identified fungal species in the endosphere were *F. acutatum* and the *Plectospherella* genus, both identified at 12% abundance. In the rhizosphere, the most abundant species was *Mortierella minutissima* at 14% abundance (Table 3.7), a common soil saprophyte. Other frequently identified genera were *Pseudombrophila* and *Plectosphaerella* both identified at 8% relative abundance. These are both common soil fungi, although several *Plectosphaerella* species cause plant disease.

Fusarium spp. and *Rhizoctonia solani* are two common pathogens known for causing seed rot and damping-off in alfalfa seedlings. Various *Fusarium* spp. were identified in both the endosphere and rhizosphere. In the endosphere, *F. waltergamsii, F. acutatum, Fusarium* sp. (ASV8), and *Fusarium* sp. (ASV32) were identified at 28%, 12%, 8%, and 1% abundance, respectively. The high abundance of *F. waltergamsii* and *F. acutatum*, not previously identified as pathogens of alfalfa, warrants further investigation to determine their potential for causing disease on alfalfa. The *Rhizoctonia solani* family, Ceratobasidiaceae (ASV13) was identified at 5% abundance in the endosphere. In the rhizosphere, *F. waltergamsii was* identified at 7% relative abundance and *F. acutatum* was identified at 4% relative abundance. Ceratobasidiaceae (ASV13) was identified at 2% abundance.

Fusarium ASV8 could not be identified down to the species level in sequencing. Berg et al. (2017) found that *F. oxysporum* and *F. incarnatum-equiseti* were the most pathogenic and widespread in causing seedling disease. *Fusarium* ASV8 that could not be identified down to the species level could potentially be *F. oxysporum,* a seedling pathogen, the Fusarium wilt pathogen or *F. incarnatum-equiseti*, a seed rot pathogen of alfalfa. On the other hand, the ASV that could not be identified down to the species level may not be pathogenic toward alfalfa and may be a *Fusarium* species that is present in the soil but not contributing to alfalfa seedling disease.

Other potentially damaging fungal pathogens were identified that have been reported in other counties as contributing to the alfalfa root rot complex. The genus

Plectosphaerella was identified in relatively high abundance in both the rhizosphere soil and endosphere samples at most locations across both years (Table 3.6 and 3.7). The first report of alfalfa root rot caused by *Plectosphaerella cucumerina* in the Inner Mongolia Autonomous region of China was published in 2021 (Zhao, Shi, Yu, & Zhang, 2021). *Paraphoma radicina* is another fungal species that was identified in relatively high abundance in endosphere samples for both years across a few locations (Table 3.6; Fig.3.23). *Paraphoma radicina* was identified as causing root rot in alfalfa in the Inner Mongolia, China (Cao, Liang, Nzabanita, & Li, 2020). In three locations, Marshfield, Unity, and West Salem in 2020, *Mycoleptodiscus terrestris* was found in endosphere samples. This fungus has a broad host range but has been identified as a root rot pathogen of alfalfa in Minnesota (Zivanov, Tancic Zivanov, & Samac, 2021).

Alpha diversity analyses for all locations

Alpha-diversity indices were estimated for each of the eight plot locations and both endosphere and rhizosphere sample types (Fig. 3.24). NMDS plots were compiled for exploratory analyses to reveal certain patterns of similarity across the plots and sample types. The NMDS plot for fungal sequences revealed similar patterns of community composition as the oomycete sequencing revealed. The NMDS plot showed that sample types were strongly clustered together at each location and by each compartment, meaning rhizosphere soil samples at each location were similar and endosphere sample community composition at each location were strongly similar (Fig. 3.25). There were differences at each location between the two sample types as the sample types at each location (rhizosphere soil vs. endosphere) were not clustered as closely together.

Additionally, the NMDS plot showed that sample composition at the Emerald and West Salem locations differed as compared to the other plots. Also, the Beaver, Unity, Spencer, Frankfort, and Marshfield plots were more closely clustered together indicating more similarity in the species at those locations. The differences in the clustering between the locations could be because the Unity, Spencer, Beaver, Frankfort, and Maple Ridge locations are all located in Marathon and Clark counties whereas Emerald and West Salem are in St. Croix and La Crosse counties, like community composition was clustered for oomycetes.

The PERMANOVA results showed the significance of location and compartment in structuring communities. The results indicated significant ($p<0.001$) correlation of location, compartment, and location:compartment in structuring the fungal communities. The beta-dispersion among groups analyses revealed that there was similar variance among communities between the rhizosphere and endosphere samples (Fig. 3.26; $p=0.0035$).

NMDS plots were constructed by compartment (endosphere and rhizosphere soil samples) among locations and cultivars. The endosphere NMDS plot revealed strong significance of location in structuring communities using permutational multivariate ANOVA (PERMANOVA) (Fig. 3.27). These results indicate that location influenced the community composition for the samples when separated by cultivar ($p<0.001$). This is consistent with the NMDS plot (Fig. 3.25) across all cultivars combined. It should be noted, as in the NMDS plot, that Beaver, Frankfort, Spencer, and Marshfield locations are grouped more closely together compared to the West Salem and Emerald locations indicating that because those plots are within the same two county region in Wisconsin their community composition is similar as opposed to different community composition at the Emerald and West Salem locations that are in different counties in different regions across Wisconsin.

The rhizosphere soil NMDS plot revealed similar results as the endosphere NMDS. The NMDS plot revealed strong significance of location in structuring communities using permutational multivariate ANOVA (PERMANOVA) (Fig. 3.28). These results indicate that location influenced the community composition for the samples when separated by cultivar ($p<0.001$). Like the endosphere NMDS, Beaver, Frankfort, Spencer, and Marshfield locations are grouped more closely together as opposed to the West Salem and Emerald locations. Additionally, the Emerald location samples are not grouped as tightly together as the other plot locations indicating variation in community composition at that location. Finally, the community composition at West Salem is probably the most different than the other locations due to the separation of West Salem from of the other locations on the plot.

Bacterial Sequencing Results

A total of 34,018 bacterial ASVs were obtained across locations and years. Communities in the rhizosphere and endosphere were distinct. However, in both the

endosphere and rhizosphere, the most abundant bacterial species were rhizobia (Table 3.8 and 3.9). In the endosphere *Ensifer, Rhizobium, Rhizobiaceae, and Bradybrhiobium* were found at 35%, 10%, 4%, and 3% abundance, respectively. The endosphere also hosted highly abundant actinomycetes, particularly *Streptomyces* (Fig. 3.29). The only abundant pathogenic genus identified was *Pseudomonas*. The alfalfa bacterial wilt pathogen, *Clavibacter insidiosus* was not specifically identified but may be grouped within sequences identified only to class in the Actinobacteria ASV8, which were highly abundant in the endosphere samples. Several specific and sensitive qPCR assays have been developed for detecting *C. insidiosus*, which could be used with endosphere DNA samples to determine if the pathogen was present in these locations.

3.3. Objective #3: Quantify the abundance of known seed rot and seedling root rot pathogens from sites with poor alfalfa establishment.

DNA was extracted from rhizosphere and bulk soil and roots of alfalfa plants from the field locations in 2020 and 2021 and used in qPCR assays to quantify *A. euteiches, P. medicaginis, Pythium irregulare, P. ultimum*, and *P. sylvaticum*. Of these, *A. euteiches* was identified in highest quantities in the endosphere and rhizosphere soil across all locations. Bulk soil qPCR data is in Appendix I. At the Unity and Spencer locations *P. medicaginis* were detected in high quantities in the endosphere and rhizosphere soil. *Pythium* spp. quantities varied by sample type and location.

Aphanomyces euteiches qPCR results

Aphanomyces euteiches was detected in all plots for 2020 and 2021 locations. In 2020, *A. euteiches* was detected in both Stratica and MegaTron roots. At the Marshfield, Beaver, and Unity locations the nanograms (ng) of *A. euteiches* DNA per gram of root were similar (Fig. 3.30). The qPCR results correspond to the relative abundance of *A. euteiches* from ITS sequencing. At the West Salem location, the nanograms of *A. euteiches* per gram of root was higher in MegaTron roots compared to Stratica roots. However, at the Emerald location, the nanograms of *A. euteiches* per gram of root was higher in Stratica roots as compared to MegaTron roots.

In 2021, *A. euteiches* was more abundant in Ameristand roots as compared to MegaTron AA roots indicating that there was a cultivar influence on pathogen abundance (Fig. 3.30), which is consistent with what was observed in ITS sequencing results. These results suggest the occurrence of race 2 strains in plot locations where

the quantity of *A. euteiches* measured was higher in Ameristand roots than MegaTron AA roots, since Ameristand is highly resistant to race 1 but not race 2. Overall, the nanograms of *A. euteiches* DNA per gram of root was much higher at the 2020 plot locations as opposed to the 2021 plot locations. This could be due to the drier spring weather in 2021 compared to 2020 resulting in less pathogen infection and therefore less *A. euteiches* DNA colonizing the alfalfa roots.

From rhizosphere soil, *A. euteiches* was detected in both Stratica and MegaTron plots at the 2020 plot locations. At the Marshfield and Emerald locations the ng of *A. euteiches* DNA per gram of rhizosphere soil were similar (Fig. 3.31). Quantities were lower at the other three locations. At all locations, the nanograms of *A. euteiches* DNA per gram of soil was higher in Stratica plot soils compared to MegaTron plot soils. The largest difference between cultivars was at the West Salem and Emerald plot locations which indicates these locations may have more Aphanomyces root rot race 2 pressure. The other locations were in Clark and Marathon counties while West Salem and Emerald plots were located in La Crosse and St. Croix counties, respectively. This could indicate that race 2 isn't as prevalent in Clark and Marathon counties and that other pathogens such as *Pythium* or *Phytophthora medicaginis* may cause more pathogen pressure, or other unknown pathogens. This is similar to ITS sequencing results where the Unity and Spencer locations had higher relative abundance of *P. medicaginis* compared to *A. euteiches* abundance.

At the 2021 plot locations, *A. euteiches* was detected in both Ameristand and MegaTron plots. At all three 2021 locations, ng of *A. euteiches* DNA per g of soil in MegaTron AA plots was similar (Fig. 3.31). In contrast, values in Ameristand plots varied by location. However, quantities were higher in the race 2 susceptible cultivar, Ameristand, as opposed to the race 1 and race 2 resistant cultivar, MegaTron AA, indicating the presence of race 2 at the 2021 plot locations.

Phytophthora medicaginis qPCR results

In 2020, *P. medicaginis* nanograms of DNA per gram of root was the highest at the Unity location while quite low at the other locations (Fig. 3.32). At the Unity location, the quantity of pathogen DNA was higher in Stratica roots as compared to MegaTron roots. This indicates that while both varieties are considered highly resistant to the pathogen, MegaTron still offers more control than Stratica to *P. medicaginis* in alfalfa

seedlings. These results are consistent with ITS sequencing results in which the Unity location had the highest abundance of *P. medicaginis* of all the five 2020 plot locations.

In 2021, *P. medicaginis* nanograms of DNA per gram of root was the highest at the Spencer location and very low in the other locations. As in 2020, this is consistent with oomycete amplicon sequencing results in which *P. medicaginis* abundance was the highest at the Spencer location for the 2021 root endosphere samples. Both the Unity and Spencer plots were both in Clark and Marathon County area, indicating that *P. medicaginis* pressure in those two counties is high. At the Spencer location, the quantity of pathogen DNA was highest in Ameristand roots as compared to MegaTron AA roots. This indicates that while both varieties have high resistance to the pathogen, MegaTron AA still offers more control than Ameristand to *P. medicaginis* in alfalfa seedlings where there is high pathogen pressure. At the Frankfort and West Salem location, the pathogen DNA quantities were similar among the two varieties.

In rhizosphere soil samples, *P. medicaginis* was detected at all locations in 2020 (Fig. 3.33). The plots with the highest *P. medicaginis* quantities were at the Unity location, which is aligned with the root qPCR results. The Beaver location also had high amounts of *P. medicaginis* in the endosphere ITS sequencing data. Other plots in that region (Beaver, Marshfield, and Spencer) also had relatively high abundance of *P. medicaginis*.

In rhizosphere soil samples, *P. medicaginis* was detected at all locations in 2021 (Fig. 3.33). The plots with the highest *P. medicaginis* quantities were at the Spencer location. This is also consistent with ITS endosphere sequencing data from the Spencer location that had high abundance of *P. medicaginis* as compared to the other plots. When comparing varieties, no major differences were observed between Ameristand and MegaTron AA at the West Salem or Frankfort plots. However, at the Spencer location, where *P. medicaginis* pressure is high, there was higher *P. medicaginis* DNA in rhizosphere soil from Ameristand plots as compared to MegaTron AA plots which could mean that when *P. medicaginis* is highly abundant MegaTron AA offers more control than Ameristand even though both have high resistance Phytophthora root rot disease ratings.

Pythium spp. qPCR results

Pythium quantities varied by species and by location. *Pythium irregulare* was detected at all plot locations by qPCR but quantities were uneven with many samples with low abundance and a few with high abundance. Quantities of *P. irregulare* varied by location and sample type. Pathogen quantities in rhizosphere soil were highest at the Frankfort and Spencer locations (Fig. 3.34). Pathogen quantities in roots were highest at the West Salem 2021, Marshfield, Frankfort, and Spencer locations (Fig. 3.35).

Of all three *Pythium* spp. qPCR assays*, Pythium ultimum* was detected the most uniformly across all locations and was present in root, rhizosphere soil (Fig.3.36), and bulk soil samples although ng of pathogen DNA per g of root or soil was not always at high levels. For roots, *P. ultimum* quantities were the highest at West Salem and Frankfort locations (Fig. 3.37). Similar to sequencing abundances, *P. ultimum* quantities were highest in the rhizosphere soil samples as opposed to the roots (Fig. 3.17 and 3.19; Table 3.4 and 3.5). This indicates that at this sampling period *P. ultimum* may not be infecting the seedlings/roots but earlier in the season when *Pythium* would cause seed rot or damping-off it would be more likely to cause infection to the seedlings.

Pythium sylvaticum was detected in the highest quantities in rhizosphere soil (Fig. 3.38 and 3.39). Highest quantities were identified at the West Salem location; however, rhizosphere soil sample quantities were similar across all plot locations across both years. Amplicon sequencing results revealed relatively high abundance of *P. sylvaticum* in both the rhizosphere samples and endosphere samples across all locations (Table 3.4 and 3.5) and the qPCR assay results align with ITS sequencing results.

Table 3.1. Rain totals (inches) per week at each plot location from planting to final harvest.

		Inches of Rain Per Week		
Dates	**Week**	**Frankfort**	**Spencer**	**West Salem**
05/05/2021-05/11/2021	Week 1	0.055	0.047	0.208
05/12/2021-05/18/2021	Week 2	0.161	0.102	0.951
05/19/2021-05/25/2021	Week 3	2.492	2.142	0.839
05/26/2021-06/01/2021	Week 4	1.17	1.865	1.225
06/02/2021-06/08/2021	Week 5	0.212	0.008	1.024
06/09/2021-06/15/2021	Week 6	1.638	0.339	0.000
06/16/2021-06/22/2021	Week 7	1.343	1.346	2.232
06/23/2021-06/29/2021	Week 8	3.237	2.598	4.725
06/30/2021-07/06/2021	Week 9	1.26	1.819	0.618
07/07/2021-07/13/2021	Week 10	0.087	0.114	0.106
07/14/2021-07/20/2021	Week 11	0.728	0.63	1.732
07/21/2021-07/27/2021	Week 12	4.775	1.802	1.677
07/28/2021-08/03/2021	Week 13	0.811	0.52	1.319
08/04/2021-08/10/2021	Week 14	2.544	4.075	6.271
08/11/2021-Final Harvest	Week 15	1.244	0.295	0.488

Table 3.2. Stand counts and yield means for 2021 locations. Pooled cultivars are both Ameristand and MegaTron AA means of seven replications. Treatments 1-5 are: Treatment 1) Untreated control-34% coat, zinc and manganese micronutrients, Ascend® plant growth regulator, and Nitragin Gold rhizobium; Treatment 2) Treatment 1 + EverGol® Energy fungicide with Metalaxyl, Penflufen, and Prothioconazole active ingredients; Treatment 3) Apron XL® fungicide containing Mefenoxam active ingredient, Stamina® fungicide which contains pyraclostrobin active ingredient, Nitragin Gold rhizobium, micronutrients zinc and manganese, and Ascend® plant growth regulator; Treatment 4) Treatment 3 + Intego Solo which contains the active ingredient Ethaboxam; Treatment 5) Treatment 3 + Intego Solo + Maxim® which contains Fludioxonil active ingredient. Stand counts are plants per linear foot. Yield is dry matter tons per acre.

Location	Cultivar	Treatment	1st Stand Count	2nd Stand Count	1st Harvest Yield	2nd Harvest Yield
Frankfort	Pooled	1	35	30	1.317	1.141
Frankfort	Pooled	2	31	30	1.298	1.232
Frankfort	Pooled	3	33	31	1.326	1.285
Frankfort	Pooled	4	32	30	1.283	1.230
Frankfort	Pooled	5	33	31	1.324	1.262
Frankfort	Ameristand	1	37	30	1.250	1.126
Frankfort	Ameristand	2	32	28	1.229	1.243
Frankfort	Ameristand	3	32	31	1.309	1.306
Frankfort	Ameristand	4	34	32	1.261	1.269
Frankfort	Ameristand	5	37	32	1.353	1.296
Frankfort	MegaTron AA	1	32	29	1.383	1.156
Frankfort	MegaTron AA	2	30	32	1.366	1.221
Frankfort	MegaTron AA	3	33	30	1.344	1.264
Frankfort	MegaTron AA	4	30	29	1.305	1.190
Frankfort	MegaTron AA	5	30	30	1.294	1.228
Spencer	Pooled	1	26	25	1.084	1.240
Spencer	Pooled	2	30	30	1.035	1.230
Spencer	Pooled	3	29	30	1.100	1.235
Spencer	Pooled	4	30	28	1.111	1.223
Spencer	Pooled	5	30	32	1.077	1.237
Spencer	Ameristand	1	23	22	1.036	1.222
Spencer	Ameristand	2	31	28	0.868	1.082
Spencer	Ameristand	3	30	31	1.075	1.235
Spencer	Ameristand	4	28	27	1.024	1.188
Spencer	Ameristand	5	31	29	1.049	1.184

Spencer	MegaTron AA	1	28	29	1.133	1.259
Spencer	MegaTron AA	2	30	31	1.202	1.377
Spencer	MegaTron AA	3	28	30	1.125	1.235
Spencer	MegaTron AA	4	33	29	1.199	1.257
Spencer	MegaTron AA	5	28	34	1.105	1.291
West Salem	Pooled	1	42	38	2.000	1.423
West Salem	Pooled	2	40	40	1.991	1.463
West Salem	Pooled	3	41	39	2.022	1.438
West Salem	Pooled	4	43	41	2.048	1.472
West Salem	Pooled	5	42	45	2.120	1.538
West Salem	Ameristand	1	44	39	2.032	1.445
West Salem	Ameristand	2	46	42	1.899	1.402
West Salem	Ameristand	3	44	38	2.095	1.496
West Salem	Ameristand	4	44	42	2.078	1.433
West Salem	Ameristand	5	45	45	2.124	1.548
West Salem	MegaTron AA	1	39	38	1.968	1.400
West Salem	MegaTron AA	2	35	38	2.084	1.525
West Salem	MegaTron AA	3	38	39	1.950	1.380
West Salem	MegaTron AA	4	42	40	2.017	1.511
West Salem	MegaTron AA	5	39	44	2.116	1.527

Table 3.3. Bioassay percent resistant plants for all plot locations. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5. Abbreviations for plot locations are as follows: Beav: Beaver, WI; Em: Emerald, WI; Marsh: Marshfield, WI; Unity: Unity, WI; WS: West Salem, WI (2020 and 2021); Frank: Frankfort, WI; S: Spencer, WI.

Tmt	% R-Unity	% R-Beav	% R-Em	% R-Marsh	% R-WS	% R-Spen	% R-Frank	% R AVG
MT1	36%	63%	42%	54%	18%	28%	64%	44%
MT2	35%	36%	49%	47%	48%	41%	67%	46%
MT3	37%	43%	52%	45%	27%	52%	71%	47%
MT4	53%	58%	61%	60%	35%	39%	76%	55%
MT5	60%	42%	49%	68%	43%	54%	76%	56%
Am1	0%	0%	0%	2%	0%	0%	18%	3%
Am2	6%	0%	0%	6%	0%	0%	11%	3%
Am3	8%	6%	7%	3%	3%	17%	34%	11%
Am4	12%	6%	0%	4%	23%	9%	53%	15%
Am5	17%	0%	13%	14%	13%	8%	37%	15%
S	0%	0%	0%	0%	0%	0%	0%	0%
A	0%	0%	0%	0%	0%	0%	0%	0%
W1	0%	0%	4%	0%	0%	0%	19%	3%
W5	15%	77%	78%	69%	66%	38%	62%	58%

Table 3.4. Relative abundance (% ± standard deviation) of most abundant oomycete OTUs in the endosphere in each location/year. Abbreviations for locations and year are as follows: B.2020: Beaver, WI 2020; E.2020: Emerald, WI 2020; M.2020: Marshfield, WI 2020; U.2020: Unity, WI 2020; W.2020: West Salem, WI 2020; F.2021: Frankfort, WI 2021; S.2021: Spencer, WI 2021; W.2021: West Salem, WI 2021.

Oomycete OTU	B.2020	E.2020	M.2020	U.2020	W.2020	F.2021	S.2021	W.2021
Aphanomyces euteiches	59.5 ± 20	76.1 ± 9.6	47 ± 19.4	38 ± 23.8	79.3 ± 13.3	60.9 ± 12.1	30.4 ± 14.4	60.5 ± 20
Phytophthora medicaginis	3.2 ± 6.7	0.9 ± 1.8	0 ± 0	43.7 ± 27.9	0 ± 0.1	3.9 ± 7.1	30.8 ± 19.5	1.9 ± 2.5
Phytophthora sansomeana	17.9 ± 21.5	0 ± 0	25.1 ± 16.4	0.3 ± 0.6	0 ± 0	0.6 ± 1.6	5.1 ± 9.7	0.7 ± 1.8
Pythium inflatum	11.7 ± 9.1	0.1 ± 0.2	11.5 ± 13.2	10.3 ± 5.6	0.1 ± 0.1	2 ± 2.2	7.1 ± 4.8	0 ± 0
Globisporangium sylvaticum	1.5 ± 0.7	3.1 ± 3.5	0.2 ± 0.3	1.1 ± 1.6	9.4 ± 5	3.8 ± 3.8	6.6 ± 5.6	6.2 ± 3.6
Globisporangium perplexum	0.8 ± 0.5	0.8 ± 1.4	5.4 ± 3.2	0 ± 0	0 ± 0	3.8 ± 4.2	5.5 ± 7.5	0 ± 0
Peronospora	0.5 ± 0.5	1.1 ± 1.1	0 ± 0	0.4 ± 0.5	0.7 ± 1.3	7 ± 13.8	1 ± 2	2.9 ± 6.4
Pythium aristosporum	0.1 ± 0.3	12 ± 8.5	0 ± 0	0.2 ± 0.2	2.7 ± 2.6	1.9 ± 5.2	0.3 ± 0.6	0.6 ± 0.9
Pythium inflatum	1.9 ± 1.4	0 ± 0	2.2 ± 3.4	3 ± 1.9	0 ± 0	0.5 ± 0.8	3.3 ± 3.2	0 ± 0
Globisporangium heterothallicum	0.3 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.5	3.9 ± 9.4	1 ± 0.8	1 ± 1.3	3 ± 3.5
Globisporangium attrantheridium	0.5 ± 0.4	0.2 ± 0.3	0.3 ± 0.2	0.2 ± 0.3	0.6 ± 0.6	3.6 ± 2.6	1.6 ± 1.7	1.2 ± 1.7
Pythium	0.1 ± 0.2	0.8 ± 2.2	1 ± 1.2	0.2 ± 0.4	0 ± 0	1.1 ± 1.1	0.1 ± 0.2	3.3 ± 6.3
Pythium conidiophorum	0.4 ± 0.5	0.7 ± 1.9	0.9 ± 1	0.2 ± 0.5	0.1 ± 0.1	1.7 ± 1.8	0.1 ± 0.1	2 ± 4.2
Pythium	0.4 ± 0.5	1 ± 0.7	0 ± 0	0.4 ± 0.6	2.4 ± 2.7	0.3 ± 0.5	0.4 ± 0.8	2 ± 2.7
Globisporangium	0 ± 0	1.8 ± 1.8	0 ± 0	0 ± 0	0.1 ± 0.3	0.8 ± 1.4	0.2 ± 0.4	2.2 ± 2.5
Globisporangium lucens	0 ± 0	1.6 ± 2	0 ± 0.1	0 ± 0	0.2 ± 0.3	0 ± 0	0 ± 0	2.9 ± 4.2
Globisporangium sylvaticum	0.5 ± 0.4	0.2 ± 0.3	0 ± 0	0.3 ± 0.3	1.6 ± 1.1	0.6 ± 1.4	0.4 ± 0.4	1.5 ± 1.2
Peronospora	0.1 ± 0.1	0.2 ± 0.2	0 ± 0	0.3 ± 0.5	0.4 ± 1	1.2 ± 2.7	0.2 ± 0.3	1.4 ± 4
Globisporangium heterothallicum	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.4	0.1 ± 0.1	0.5 ± 0.4	0.6 ± 0.5	0.6 ± 0.8	1.3 ± 1.7
Globisporangium	0.3 ± 0.4	0 ± 0	0 ± 0.1	0 ± 0.1	0 ± 0	0.4 ± 1.4	2 ± 2.3	0.3 ± 1.1
Pythiaceae	0 ± 0	0.2 ± 0.4	2.1 ± 3.8	0 ± 0	0 ± 0	1 ± 1.1	0.4 ± 0.7	0 ± 0
Globisporangium	0 ± 0	0 ± 0	2.5 ± 4.4	0 ± 0	0 ± 0	0.9 ± 1.2	0 ± 0	0 ± 0
Globisporangium pleroticum	0.1 ± 0.2	0.2 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.6 ± 1.5	0.2 ± 0.6	0.2 ± 0.3	1.2 ± 2.8
Globisporangium nov.	0 ± 0	0.5 ± 0.7	0 ± 0	0 ± 0	0.1 ± 0.2	0 ± 0	0 ± 0.1	1.3 ± 1.7
Pythiaceae	0 ± 0.1	0 ± 0	1.7 ± 3.3	0 ± 0	0 ± 0	0.5 ± 0.8	0 ± 0	0 ± 0
Pythium	0 ± 0.1	0 ± 0	0 ± 0	1.6 ± 1.6	0 ± 0	0 ± 0	0.4 ± 1	0 ± 0

Pythium	0.9 ± 1.1	0.2 ± 0.3	0 ± 0.1	0.3 ± 0.4	0 ± 0	0.4 ± 0.7	0.2 ± 0.2	0 ± 0
Globisporangium ultimum	0 ± 0	0 ± 0.1	0 ± 0	0 ± 0	0.3 ± 0.7	0 ± 0	0 ± 0	1.1 ± 2.7
Globisporangium	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.2 ± 4.3	0 ± 0
Oomycetes	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.3 ± 0.5	0 ± 0	0.9 ± 1.3

Table 3.5. Relative abundance (% ± standard deviation) of most abundant oomycete OTUs in the rhizosphere in each location/year. Abbreviations for locations and year are as follows: B.2020: Beaver, WI 2020; E.2020: Emerald, WI 2020; M.2020: Marshfield, WI 2020; U.2020: Unity, WI 2020; W.2020: West Salem, WI 2020; F.2021: Frankfort, WI 2021; S.2021: Spencer, WI 2021; W.2021: West Salem, WI 2021.

Oomycete OTU	B.2020	E.2020	M.2020	U.2020	W.2020	F.2021	S.2021	W.2021
Aphanomyces euteiches	37.4 ± 13.7	44 ± 8.9	43.1 ± 18.6	38.5 ± 14.7	23.4 ± 17.3	33.7 ± 16.4	41.4 ± 14.4	47.8 ± 19.2
Phytophthora medicaginis	7.1 ± 1.6	10 ± 3.9	14.3 ± 12.8	7.6 ± 5.4	10.2 ± 4.5	16.1 ± 14.7	16 ± 7.3	7.5 ± 3.7
Phytophthora sansomeana	0.9 ± 2.5	0.1 ± 0.3	0.1 ± 0.1	14.2 ± 16.3	21 ± 11.1	6.5 ± 10	0.3 ± 0.6	0.6 ± 0.8
Pythium inflatum	4.2 ± 3.4	9.7 ± 9.2	10.3 ± 17.3	6.7 ± 15.4	1.1 ± 0.5	1.2 ± 1.9	2.1 ± 2.8	5.6 ± 10.9
Globisporangium sylvaticum	3.3 ± 0.7	3.5 ± 1.5	4.7 ± 2.2	7.7 ± 10	3 ± 2	2.8 ± 4	6.6 ± 3.2	5.2 ± 2.3
Globisporangium perplexum	4.4 ± 2.4	3.9 ± 2.3	1.4 ± 0.8	2.3 ± 1.1	4.4 ± 3.2	2 ± 2.1	8.1 ± 4.8	6.2 ± 2.6
Peronospora	2.1 ± 1	3 ± 1.6	2.1 ± 1.6	1.5 ± 1.3	3.5 ± 4.9	4.8 ± 3.5	3 ± 1.9	4.7 ± 2.5
Pythium aristosporum	9.1 ± 17	0 ± 0	6.3 ± 14.2	0.6 ± 1.1	3.2 ± 7	7 ± 8.5	0.6 ± 2.1	0.4 ± 0.4
Pythium inflatum	1.7 ± 1.3	0.1 ± 0.2	0.7 ± 1.1	9 ± 10.9	9 ± 5.2	3.6 ± 4.1	0.5 ± 0.9	0.4 ± 1.4
Globisporangium heterothallicum	1.9 ± 0.5	2.3 ± 1.1	3.2 ± 1.6	3.7 ± 4.5	1.9 ± 1.3	1.5 ± 1.9	3.4 ± 1.3	2.9 ± 1.3
Globisporangium attrantheridium	0.1 ± 0.1	3.8 ± 2.8	0.7 ± 1	0.2 ± 0.2	0.3 ± 0.6	1.2 ± 2.5	5.3 ± 7.8	1.2 ± 0.8
Pythium	0.2 ± 0.1	0.2 ± 0.2	0.9 ± 1	0.7 ± 0.9	0.9 ± 1.4	2.8 ± 3.9	0.6 ± 1.2	5.7 ± 8.1
Pythium conidiophorum	1.1 ± 1.2	0.4 ± 0.2	1.3 ± 1.7	0.7 ± 1	0.4 ± 0.7	3.7 ± 4.5	0.5 ± 0.8	3.2 ± 3.2
Pythium	6.6 ± 5.6	0.1 ± 0.2	1.3 ± 1.6	0.8 ± 0.6	3.2 ± 2.6	2.1 ± 4.9	0.3 ± 0.4	0.1 ± 0.2
Globisporangium	0.3 ± 0.6	0.1 ± 0.2	1.4 ± 1.3	1.6 ± 2.1	5.9 ± 6.7	2.4 ± 3.7	0.2 ± 0.5	0 ± 0
Globisporangium lucens	2 ± 2.3	0.4 ± 0.7	4.3 ± 3.7	0.9 ± 1.5	2 ± 2.3	1 ± 1.1	0.5 ± 0.6	0.2 ± 0.2
Globisporangium sylvaticum	0.2 ± 0.4	3.1 ± 5.8	0.2 ± 0.3	1.2 ± 2.9	0.5 ± 0.4	0.2 ± 0.3	1.4 ± 1.3	2.3 ± 2.7
Peronospora	1 ± 0.7	2.1 ± 1.4	1.1 ± 0.6	0.8 ± 1	0.4 ± 0.4	0.6 ± 0.9	1.5 ± 3.3	0.9 ± 1.2
Globisporangium heterothallicum	0.2 ± 0.5	2.4 ± 1.5	0.5 ± 1	0.1 ± 0.1	0.1 ± 0.2	1.7 ± 3.2	1.1 ± 2.3	0.8 ± 0.4
Globisporangium	0.8 ± 0.6	0.2 ± 0.2	0.2 ± 0.3	0.1 ± 0.1	0.2 ± 0.1	0.6 ± 0.7	1.7 ± 0.9	1.8 ± 1
Pythiaceae	1.2 ± 0.8	0 ± 0	4.5 ± 4.3	0.5 ± 0.8	0.2 ± 0.2	0.2 ± 0.4	1 ± 2	0 ± 0.1
Globisporangium	0.8 ± 0.3	1 ± 0.4	0.6 ± 0.4	0.7 ± 0.6	0.8 ± 0.7	0.6 ± 0.9	1 ± 0.5	0.7 ± 0.4
Globisporangium pleroticum	0.5 ± 0.4	0.5 ± 0.3	0.3 ± 0.2	0.3 ± 0.5	0.5 ± 0.7	1.3 ± 1.7	0.7 ± 0.4	1.1 ± 0.7
Globisporangium nov.	0.6 ± 1	1.4 ± 0.6	0.7 ± 1.4	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	1.2 ± 2.7	0.9 ± 0.7
Pythiaceae	0.3 ± 0.4	0 ± 0	0.1 ± 0.1	2.1 ± 2.1	2 ± 1.3	1 ± 1.7	0.1 ± 0.3	0 ± 0.1
Pythium	0 ± 0	0 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0.4 ± 1.5	1 ± 1.7	1.9 ± 1.4

Pythium	5.4 ± 5.2	0 ± 0	0 ± 0.1	0.1 ± 0.1	0.3 ± 0.5	0 ± 0.1	0 ± 0	0 ± 0
Globisporangium ultimum	0 ± 0	1.7 ± 1.1	0 ± 0	0 ± 0	0.1 ± 0.2	0 ± 0	0.7 ± 0.8	1.1 ± 1.4
Globisporangium	0.5 ± 1.2	0 ± 0	0 ± 0	1.3 ± 2.5	1.5 ± 1.8	0.5 ± 1.8	0.1 ± 0.5	0 ± 0.1
Oomycetes	0.3 ± 0.2	0.2 ± 0.1	0.5 ± 1.2	2.8 ± 7.8	0.1 ± 0.2	0.3 ± 0.5	0 ± 0.1	0 ± 0

Table 3.6. Relative abundance (% ± standard deviation) of most abundant fungal ASV's in the endosphere in each location/year. Abbreviations for locations and year are as follows: B.2020: Beaver, WI 2020; E.2020: Emerald, WI 2020; M.2020: Marshfield, WI 2020; U.2020: Unity, WI 2020; W.2020: West Salem, WI 2020; F.2021: Frankfort, WI 2021; S.2021: Spencer, WI 2021; W.2021: West Salem, WI 2021.

Fungal ASV	B.2020	E.2020	M.2020	U.2020	W.2020	F.2021	S.2021	W.2021
Fusarium waltergamsii	24.31 +/- 9.1	43.25 +/- 21.12	22.6 +/- 20.13	25.96 +/- 16.73	19.21 +/- 12.01	39.14 +/- 23.25	20.16 +/- 17.3	28.19 +/- 23.1
Plectosphaerella	5.79 +/- 0.6	4.15 +/- 3.51	14.5 +/- 12.91	8.44 +/- 14.46	11.55 +/- 12.2	9.02 +/- 7.5	16.56 +/- 10.12	13.08 +/- 8.52
Fusarium acutatum	15.53 +/- 5.73	20.84 +/- 5.25	8.82 +/- 5.34	14.14 +/- 10.68	11.29 +/- 7.8	9.23 +/- 7.9	11.13 +/- 9.28	12.16 +/- 5.47
Fusarium	14.08 +/- 8.73	13.93 +/- 7.92	7.36 +/- 7.33	5.96 +/- 2.54	16.03 +/- 20.76	6.28 +/- 8.32	7.66 +/- 7.64	4.23 +/- 4.91
Ceratobasidiaceae	0.88 +/- 1.51	0 +/- 0	8.25 +/- 15.01	2.02 +/- 3.82	3.47 +/- 8.58	12.37 +/- 17.59	7 +/- 7.98	9.32 +/- 12.1
Paraphoma radicina	2.58 +/- 1.52	1.4 +/- 2.18	7.09 +/- 8.24	7.52 +/- 9.58	7.02 +/- 4.3	3.83 +/- 3.24	4.37 +/- 6.23	1.42 +/- 1.89
Setophoma terrestris	0 +/- 0	0.05 +/- 0.08	3.97 +/- 5.03	11.76 +/- 7.34	14.23 +/- 15.27	0.82 +/- 1.21	0.67 +/- 1.97	0 +/- 0.01
Apodus deciduus	1.62 +/- 0.42	3.26 +/- 2.97	2.8 +/- 6.93	0 +/- 0	0 +/- 0	3.47 +/- 6.27	1.13 +/- 2.73	3.12 +/- 3.91
Ceratobasidiaceae	7.42 +/- 8.15	0.68 +/- 1.24	1.46 +/- 3.4	0 +/- 0	0 +/- 0	0.3 +/- 0.85	0.93 +/- 2.94	6.63 +/- 6.77
Glomeraceae	3.9 +/- 1.13	1.53 +/- 0.98	0.37 +/- 0.72	0.07 +/- 0.14	0.01 +/- 0.02	2.94 +/- 4.73	3.71 +/- 2.88	1.74 +/- 1.43
s__*Ilyonectria macrodidyma*|ASV40	1.37 +/- 1.13	0.19 +/- 0.13	0.1 +/- 0.08	0.72 +/- 1.57	0.38 +/- 0.69	2.07 +/- 2.81	3.44 +/- 4.78	3.44 +/- 5.8
Setophoma terrestris	1.51 +/- 0.87	3 +/- 1.75	1.65 +/- 1.63	2.1 +/- 4.02	2.07 +/- 2.28	1.37 +/- 2.8	1.09 +/- 1.19	1.4 +/- 1.93
Alternaria	0.73 +/- 0.4	2.37 +/- 4.63	5.08 +/- 10.99	0.61 +/- 0.63	3.29 +/- 6.12	1.33 +/- 2.29	0.8 +/- 0.8	0.89 +/- 1.02
Nectriaceae	0.06 +/- 0.04	0.41 +/- 0.62	5.07 +/- 7.27	3.43 +/- 4.01	1.87 +/- 2.46	1.43 +/- 2.49	0.57 +/- 0.64	0.2 +/- 0.28
Ascomycota	9.98 +/- 0.88	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	0.98 +/- 3.65	4.86 +/- 7.12
Didymella	0.48 +/- 0.16	2.4 +/- 1.92	2.08 +/- 5.23	0.03 +/- 0.03	0.02 +/- 0.02	1.49 +/- 1.57	1.67 +/- 2.29	1.57 +/- 3.06
Paraphoma pye	4.91 +/- 4.22	0.4 +/- 0.58	0.07 +/- 0.15	0.02 +/- 0.05	0.01 +/- 0.03	0.47 +/- 0.74	0.98 +/- 1.24	4.06 +/- 6.27
Fusarium	0.63 +/- 0.55	4.14 +/- 3.79	2.05 +/- 4.98	0.01 +/- 0.02	0.02 +/- 0.02	0.98 +/- 1.1	1.24 +/- 1.13	0.41 +/- 0.35

Ceratobasidiaceae	1.07 +/- 1.26	0 +/- 0	0.26 +/- 0.44	1.74 +/- 3.04	0.83 +/- 1.39	2.53 +/- 5.97	0.95 +/- 1.56	0.5 +/- 0.68
Colletotrichum	0.09 +/- 0.01	0.07 +/- 0.08	1.39 +/- 2.24	0.26 +/- 0.48	0.05 +/- 0.13	0.4 +/- 1.03	3.4 +/- 6.96	0.77 +/- 1.54
Sordariomycetes	0.15 +/- 0.02	0.07 +/- 0.09	1.32 +/- 2.16	1.62 +/- 2.13	2.96 +/- 6.3	0.58 +/- 1.2	1.36 +/- 2.32	0.61 +/- 1.08
Dothideomycetes	0 +/- 0	0 +/- 0	0.08 +/- 0.16	4.64 +/- 10.35	5.74 +/- 6.91	0.28 +/- 0.37	0 +/- 0.01	0.01 +/- 0.02
Mycoleptodiscus terrestris	0 +/- 0	0 +/- 0	2.83 +/- 5.21	4.08 +/- 8.15	2.27 +/- 5.91	0.2 +/- 0.31	0 +/- 0	0 +/- 0.01
Nectriaceae	0.42 +/- 0.47	0.25 +/- 0.26	1.76 +/- 2.3	0.82 +/- 0.69	0.69 +/- 0.61	0.76 +/- 1.23	0.83 +/- 0.85	1.07 +/- 2.69
Corynespora cassiicola	1.26 +/- 0.48	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	1.02 +/- 2.64	2.25 +/- 2.96	0.62 +/- 0.6
Mortierella minutissima	0.95 +/- 0.57	0.12 +/- 0.28	0.71 +/- 1.02	0.35 +/- 0.31	0.12 +/- 0.18	0.97 +/- 1.46	1.77 +/- 2.48	0.38 +/- 0.39
Paraphoma rhaphiolepidis	4.47 +/- 3.75	0.37 +/- 0.36	0.03 +/- 0.06	0 +/- 0	0.08 +/- 0.21	0.56 +/- 0.85	0.67 +/- 1	1.33 +/- 1.4
Paraphoma radicina	2.9 +/- 1.71	1.12 +/- 1.21	0.29 +/- 0.61	0.07 +/- 0.12	0.04 +/- 0.07	0.13 +/- 0.17	0.35 +/- 0.49	1.88 +/- 1.78
Paraphoma radicina	1.94 +/- 0.58	0.41 +/- 0.61	0.18 +/- 0.27	0.41 +/- 0.61	0.99 +/- 1.55	0.55 +/- 1.72	0.85 +/- 1.28	1.34 +/- 1.48
Rhizoctonia	0.45 +/- 0.9	0 +/- 0	2.06 +/- 5.82	0.82 +/- 1.51	0.03 +/- 0.07	0.84 +/- 2.26	0.56 +/- 1.28	0.5 +/- 0.87

Table 3.7. Relative abundance (% ± standard deviation) of most abundant fungal ASV's in the rhizosphere in each location/year. Abbreviations for locations and year are as follows: B.2020: Beaver, WI 2020; E.2020: Emerald, WI 2020; M.2020: Marshfield, WI 2020; U.2020: Unity, WI 2020; W.2020: West Salem, WI 2020; F.2021: Frankfort, WI 2021; S.2021: Spencer, WI 2021; W.2021: West Salem, WI 2021.

Fungal ASV	B.2020	E.2020	M.2020	U.2020	W.2020	F.2021	S.2021	W.2021
Mortierella minutissima	13 +/- 6.26	11.7 +/- 5.14	11.24 +/- 6.54	29.94 +/- 16.82	22.56 +/- 12.79	6.87 +/- 2.83	8.73 +/- 4.81	9.14 +/- 5.12
Pseudombrophila	6.86 +/- 5.06	5.41 +/- 4.83	1.91 +/- 3.32	0.38 +/- 0.33	0.6 +/- 0.95	19.7 +/- 21.68	14.29 +/- 6.66	20.75 +/- 13.04
Plectosphaerella	5.41 +/- 3.36	0.86 +/- 1.17	13.23 +/- 14.84	7.63 +/- 8.86	14.65 +/- 17.2	2.13 +/- 1.06	8.85 +/- 5.8	10.27 +/- 8.04
Fusarium waltergamsii	5.23 +/- 3.68	9.7 +/- 7.66	2.8 +/- 1.8	5.8 +/- 4.93	17.3 +/- 32.24	5.61 +/- 8	3.69 +/- 4.7	4.37 +/- 5.09
Nectriaceae	0.6 +/- 0.5	1.1 +/- 0.77	11.96 +/- 11.7	10.14 +/- 5.38	10.96 +/- 9.32	2.16 +/- 3.23	3.43 +/- 3.54	0.78 +/- 0.56
Sordariomycetes	1.69 +/- 0.45	1.02 +/- 0.62	11.31 +/- 16.01	10.34 +/- 4.25	10.12 +/- 5.48	0.67 +/- 0.5	3.48 +/- 3.08	1.92 +/- 1.09
Sordariales	11.6 +/- 7.36	3.89 +/- 3.14	1.73 +/- 4.13	0 +/- 0	0.1 +/- 0.28	3.8 +/- 3.58	3.26 +/- 3.47	7.88 +/- 5.56
Fusarium acutatum	3.94 +/- 2.57	5.23 +/- 3.73	4.7 +/- 5.02	3.96 +/- 2.17	4.1 +/- 3.48	3.24 +/- 2.35	3.44 +/- 2.88	3.47 +/- 5.39
Alternaria	1.82 +/- 1.55	3.95 +/- 6.3	7.83 +/- 7.87	6.71 +/- 7.07	4.88 +/- 4.61	3.47 +/- 5.98	3.22 +/- 2.32	2.63 +/- 1.98
Mortierella	5.95 +/- 2.11	2.14 +/- 2.86	0.34 +/- 0.72	0.01 +/- 0.01	0.02 +/- 0.06	0.31 +/- 0.4	7.46 +/- 4.1	8.99 +/- 4.52
Didymella	4.95 +/- 2.79	5.85 +/- 5.55	0.97 +/- 1.92	0.57 +/- 1.23	0.11 +/- 0.1	4.23 +/- 3.9	4.62 +/- 2.83	4.13 +/- 3.24
Lasiosphaeriaceae	0.8 +/- 0.44	3.09 +/- 2.21	0.98 +/- 1.93	0.01 +/- 0.02	0.04 +/- 0.07	11.62 +/- 8.91	3.07 +/- 2.48	1.32 +/- 1.37
Cladorrhinum foecundissimum	7.66 +/- 3.95	5.58 +/- 6.05	2.11 +/- 4.26	0 +/- 0	0.07 +/- 0.2	5.05 +/- 6.31	2.01 +/- 2.2	2.82 +/- 3.6
Nectriaceae	1 +/- 0.59	1.06 +/- 0.69	3.17 +/- 1.93	6.31 +/- 3.38	6.09 +/- 5.58	2 +/- 2.99	5.2 +/- 3.64	0.92 +/- 1
Thelebolus globosus	6.59 +/- 1.97	2.23 +/- 4.27	0.17 +/- 0.42	0.01 +/- 0.02	0.05 +/- 0.13	3.97 +/- 4.86	1.34 +/- 0.81	8.41 +/- 5.5
Lasiosphaeriaceae	2.79 +/- 1.16	9.78 +/- 5.95	2.44 +/- 5.23	0.01 +/- 0.01	0.03 +/- 0.09	0.11 +/- 0.17	1.52 +/- 1.25	6.61 +/- 4.62
Cladosporium delicatulum	1.76 +/- 1.31	0.94 +/- 0.61	2.38 +/- 1.76	3.52 +/- 2.85	2.59 +/- 2.45	2.62 +/- 3.8	4.21 +/- 3.04	2.9 +/- 2.91
Sordariales	4.14 +/- 2.14	1.67 +/- 2.72	0.07 +/- 0.12	0 +/- 0.01	0.02 +/- 0.05	1.55 +/- 2.33	3.48 +/- 3.16	3.03 +/- 1.43

Ceratobasidiaceae	3.18 +/- 4.06	0.02 +/- 0.05	4.14 +/- 7.14	0.02 +/- 0.02	2.49 +/- 6.17	2.18 +/- 4.86	2.66 +/- 3.31	1.12 +/- 2.64
Mortierella	4.22 +/- 2.24	1.76 +/- 1.3	2.41 +/- 1.24	4.57 +/- 3.31	3.83 +/- 1.85	0.03 +/- 0.04	0.27 +/- 0.39	1.16 +/- 1.59
Leotiomycetes	0.82 +/- 0.49	1.56 +/- 0.8	2.68 +/- 4.23	2 +/- 3.86	1.36 +/- 1.71	1.65 +/- 2.36	1.44 +/- 1.51	2.11 +/- 3.45
Didymellaceae	0.05 +/- 0.05	0.05 +/- 0.06	4.81 +/- 12.98	0.24 +/- 0.43	0.51 +/- 0.95	0.53 +/- 0.85	3.95 +/- 9.79	1.3 +/- 2.62
Mortierella exigua	0.02 +/- 0.03	0.81 +/- 0.96	2.19 +/- 1.62	4.22 +/- 2.9	4.22 +/- 2.25	1 +/- 1.56	1.11 +/- 1.01	0.2 +/- 0.35
Mortierella hyalina	2.57 +/- 4.58	1.29 +/- 0.96	0.73 +/- 0.52	0.7 +/- 0.42	2.23 +/- 4.8	2.34 +/- 2.81	0.64 +/- 0.63	1.1 +/- 0.51
Mortierella	0.06 +/- 0.04	1.53 +/- 1.51	3.34 +/- 1.61	5.14 +/- 3.49	4.05 +/- 2.25	0.23 +/- 0.24	0.06 +/- 0.05	0.06 +/- 0.06
Podospora	0.66 +/- 0.3	1.59 +/- 0.75	1.32 +/- 0.61	1.29 +/- 0.83	1.17 +/- 0.93	2.55 +/- 1.23	1.36 +/- 0.65	0.7 +/- 0.31
Apodus deciduus	0.67 +/- 0.47	0.64 +/- 0.46	0.19 +/- 0.41	0 +/- 0	0.03 +/- 0.08	8.19 +/- 21.63	0.05 +/- 0.09	1.32 +/- 2.32
Sporormiaceae	4.41 +/- 1.62	1.34 +/- 2.22	0.04 +/- 0.08	0 +/- 0.01	0.01 +/- 0.02	0.61 +/- 0.94	2.22 +/- 2.39	1.38 +/- 1.93
Podospora multipilosa	0.87 +/- 0.36	2.24 +/- 1	1.32 +/- 2.46	0.03 +/- 0.03	0.02 +/- 0.03	2.29 +/- 2.06	0.91 +/- 0.68	1.13 +/- 0.79
Colletotrichum	2.13 +/- 3.36	0.75 +/- 0.8	2.59 +/- 3.72	2.24 +/- 4.16	0.5 +/- 1.13	0.43 +/- 1.25	0.79 +/- 0.57	0.63 +/- 0.81

Table 3.8. Relative abundance (% ± standard deviation) of most abundant bacterial ASV's in the endosphere in each location/year. Abbreviations for locations and year are as follows: B.2020: Beaver, WI 2020; E.2020: Emerald, WI 2020; M.2020: Marshfield, WI 2020; U.2020: Unity, WI 2020; W.2020: West Salem, WI 2020; F.2021: Frankfort, WI 2021; S.2021: Spencer, WI 2021; W.2021: West Salem, WI 2021.

Bacterial ASV	B.2020	E.2020	M.2020	U.2020	W.2020	F.2021	S.2021	W.2021
Ensifer	47.75 +/- 28.79	87.32 +/- 49.61	25.16 +/- 37.16	21.27 +/- 37.81	54.97 +/- 53.93	11.29 +/- 16.36	39.7 +/- 47.2	31.43 +/- 46.71
Streptomyces	12.87 +/- 10.07	5.81 +/- 0.96	6.92 +/- 2.59	7.52 +/- 4.76	6.52 +/- 4.01	7.67 +/- 6.76	13.41 +/- 7.88	7.8 +/- 5.53
Rhizobium	24.85 +/- 21.15	0.11 +/- 0.1	23.03 +/- 16.62	12.55 +/- 17.56	3.36 +/- 4.29	1.94 +/- 2.87	11.38 +/- 20.82	0.15 +/- 0.08
Micromonosporaceae	3.14 +/- 1.62	4.28 +/- 1.51	6.05 +/- 2.14	7.37 +/- 3.27	4.15 +/- 1.4	3.3 +/- 2.12	3.1 +/- 2.12	4.85 +/- 2.51
Actinobacteria	6.15 +/- 2.97	3.23 +/- 1.15	3.35 +/- 1.53	3.82 +/- 2.08	3.11 +/- 2.28	2.04 +/- 1.39	5.27 +/- 3.11	4.77 +/- 3.57
Pseudomonas	1.51 +/- 1.02	1.53 +/- 1.12	2.46 +/- 0.98	2.4 +/- 1.68	2.99 +/- 2.02	9 +/- 14.11	2.88 +/- 3.13	3.14 +/- 5.16
Rhizobiaceae	1.84 +/- 1.11	0.98 +/- 0.4	4.53 +/- 3.16	3.83 +/- 1.66	3.69 +/- 2.47	4.2 +/- 2.52	3.71 +/- 2.58	4.36 +/- 3.5
Streptomyces	4.96 +/- 0.89	1.52 +/- 0.55	2.77 +/- 1.68	2.88 +/- 0.75	5.69 +/- 2.97	6.7 +/- 4.58	2.57 +/- 1.41	1.41 +/- 1.01
Bradyrhizobium	3.8 +/- 2.2	1.92 +/- 0.7	2.31 +/- 1.18	2.87 +/- 1.66	2.16 +/- 0.78	2.89 +/- 1.46	3.81 +/- 1.56	3.43 +/- 1.49
Steroidobacter	0.86 +/- 0.59	0.54 +/- 0.58	3.75 +/- 2.16	2.55 +/- 1.9	2.16 +/- 3.77	6 +/- 4.59	2.62 +/- 1.29	2.6 +/- 1.8
Novosphingobium	1.49 +/- 0.83	1.09 +/- 0.66	2.95 +/- 2.17	5.14 +/- 3.76	3.4 +/- 2.03	3.38 +/- 1.28	1.95 +/- 0.97	2.7 +/- 1.51
Streptomyces	0.22 +/- 0.1	4.12 +/- 2.29	2.26 +/- 2.07	4.21 +/- 3.45	5.42 +/- 2.64	2.11 +/- 1.72	1.38 +/- 1.48	2 +/- 1.11
Saccharibacteria_genera_incertae_sedis	1.15 +/- 0.38	1.35 +/- 0.53	1.22 +/- 0.57	0.88 +/- 0.61	0.97 +/- 0.58	2.7 +/- 2.9	6.34 +/- 5.04	2.26 +/- 1.27
Niastella	2.13 +/- 0.96	3.08 +/- 1.79	4.23 +/- 3.08	1.89 +/- 1.44	1.77 +/- 1.87	4.49 +/- 3.17	0.79 +/- 0.54	1.38 +/- 1.28
Micromonosporaceae	1.41 +/- 0.76	3.87 +/- 1.07	0.68 +/- 0.58	3 +/- 4.28	8.95 +/- 5.76	0.92 +/- 0.65	0.43 +/- 0.79	0.81 +/- 0.64
Streptomyces	2.64 +/- 0.75	1.01 +/- 0.53	1.72 +/- 1.06	1.31 +/- 0.76	1.25 +/- 1.45	3.32 +/- 1.37	2.55 +/- 1.75	1.37 +/- 0.76
Saccharibacteria_genera_incertae_sedis	3.09 +/- 1.44	0.23 +/- 0.27	0.22 +/- 0.29	0.49 +/- 0.92	1.62 +/- 1.5	2.91 +/- 1.85	3.99 +/- 3.33	1.32 +/- 1.32
Actinoplanes	3.32 +/- 1.66	3.53 +/- 0.91	1.93 +/- 0.99	1.16 +/- 0.77	1.04 +/- 2.06	0.64 +/- 0.52	1.73 +/- 2.37	2.28 +/- 1.32

Actinoplanes	14.19 +/- 4.49	0.03 +/- 0.04	0.28 +/- 0.28	0.07 +/- 0.12	0.58 +/- 1.17	1.29 +/- 1.08	2.4 +/- 2.25	0.61 +/- 0.78
Steroidobacter	0.83 +/- 0.61	3.4 +/- 1.71	1.72 +/- 0.94	1.77 +/- 1.48	0.82 +/- 0.93	1.3 +/- 1.11	1.78 +/- 1.77	3.08 +/- 1.76
Flavobacterium	0.1 +/- 0.09	0.17 +/- 0.21	3.47 +/- 2.73	3.46 +/- 3.51	0.94 +/- 1.18	1.66 +/- 1.15	0.95 +/- 1.06	1.46 +/- 1.38
Aquabacterium	0.5 +/- 0.48	0.46 +/- 0.23	1.46 +/- 0.71	1.46 +/- 0.64	0.86 +/- 0.77	1.67 +/- 0.78	1.89 +/- 2.1	2.43 +/- 1.4
Steroidobacter	0.49 +/- 0.41	1.98 +/- 0.81	2.17 +/- 1.57	2.26 +/- 1.83	0.92 +/- 0.41	1.76 +/- 1.66	0.65 +/- 1.04	1.24 +/- 0.94
Myxococcales	0.13 +/- 0.22	0.5 +/- 0.34	0 +/- 0	0.05 +/- 0.13	0.4 +/- 0.85	0.06 +/- 0.16	1.75 +/- 3.58	5.44 +/- 5.83
Acidovorax	0.64 +/- 0.33	0.16 +/- 0.07	2.61 +/- 1.24	1.69 +/- 0.7	2.17 +/- 2.32	1.93 +/- 1.21	0.71 +/- 0.65	0.5 +/- 0.28
Nonomuraea	1.5 +/- 1.12	0.07 +/- 0.05	1.55 +/- 0.6	1.18 +/- 1.38	0.25 +/- 0.48	3.2 +/- 2.18	0.78 +/- 0.95	0.16 +/- 0.15
Niastella	0.93 +/- 0.49	0.66 +/- 0.33	1.73 +/- 1.08	1.27 +/- 0.66	1.06 +/- 0.7	1.88 +/- 1.29	0.79 +/- 0.47	0.85 +/- 0.69
Niastella	2.3 +/- 1.02	0.43 +/- 0.42	2.06 +/- 0.77	1.46 +/- 1.36	0.58 +/- 0.5	1.62 +/- 1.16	0.7 +/- 0.95	0.17 +/- 0.16
Rhizobacter	0.78 +/- 0.18	0.29 +/- 0.17	2.52 +/- 1.14	3.04 +/- 1.89	1.33 +/- 1.2	0.56 +/- 0.27	0.42 +/- 0.34	0.51 +/- 0.65
Flavobacterium	0.11 +/- 0.26	0.52 +/- 0.47	1.7 +/- 0.94	1.62 +/- 0.97	0.48 +/- 0.67	1.05 +/- 1.37	1.79 +/- 3.38	1.1 +/- 1.76

Table 3.9. Relative abundance (% ± standard deviation) of most abundant bacterial ASV's in the rhizosphere in each location/year. Abbreviations for locations and year are as follows: B.2020: Beaver, WI 2020; E.2020: Emerald, WI 2020; M.2020: Marshfield, WI 2020; U.2020: Unity, WI 2020; W.2020: West Salem, WI 2020; F.2021: Frankfort, WI 2021; S.2021: Spencer, WI 2021; W.2021: West Salem, WI 2021.

Bacterial ASV	B.2020	E.2020	M.2020	U.2020	W.2020	F.2021	S.2021	W.2021
Ensifer	32.32 +/- 31.27	45.42 +/- 62.54	73.66 +/- 79.67	38.42 +/- 39.16	43.98 +/- 65.18	53.72 +/- 47.91	10.66 +/- 12.87	26.06 +/- 33.11
Rhizobium	7.09 +/- 17.15	0.18 +/- 0.13	25.91 +/- 23.09	24.12 +/- 19.98	5.57 +/- 14.4	2.04 +/- 3.84	1.87 +/- 3.97	0.47 +/- 0.26
Micrococcaceae	7.78 +/- 3.62	4.86 +/- 1.91	7.83 +/- 3.32	11.74 +/- 6.42	11.07 +/- 7.68	6.96 +/- 3.29	5.84 +/- 3.44	6.5 +/- 4.89
Bradyrhizobium	5.3 +/- 1.73	5.33 +/- 2.09	5.95 +/- 4.52	4 +/- 2.11	4.18 +/- 1.77	5.58 +/- 4.03	6.83 +/- 3.53	4.4 +/- 1.77
Pseudomonas	5.31 +/- 13.15	8.26 +/- 19.32	7.78 +/- 9.13	2.53 +/- 3.08	2.36 +/- 4.44	4.7 +/- 3.88	2.38 +/- 3.74	1.04 +/- 1.28
Micrococcaceae	2.62 +/- 1.14	2.15 +/- 1.62	4.59 +/- 1.78	3.98 +/- 1.66	1.54 +/- 0.75	2.54 +/- 1.32	3.33 +/- 1.81	2.82 +/- 1.48
Bacillus	0.1 +/- 0.11	0.32 +/- 0.22	1.7 +/- 2.05	0.55 +/- 0.54	0.11 +/- 0.11	0.52 +/- 0.53	6.24 +/- 7.81	8.52 +/- 10.65
Acidobacteria	3.78 +/- 2.27	3.72 +/- 1.18	1.63 +/- 0.42	1.77 +/- 0.99	1.87 +/- 1.16	2.38 +/- 1.48	3.51 +/- 1.48	4.24 +/- 1.8
Nitrospira	2.65 +/- 1.31	2.76 +/- 1.31	1.84 +/- 0.81	1.3 +/- 0.65	2.09 +/- 1.12	3.18 +/- 1.87	4.11 +/- 2.04	4.63 +/- 3.02
Ilumatobacter	4.4 +/- 2.09	2.37 +/- 0.77	3.84 +/- 1.58	5.03 +/- 3.1	3.58 +/- 2.04	3.32 +/- 1.91	1.14 +/- 0.55	1.56 +/- 0.98
Flavobacterium	0.2 +/- 0.16	0.94 +/- 1.59	8.1 +/- 8.62	5.29 +/- 5	0.63 +/- 0.84	4.71 +/- 5.87	2.33 +/- 2.75	1.49 +/- 1.06
Fulvivirgaceae	0.51 +/- 0.3	4.15 +/- 2.12	2.69 +/- 1.2	4.96 +/- 3.76	6.53 +/- 4.82	3.11 +/- 2.08	0.4 +/- 0.22	0.63 +/- 0.41
Saccharibacteria_genera_incertae_sedis	0.47 +/- 0.25	3.55 +/- 2.79	2.09 +/- 1.53	1.02 +/- 1.39	0.78 +/- 1.46	2.47 +/- 3.16	5.06 +/- 3.91	4.33 +/- 7.19
Rhizobiales	2.65 +/- 1.63	2.91 +/- 1.85	1.26 +/- 0.6	1.71 +/- 0.4	2.15 +/- 1.02	2.05 +/- 1.01	3.11 +/- 1.44	2.58 +/- 1.24
Saccharibacteria_genera_incertae_sedis	23.66 +/- 22.92	0.05 +/- 0.08	1.93 +/- 3.76	2.44 +/- 4.08	0.02 +/- 0.04	0.51 +/- 1.72	0.03 +/- 0.06	0.02 +/- 0.08
Betaproteobacteria	2.51 +/- 0.79	2.43 +/- 1.07	1.29 +/- 0.41	2.2 +/- 0.66	2.62 +/- 1.29	1.33 +/- 0.46	1.95 +/- 1.12	3.83 +/- 2.17
Neobacillus	0.13 +/- 0.04	0.59 +/- 0.32	0.35 +/- 0.24	0.33 +/- 0.27	0.14 +/- 0.12	0.53 +/- 0.39	4.98 +/- 7.57	7.83 +/- 7.47
Flavobacterium	0.04 +/- 0.1	0.67 +/- 0.94	5.71 +/- 4.36	4.06 +/- 4.45	0.14 +/- 0.07	2.48 +/- 4.48	1.72 +/- 2.08	2.86 +/- 3.43

Novosphingobium	0.95 +/- 0.24	1.38 +/- 1.3	4.2 +/- 3.82	2.85 +/- 2.18	1.46 +/- 1.11	2.58 +/- 2.13	2 +/- 1.91	2.34 +/- 1.64
Stenotrophobacter	2.14 +/- 0.88	3.22 +/- 2.14	1.56 +/- 0.57	1.49 +/- 0.46	2.18 +/- 0.89	2.65 +/- 1.36	2.3 +/- 2.33	1.17 +/- 0.78
Streptomyces	1.1 +/- 1.13	1.01 +/- 0.56	5.96 +/- 6.82	1.22 +/- 1.07	1.08 +/- 1.05	1.78 +/- 1.87	1.53 +/- 0.83	2.99 +/- 3.74
Aeromicrobium	5.22 +/- 3.45	1.27 +/- 0.72	3.38 +/- 2.41	2.37 +/- 0.84	1.7 +/- 0.93	2.06 +/- 1.63	0.39 +/- 0.26	0.53 +/- 0.48
Gp7	2.04 +/- 1.27	1.02 +/- 0.78	1.02 +/- 0.49	1.26 +/- 1.07	0.98 +/- 1.53	2.49 +/- 1.83	3.66 +/- 3.25	2.26 +/- 1.71
Ilumatobacteraceae	2.49 +/- 1.09	2.39 +/- 1.2	0.84 +/- 0.5	1.81 +/- 1.1	5.05 +/- 4.02	1.63 +/- 1.4	1.38 +/- 0.81	1.08 +/- 0.81
Gp16	2.47 +/- 0.86	0.93 +/- 0.27	1.8 +/- 0.9	2.45 +/- 0.92	2.51 +/- 1.66	2.53 +/- 1.32	1.81 +/- 1.75	1.16 +/- 0.78
Rhizobiaceae	0.96 +/- 0.68	1.15 +/- 0.73	2.83 +/- 2.19	1.99 +/- 0.53	1.13 +/- 1.07	1.51 +/- 1.17	2.4 +/- 2	1.88 +/- 1.07
Chryseolinea	2.08 +/- 1.02	1.5 +/- 1.04	2.79 +/- 1.53	4.17 +/- 1.88	4.13 +/- 3.06	1.64 +/- 0.63	0.87 +/- 0.66	0.44 +/- 0.28
Micromonosporaceae	1.59 +/- 0.53	1.71 +/- 1.74	2.37 +/- 1.34	2.4 +/- 1.55	0.86 +/- 0.37	2.11 +/- 1.32	1.48 +/- 0.87	1.1 +/- 0.66
Gp6	2.85 +/- 1.54	1.7 +/- 1.3	1.6 +/- 0.99	2.7 +/- 1.53	2.13 +/- 1.16	1.31 +/- 0.56	1.33 +/- 0.69	1.47 +/- 0.77
Gammaproteobacteria_incertae_sedis	1.07 +/- 0.51	1.23 +/- 0.96	1.93 +/- 1.01	1.58 +/- 0.27	2.23 +/- 0.96	2.03 +/- 1.03	1.3 +/- 0.97	1.61 +/- 1.41

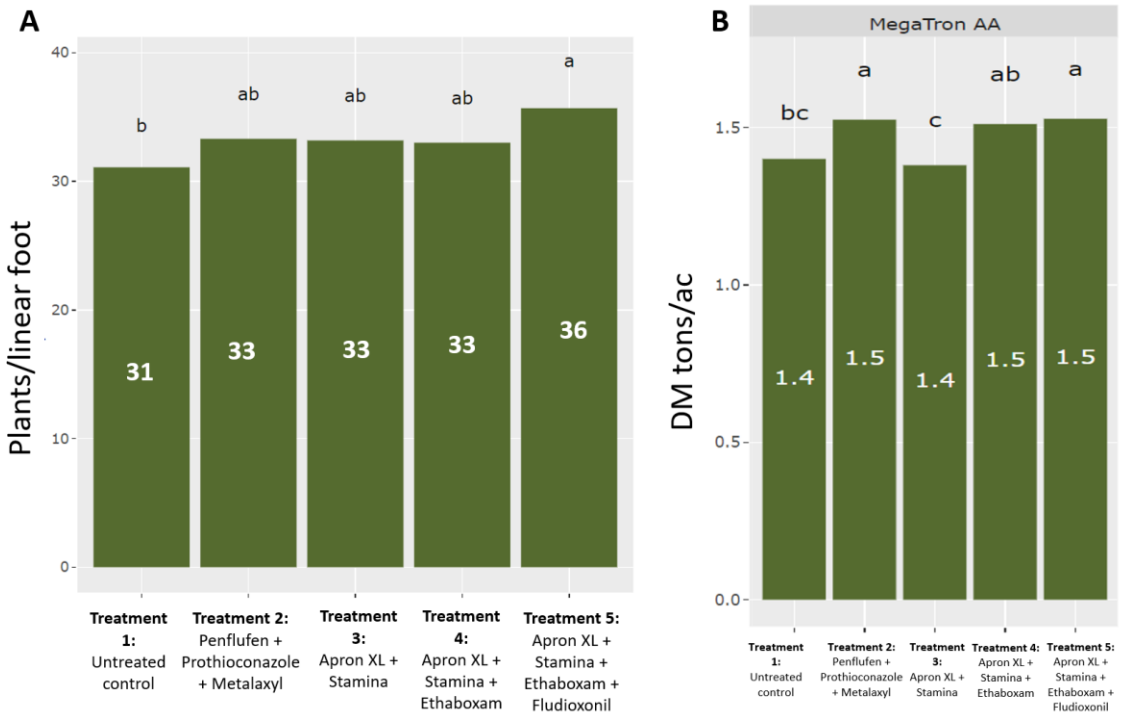


Figure 3.1 Stand counts in plants per linear foot and yield in dry matter (DM) tons per acre in which significant differences among treatments were observed. A) Second stand count per linear foot over all locations and both cultivars (p=0.067). B) Second harvest yield in dry matter tons per acre in MegaTron AA cultivar at West Salem, WI location (p=0.043). Different letters above bars indicate significant differences.

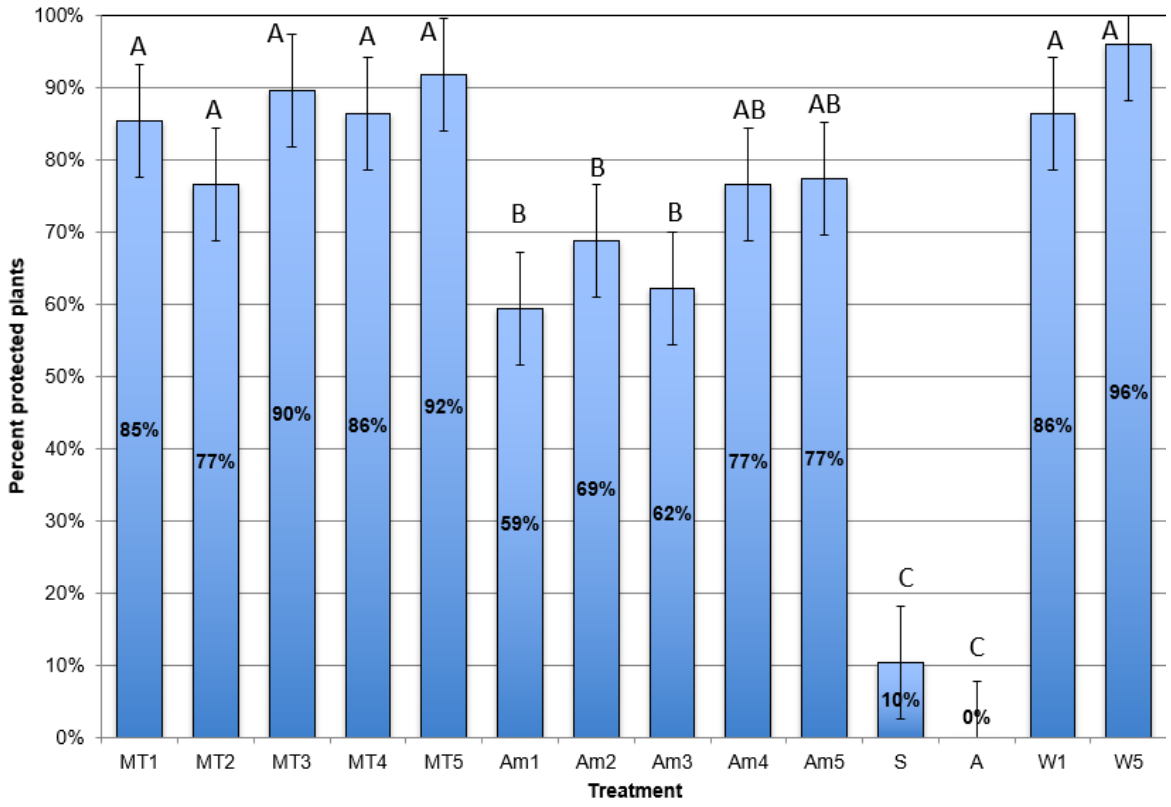


Figure 3.2. Percent protected plants from *Aphanomyces euteiches* race 1. Different letters above bars indicate significant differences (p=0.0001). Bars are means of three replications with standard deviation. Resistant plants were scored 1 or 2 with no or minimal disease symptoms. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

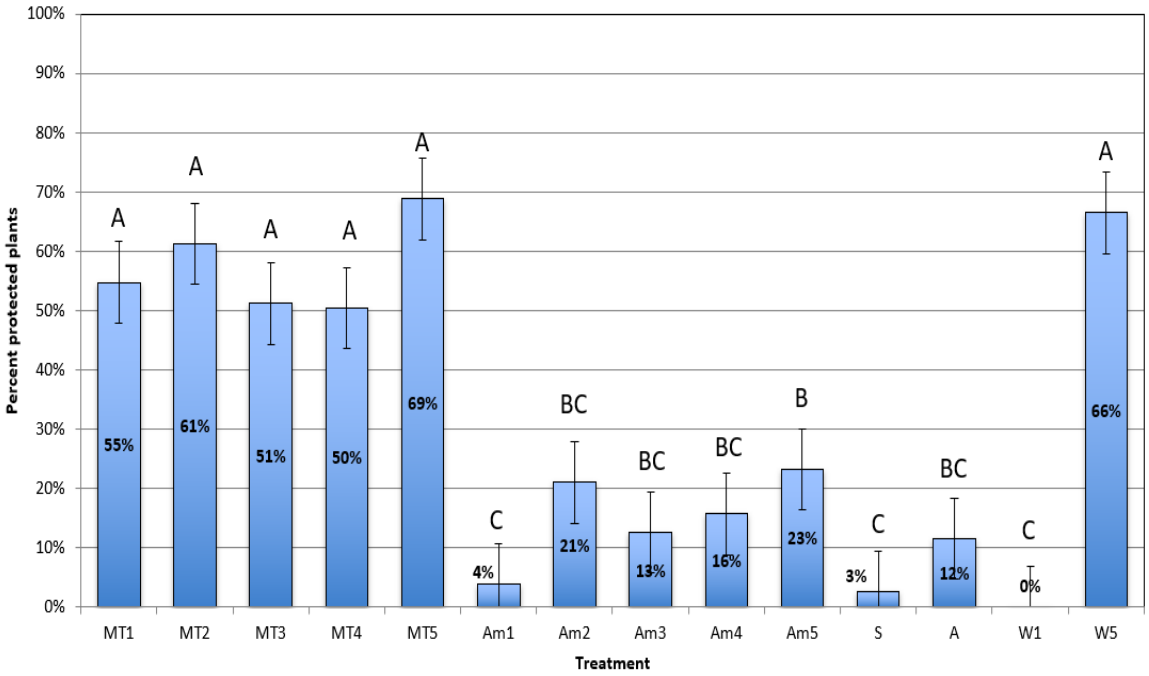


Figure 3.3. Percent protected plants from *Aphanomyces euteiches* race 2-MER4 isolate. Different letters above bars indicate significant differences (p=0.0001). Bars are means of three replications with standard deviation. Resistant plants were scored 1 or 2 with no or minimal disease symptoms. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

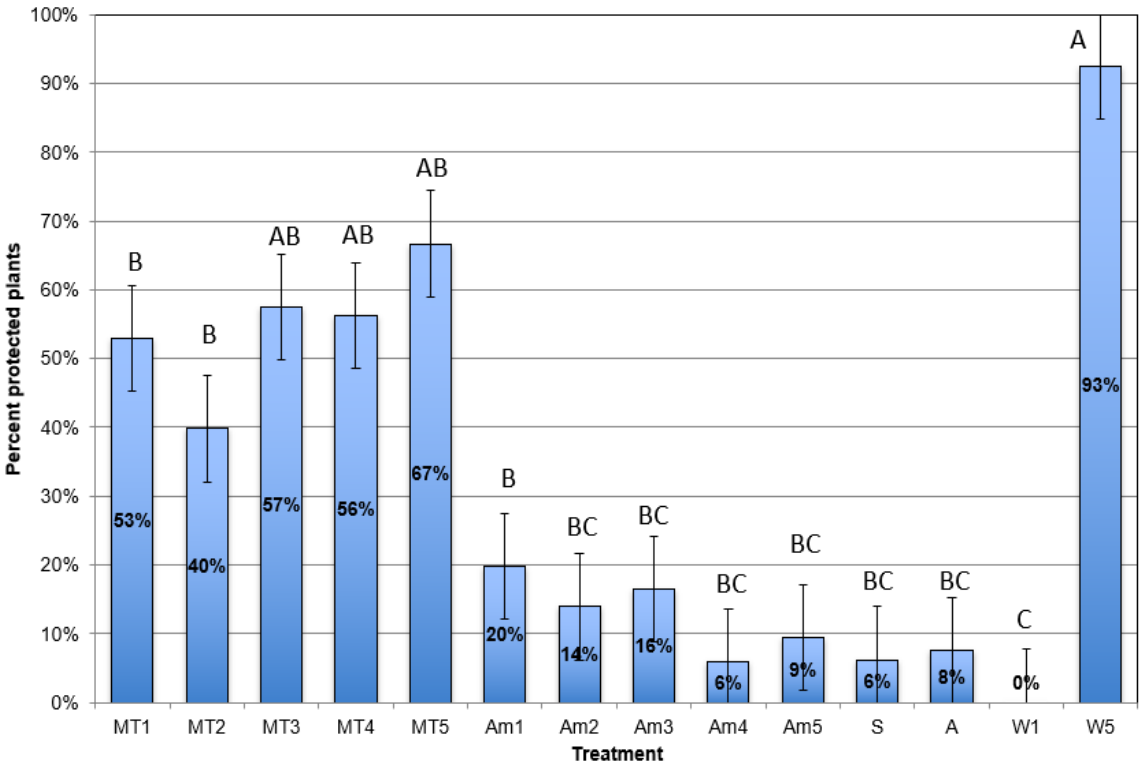


Figure 3.4. Percent protected plants from *Aphanomyces euteiches* race 2-NC1 isolate. Different letters above bars indicate significant differences (p=0.0001). Bars are means of three replications with standard deviation. Resistant plants were scored 1 or 2 with no or minimal disease symptoms. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

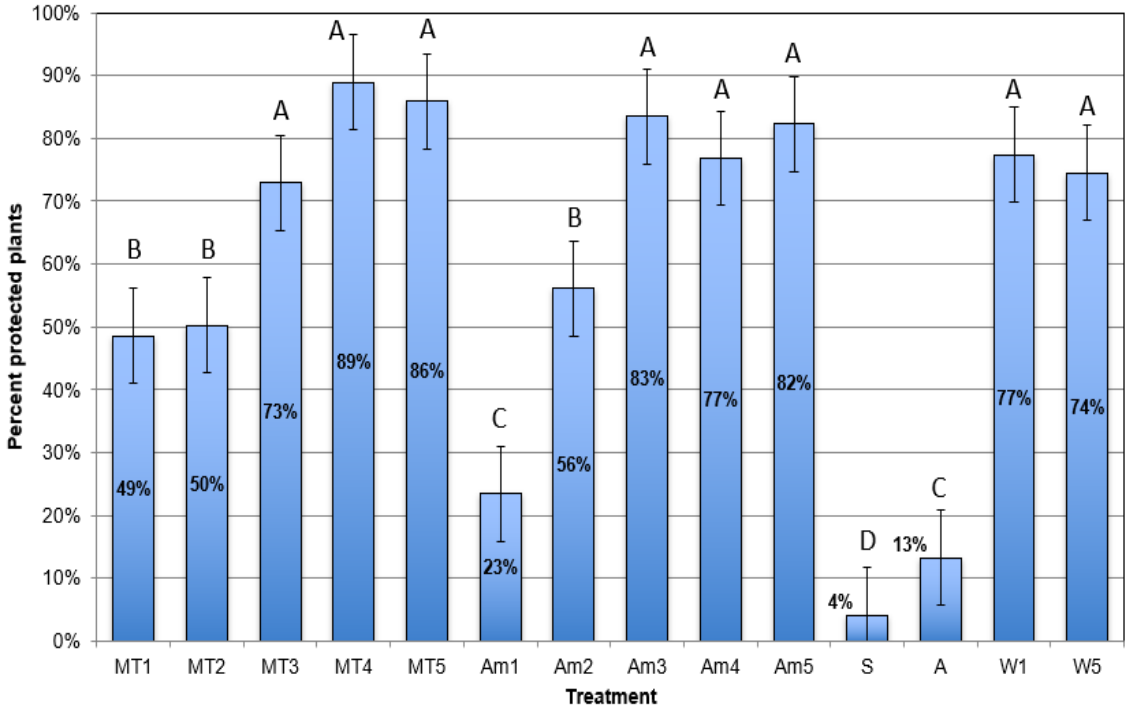


Figure 3.5. Percent protected plants from *Phytophthora medicaginis*. Different letters above bars indicate significant differences (p=0.0001). Bars are means of three replications with standard deviation. Resistant plants were scored 1 or 2 with no or minimal disease symptoms. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

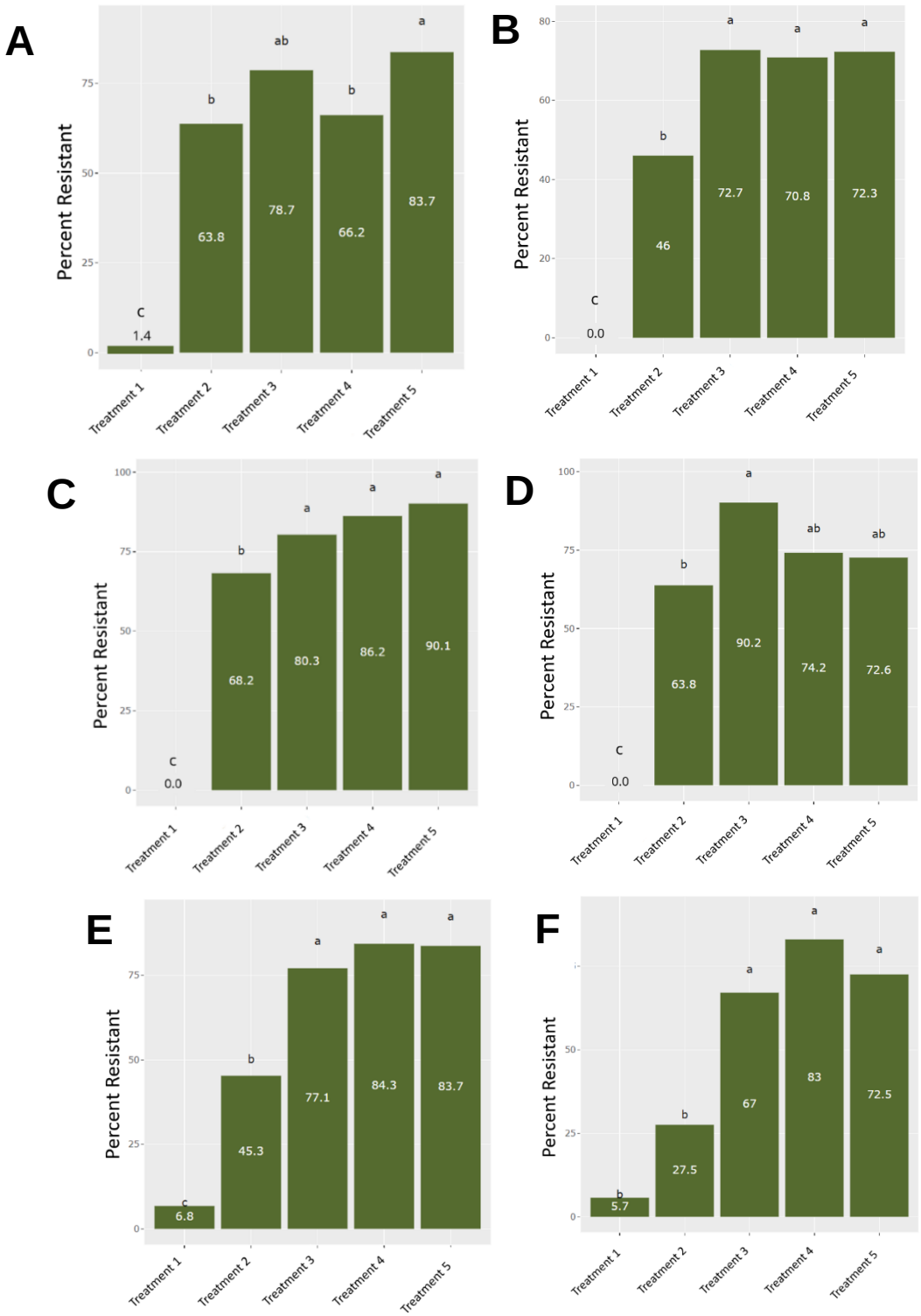


Figure 3.6. *Pythium* spp. standard test results. Different letters above bars indicate significant differences (p=0.0001). Bars are means of three replications. Resistant plants were scored 1 or 2 with no or minimal disease symptoms. A) Percent resistant plants to *Pythium irregulare* in MegaTron AA treatments 1 through 5. B) Percent resistant plants

to *Pythium irregulare* in Ameristand treatments 1 through 5. C) Percent resistant plants to *Pythium ultimum* in Megatron AA treatments 1 through 5. D) Percent resistant plants to *Pythium ultimum* in Ameristand treatments 1 through 5. E) Percent resistant plants to *Pythium paroecandrum* in Megatron AA treatments 1 through 5. F) Percent resistant plants to *Pythium paroecandrum* in Ameristand treatments 1 through 5. Treatments are as follows: Treatment 1: Untreated Control (Nitragen rhizobia, Zn, Mn); Treatment 2: Penflufen + Prothioconazole + Metalaxyl (EverGol Energy); Treatment 3: Mefenoxam + Pyraclostrobin (Apron XL + Stamina); Treatment 4: Treatment 3 + Ethaboxam (Treatment 3 + Intego Solo); Treatment 5: Treatment 3 + Ethaboxam + Fludioxonil (Treatment 3 + Intego Solo + Maxim).

Figure 3.7. Photos of *Phytophthora sansomeana* symptoms on 26-day old, infected alfalfa seedlings from the standard pathogen test where pathogenicity of *P. sansomeana* was evaluated on alfalfa seedlings using the *P. medicaginis* standard test protocol. A) Infected seedlings. B) Three infected seedlings on the left with one healthy seedling on the right.

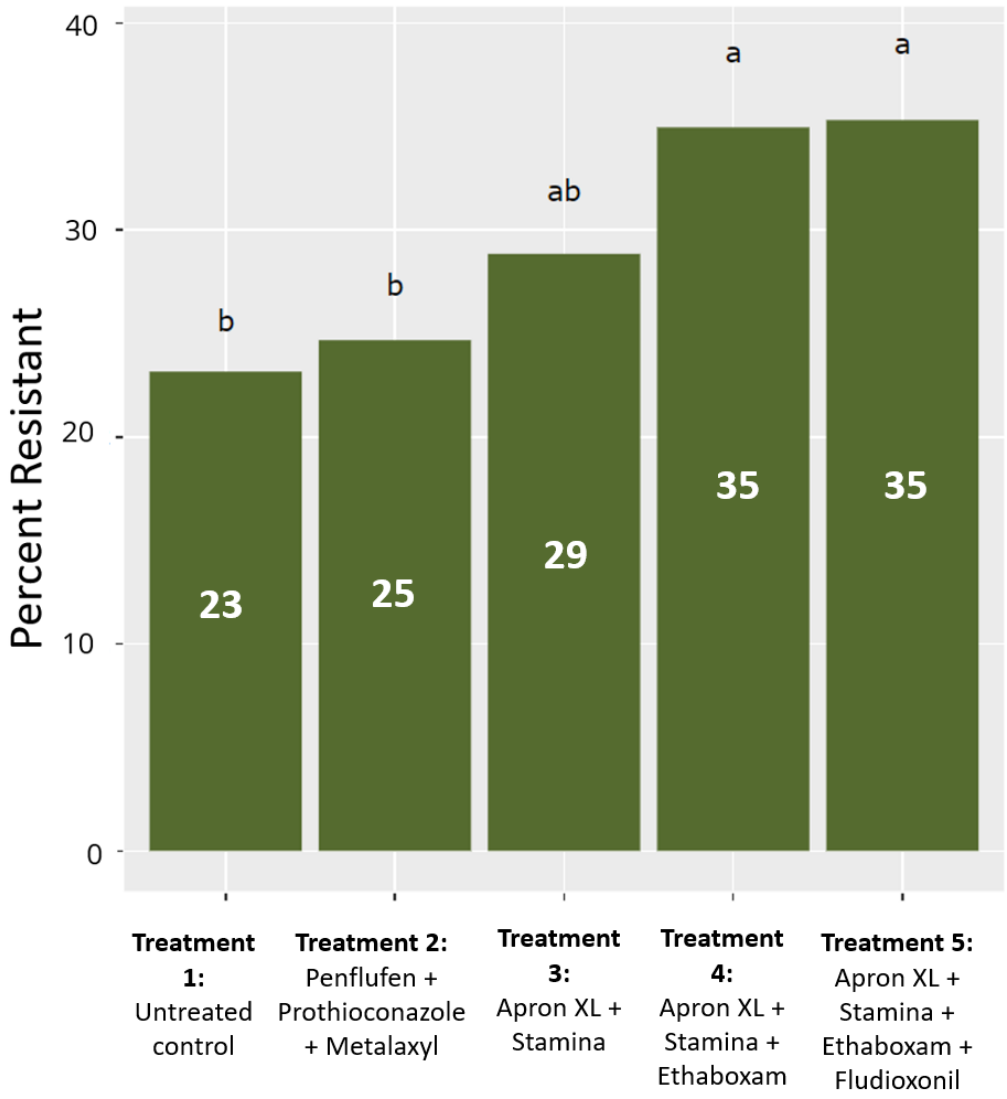


Figure 3.8. Percent protected plants to pathogens in growth chamber soil bioassays over all seven locations and both cultivars. Different letters above bars indicate significant differences (p=0.0001). Bars are means of three replications.

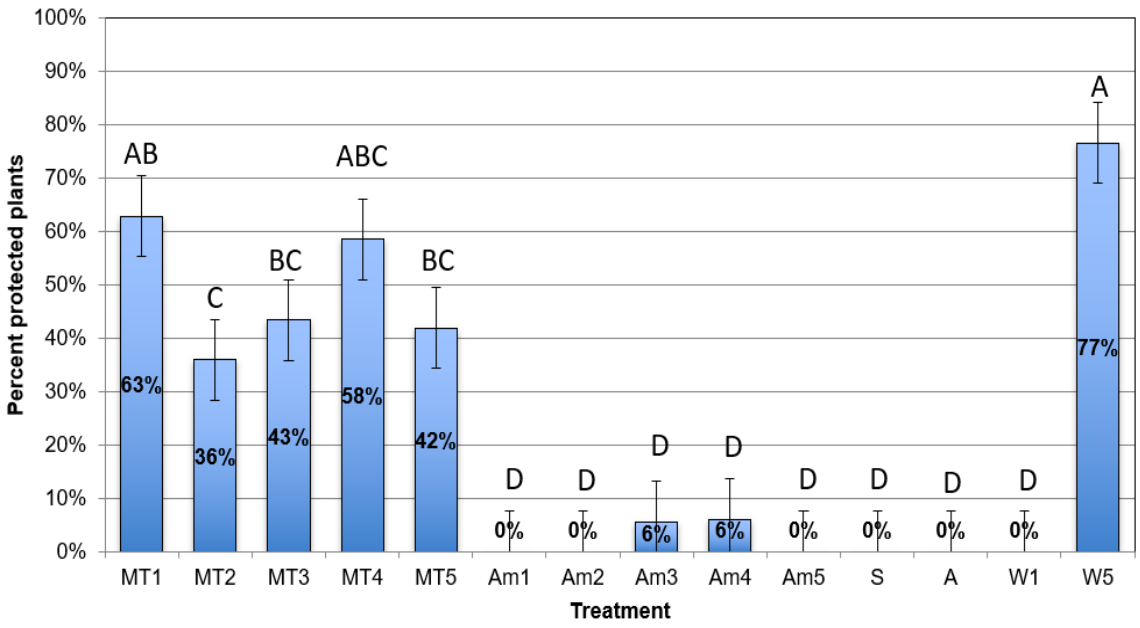


Figure 3.9. Percent protected plants to pathogens found at the Beaver, WI location in growth chamber soil bioassays. Different letters above bars indicate significant differences (p=0.0001). Bars are means of three replications with standard deviation. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

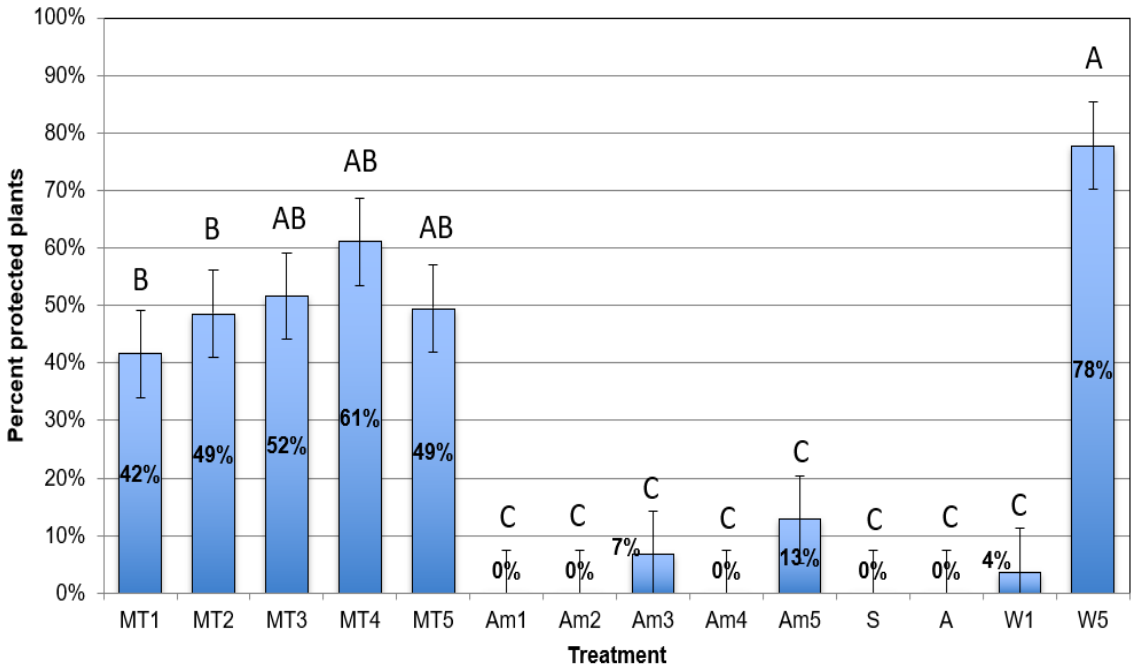


Figure 3.10. Percent protected plants to pathogens found at the Emerald, WI location in growth chamber soil bioassays. Different letters above bars indicate significant differences (p=0.0001). Bars are means of three replications with standard deviation. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

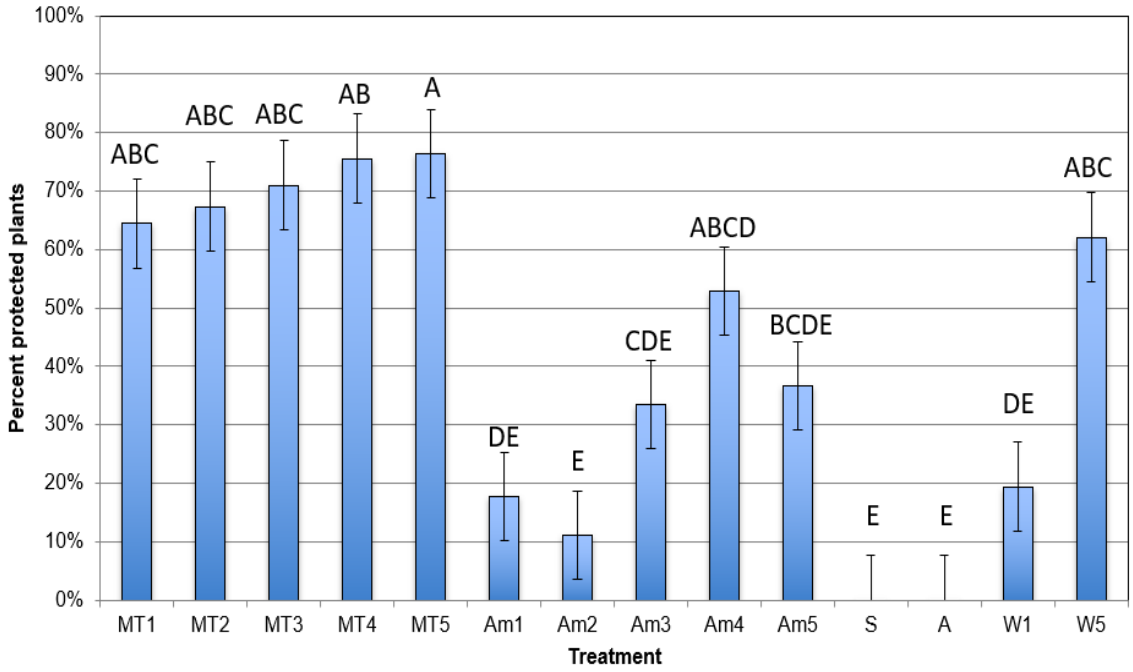


Figure 3.11. Percent protected plants to pathogens found at Frankfort, WI location in growth chamber soil bioassays. Different letters above bars indicate significant differences (p=0.001). Bars are means of three replications with standard deviation. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

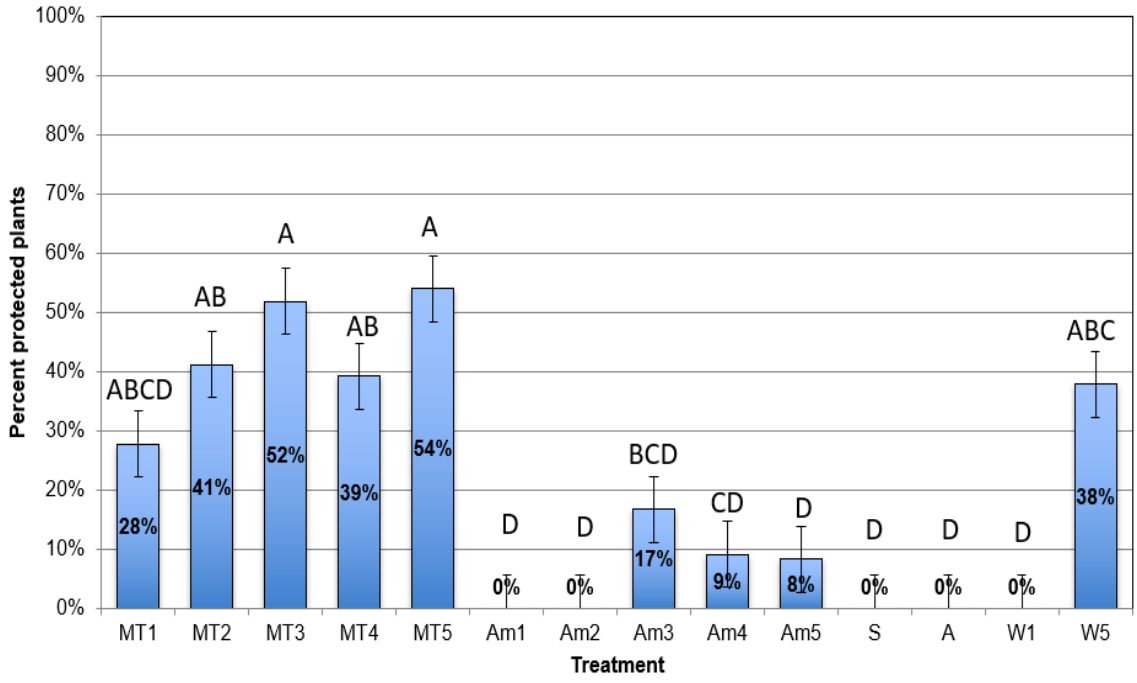


Figure 3.12. Percent protected plants to pathogens found at the Spencer, WI location in growth chamber soil bioassays. Different letters above bars indicate significant differences (p=0.001). Bars are means of three replications with standard deviation. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

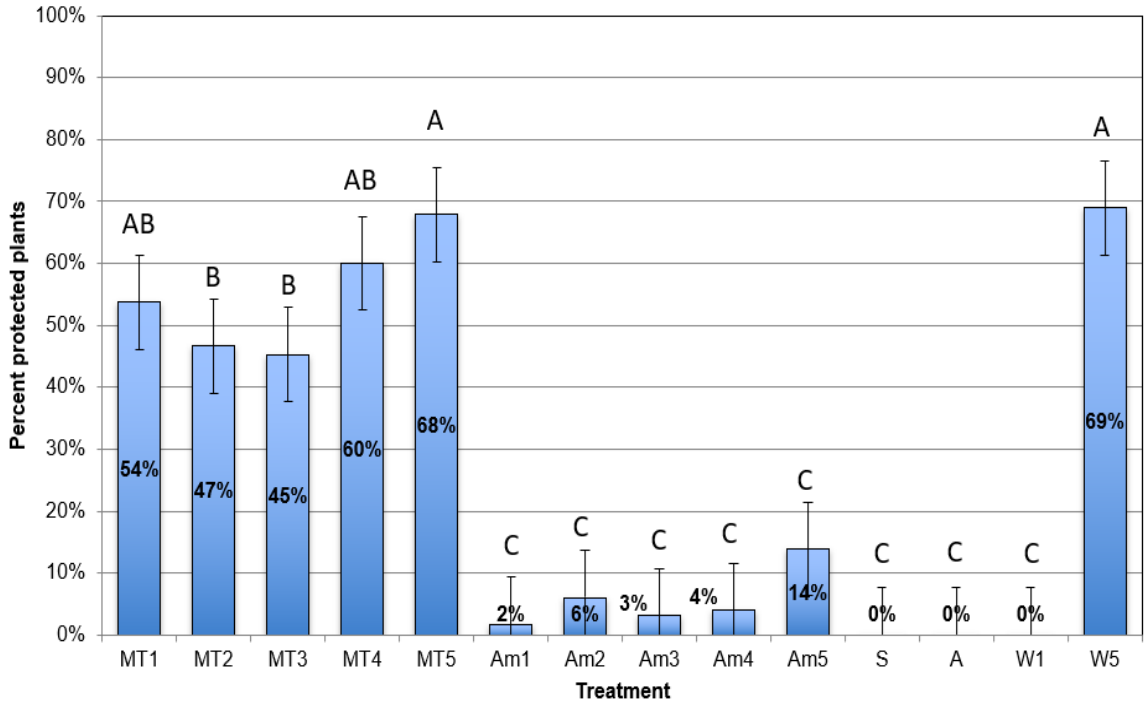


Figure 3.13. Percent protected plants to pathogens found at the Marshfield, WI location in growth chamber soil bioassays. Different letters above bars indicate significant differences (p=0.001). Bars are means of three replications with standard deviation. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

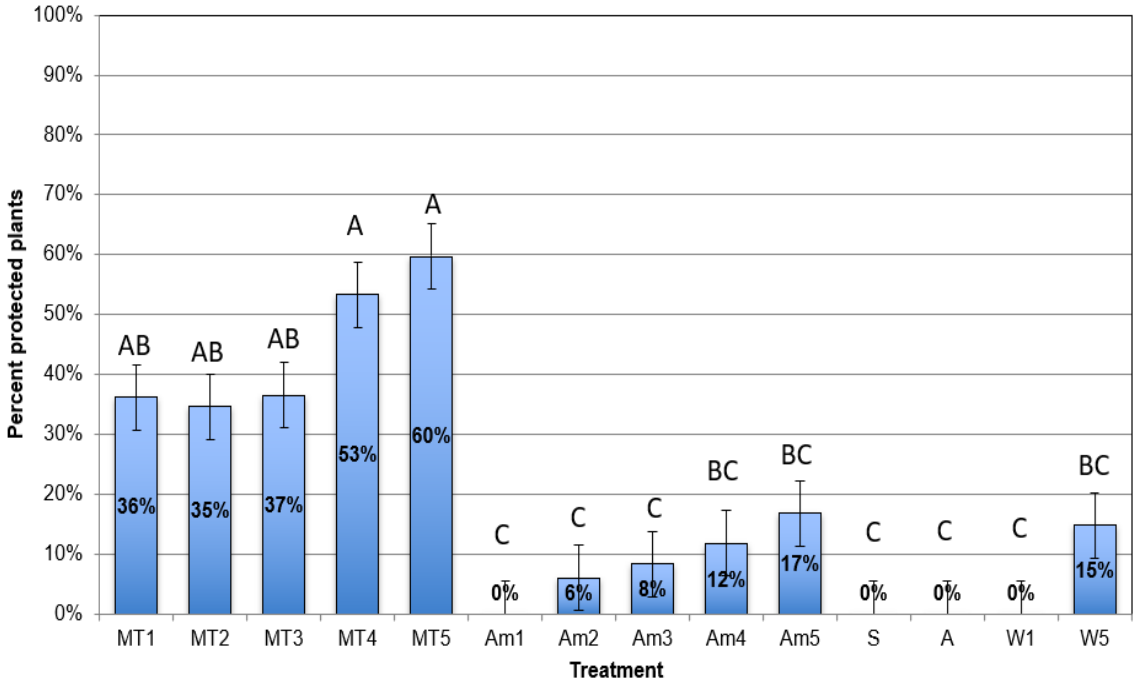


Figure 3.14. Percent protected plants to pathogens found at the Unity, WI location in growth chamber soil bioassays. Different letters above bars indicate significant differences (p=0.0001). Bars are means of three replications with standard deviation. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

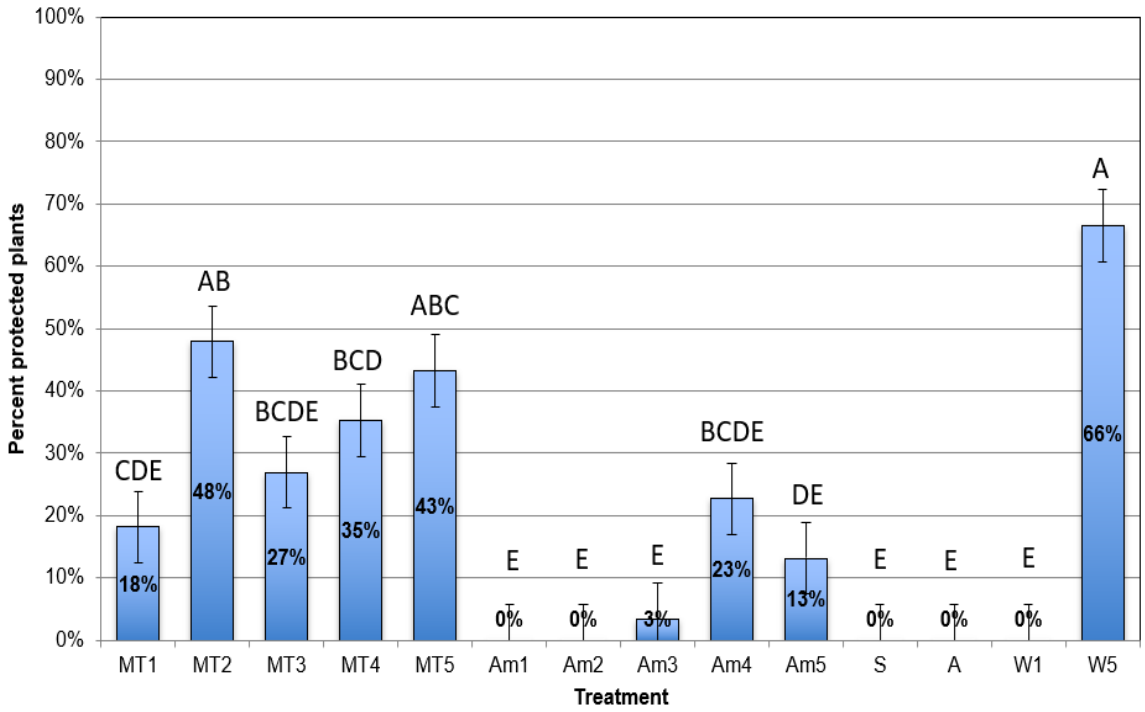


Figure 3.15. Percent protected plants to pathogens found at the West Salem, WI location in growth chamber soil bioassays. Different letters above bars indicate significant differences (p=0.0001). Bars are means of three replications with standard deviation. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

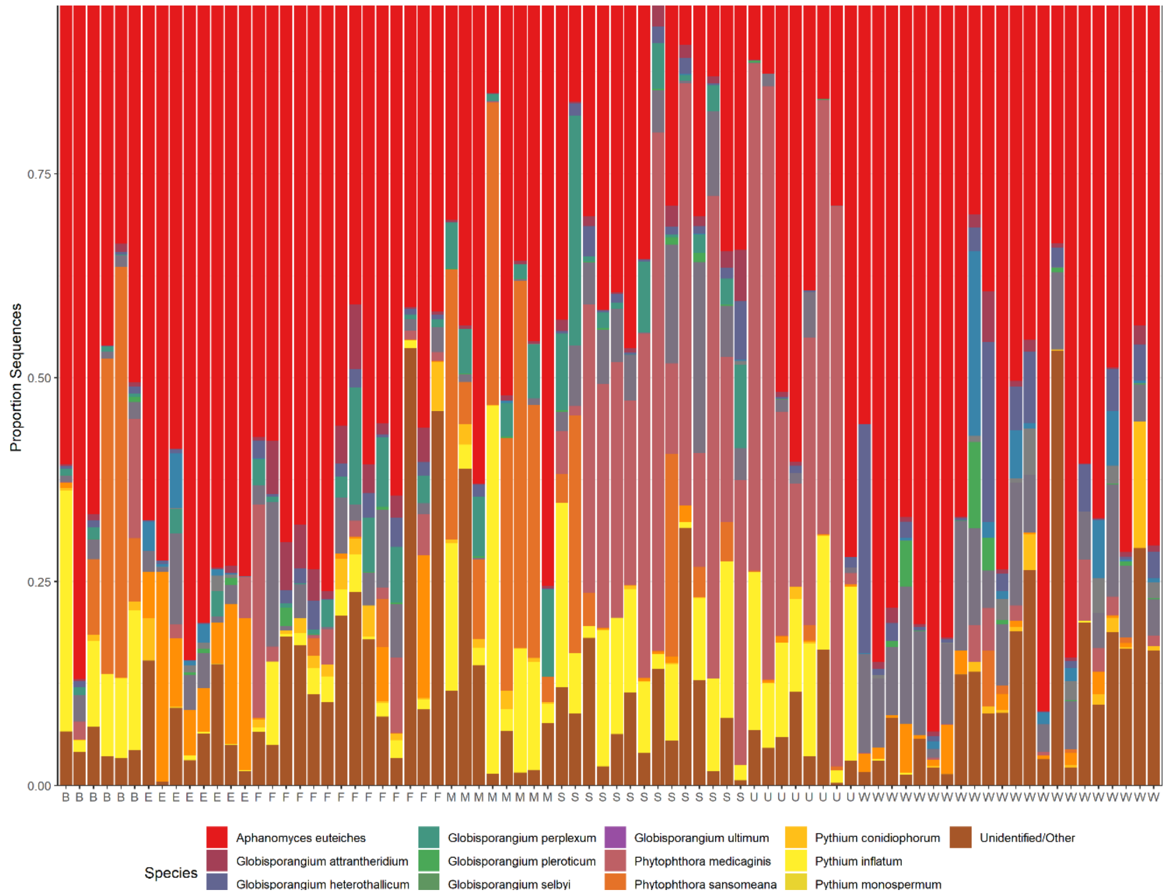


Figure 3.16. Proportion of oomycete species sequences found in the endosphere for each of the eight plot locations. Abbreviations for locations are as follows: B: Beaver, WI; E: Emerald, WI; M: Marshfield, WI; U: Unity, WI; W: West Salem, WI (2020 and 2021); F: Frankfort, WI; S: Spencer, WI.

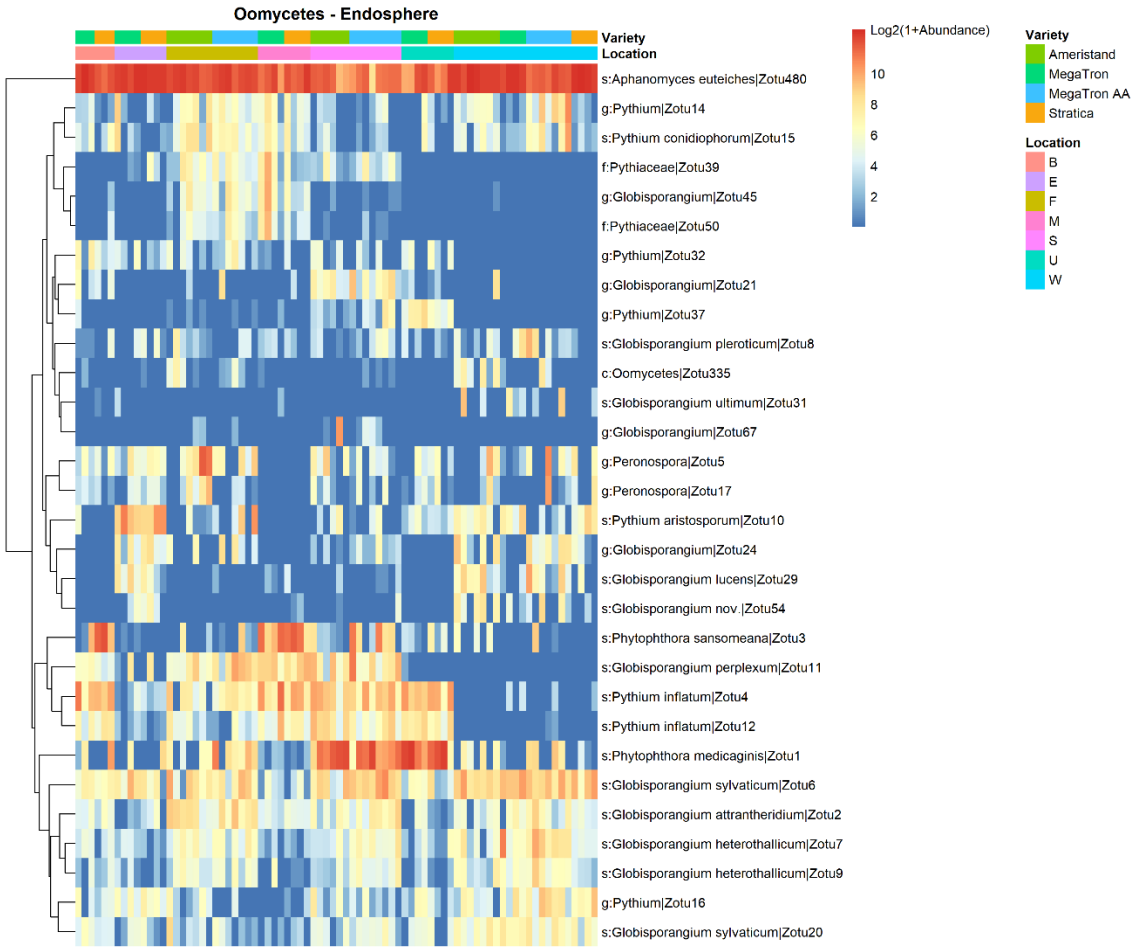


Figure 3.17. Heat map of the most abundant oomycete species found in the endosphere at the eight plot locations. Abbreviations for locations are as follows: B: Beaver, WI; E: Emerald, WI; M: Marshfield, WI; U: Unity, WI; W: West Salem, WI (2020 and 2021); F: Frankfort, WI; S: Spencer, WI.

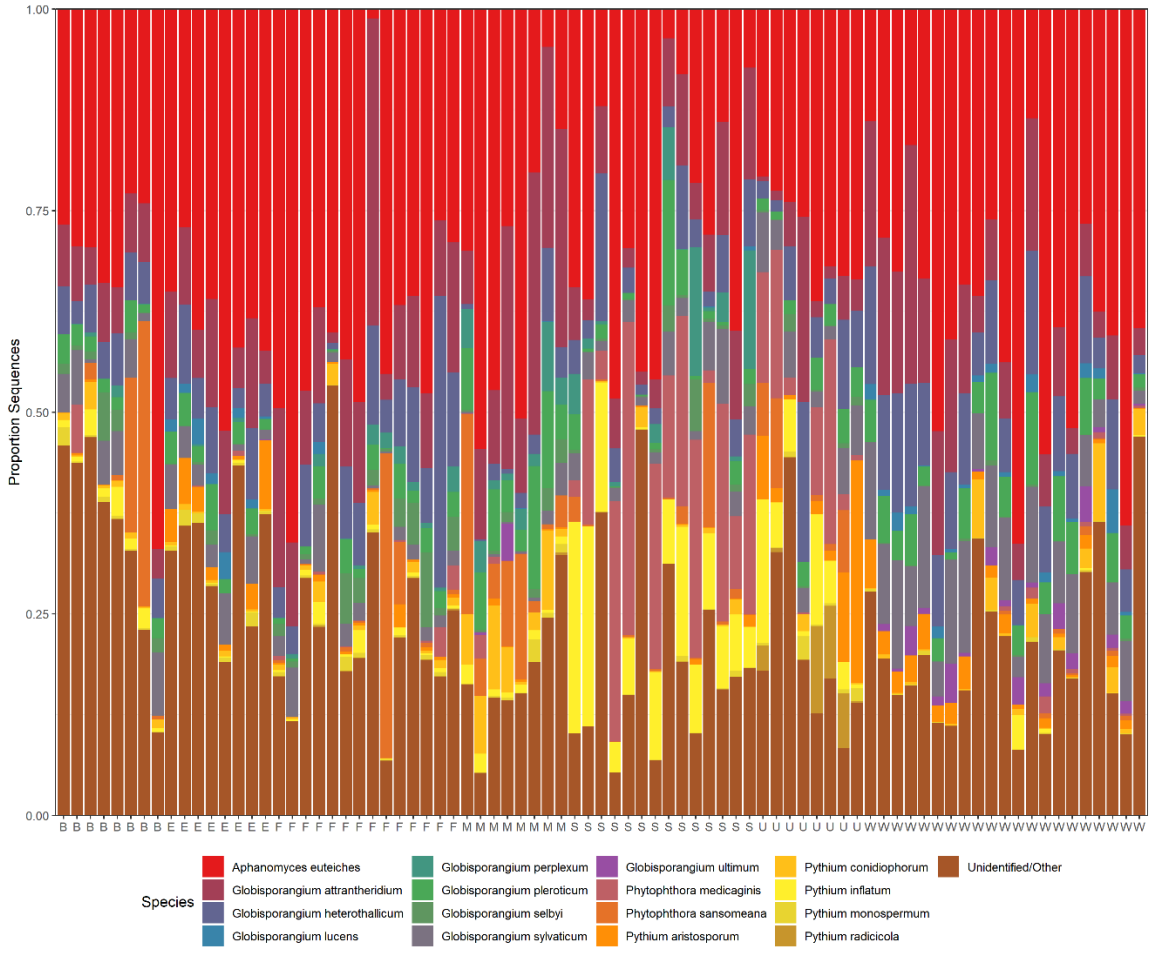


Figure 3.18. Proportion of oomycete species sequences found in the rhizosphere soil for each of the eight plot locations. Abbreviations for locations are as follows: B: Beaver, WI; E: Emerald, WI; M: Marshfield, WI; U: Unity, WI; W: West Salem, WI 2020 and 2021; F: Frankfort, WI; S: Spencer, WI.

Figure 3.19. Heat map of most abundant oomycete species found in the rhizosphere at the eight plot locations. Abbreviations for locations are as follows: B: Beaver, WI; E: Emerald, WI; M: Marshfield, WI; U: Unity, WI; W: West Salem, WI (2020 and 2021); F: Frankfort, WI; S: Spencer, WI.

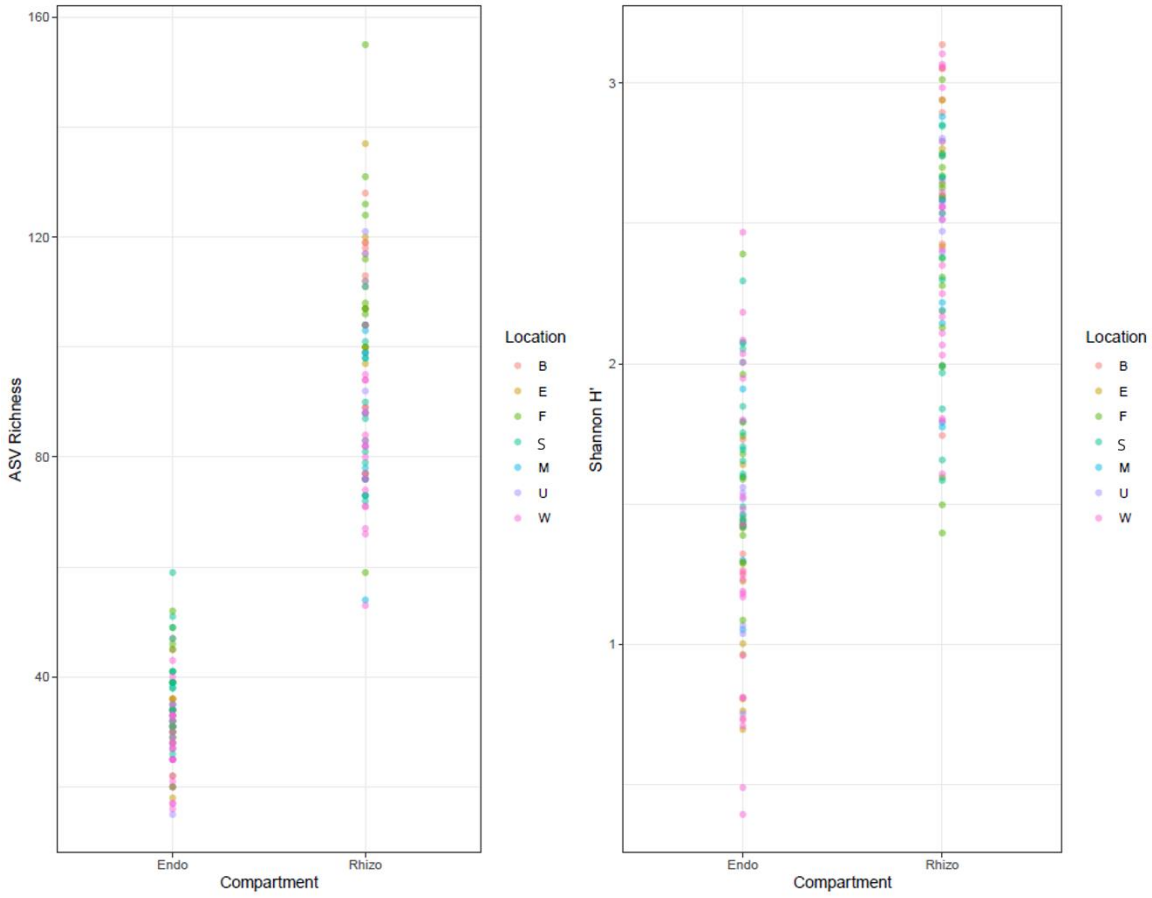


Figure 3.20. Alpha-diversity indices (ASV richness and Shannon diversity) were estimated for each of the eight plot locations and both endosphere and rhizosphere sample types. Abbreviations for locations are as follows: B: Beaver, WI; E: Emerald, WI; M: Marshfield, WI; U: Unity, WI; W: West Salem, WI (2020 and 2021); F: Frankfort, WI; S: Spencer, WI.

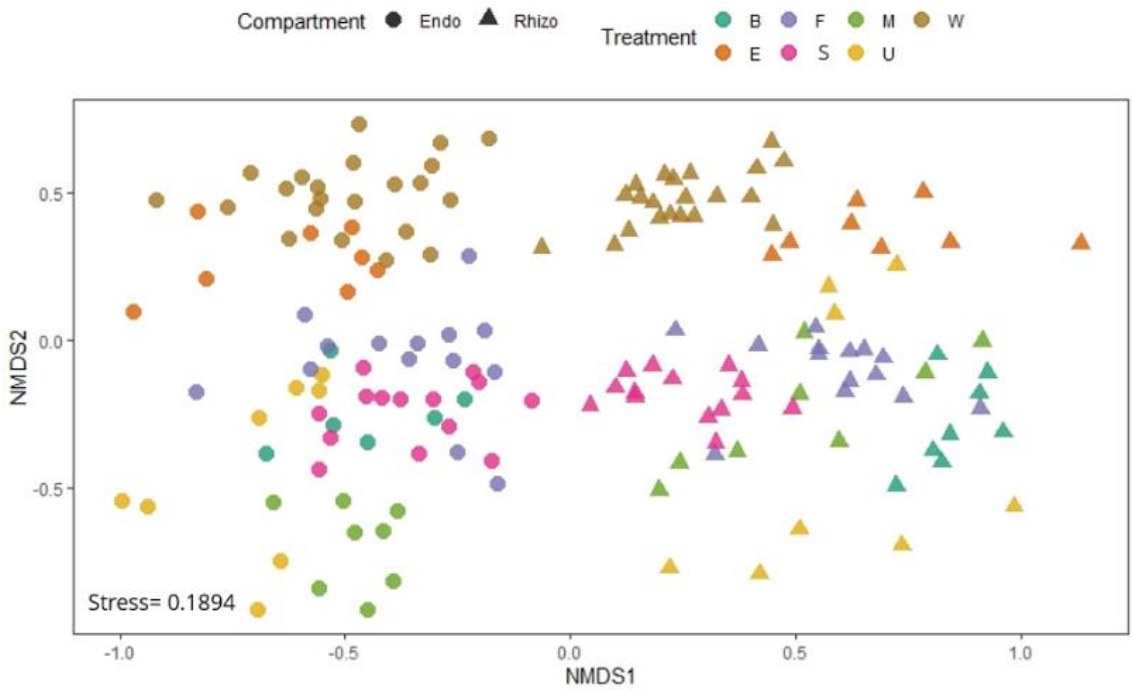


Figure 3.21. Oomycete NMDS plot for all eight locations. Abbreviations for locations are as follows: B: Beaver, WI; E: Emerald, WI; M: Marshfield, WI; U: Unity, WI; W: West Salem, WI (2020 and 2021); F: Frankfort, WI; S: Spencer, WI.

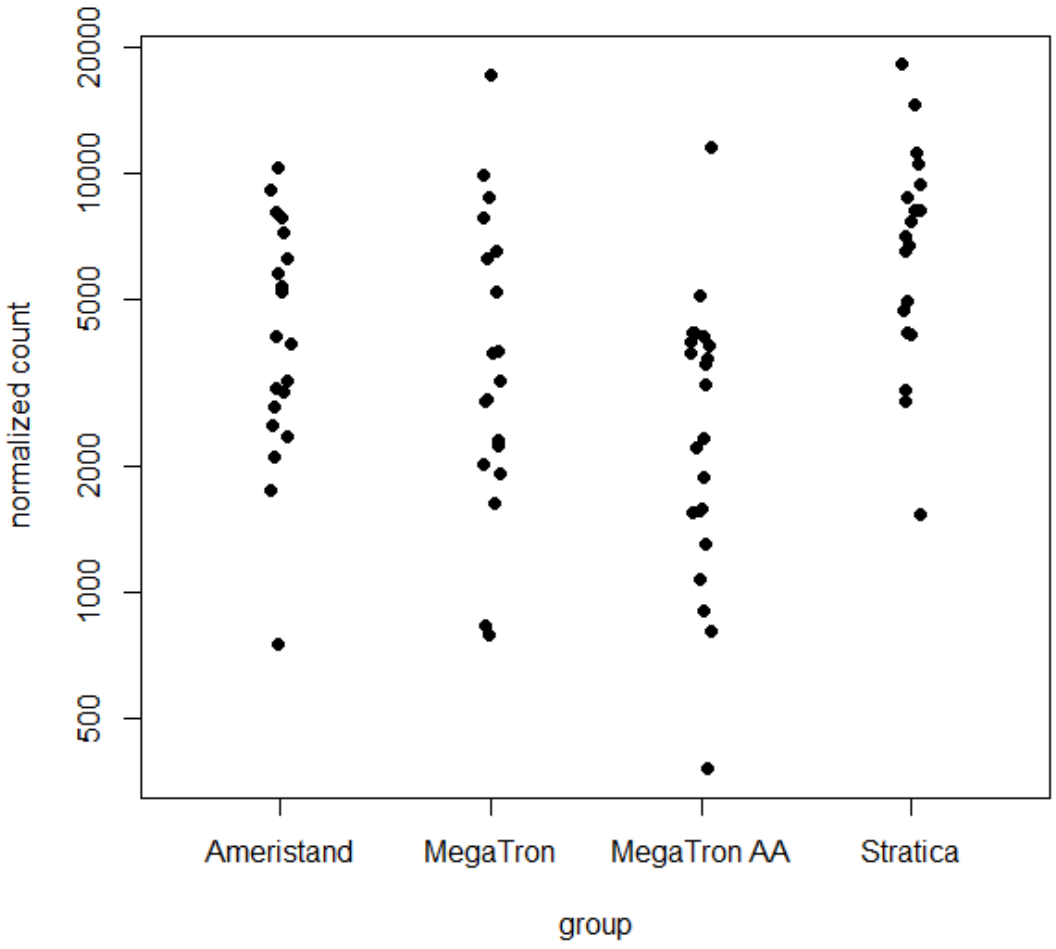


Figure 3.22. Differential abundance plot comparing the relative abundance of *Aphanomyces euteiches* (Zotu 480) in each of the four cultivars for all eight locations. Y-axis is normalized sequencing count. Stratica and MegaTron were used in 2020. Ameristand and MegaTron AA were used in 2021. Significant differences ($p<0.001$) were found in *Aphanomyces euteiches* abundance in the Aphanomyces root rot race 2 susceptible (Ameristand and Stratica) cultivars as compared to the Aphanomyces root rot race 2 resistant (MegaTron and MegaTron AA) cultivars.

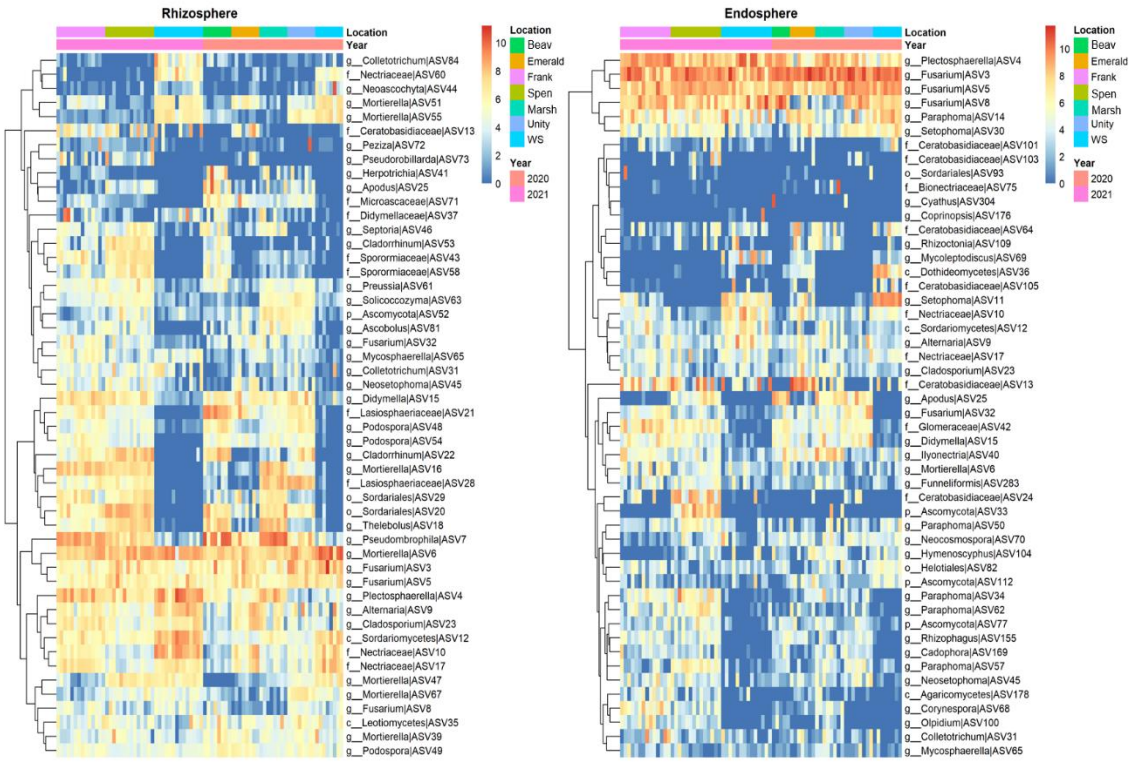


Figure 3.23. Heat map of most abundant fungal species found in the endosphere and rhizosphere soil at the eight plot locations. Abbreviations for locations are as follows: Beav: Beaver, WI; Emerald: Emerald, WI; Marsh: Marshfield, WI; Unity: Unity, WI; WS: West Salem, WI (2020 and 2021); Frank: Frankfort, WI; Spen: Spencer, WI.

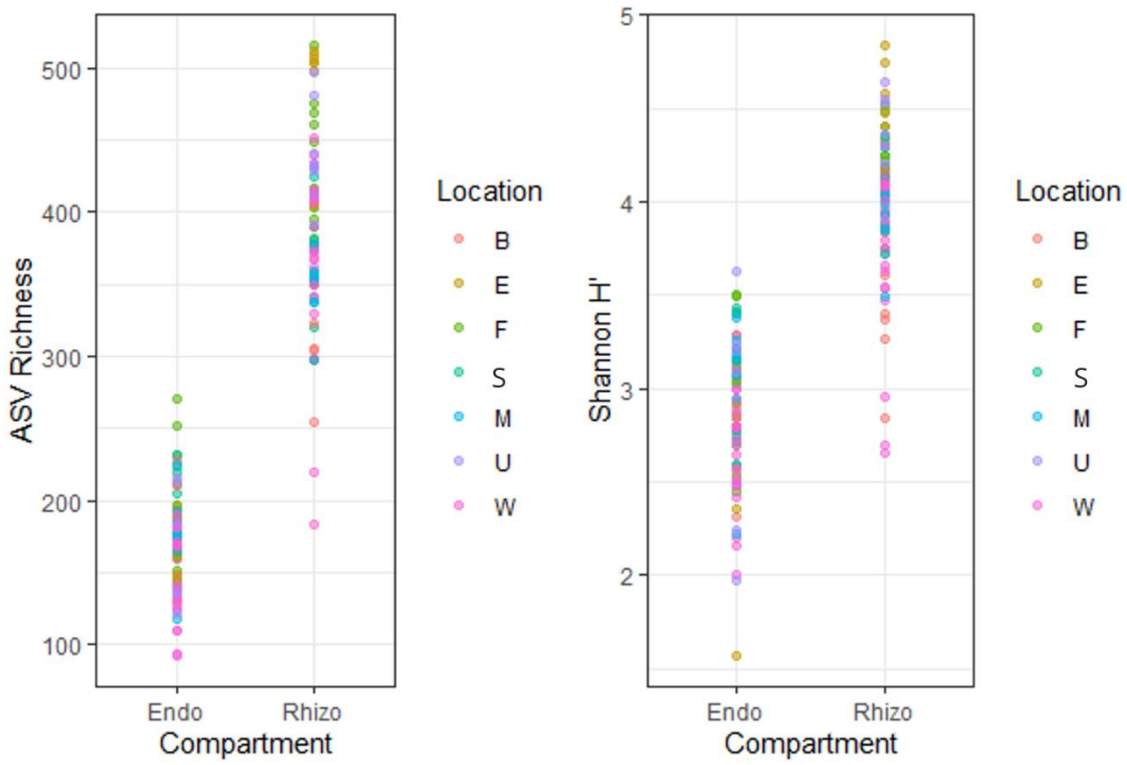


Figure 3.24. Fungal alpha-diversity indices (ASV richness and Shannon diversity) were estimated for each of the eight plot locations and both endosphere and rhizosphere sample types. Abbreviations for locations are as follows: B: Beaver, WI; E: Emerald, WI; M: Marshfield, WI; U: Unity, WI; W: West Salem, WI (2020 and 2021); F: Frankfort, WI; S: Spencer, WI.

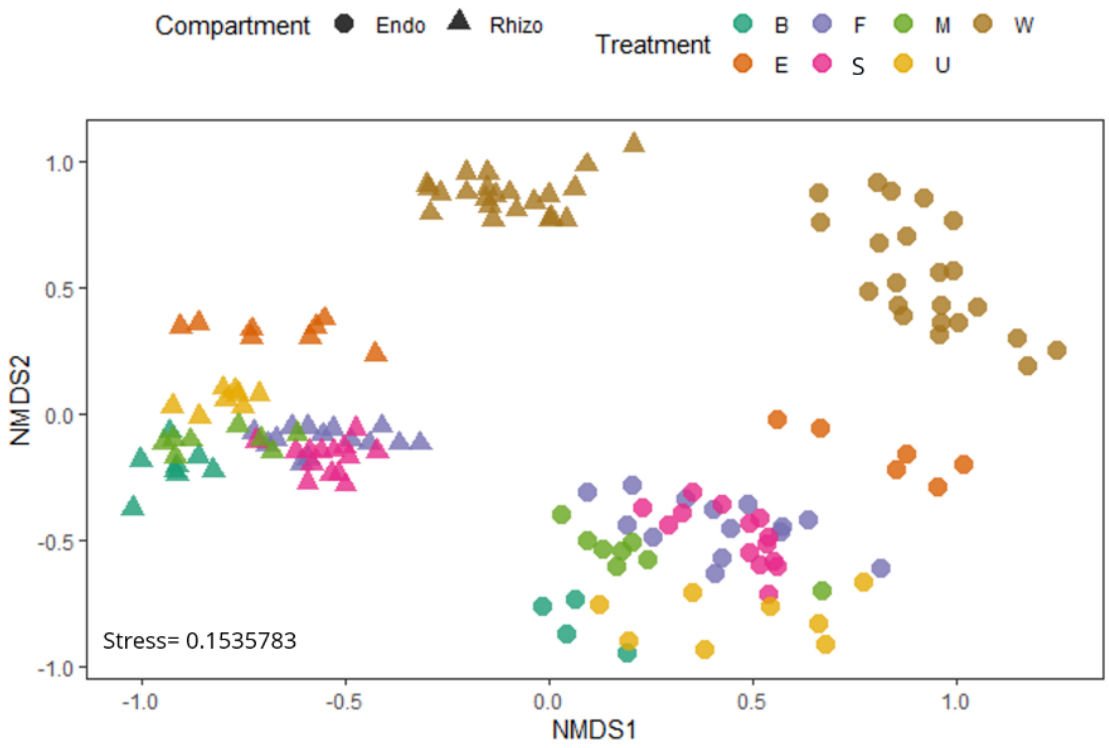


Figure 3.25. Fungal NMDS plot for all eight locations. Abbreviations for locations are as follows: B: Beaver, WI; E: Emerald, WI; M: Marshfield, WI; U: Unity, WI; W: West Salem, WI (2020 and 2021); F: Frankfort, WI; S: Spencer, WI.

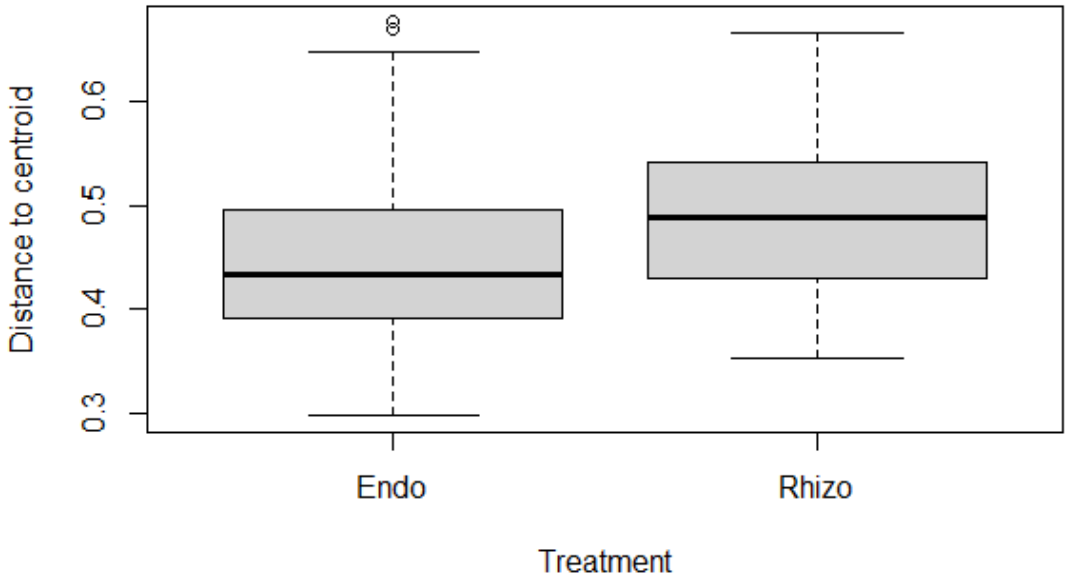


Figure 3.26. Beta dispersion among groups showed similar variance among communities between the rhizosphere and endosphere samples (p=0.0035).

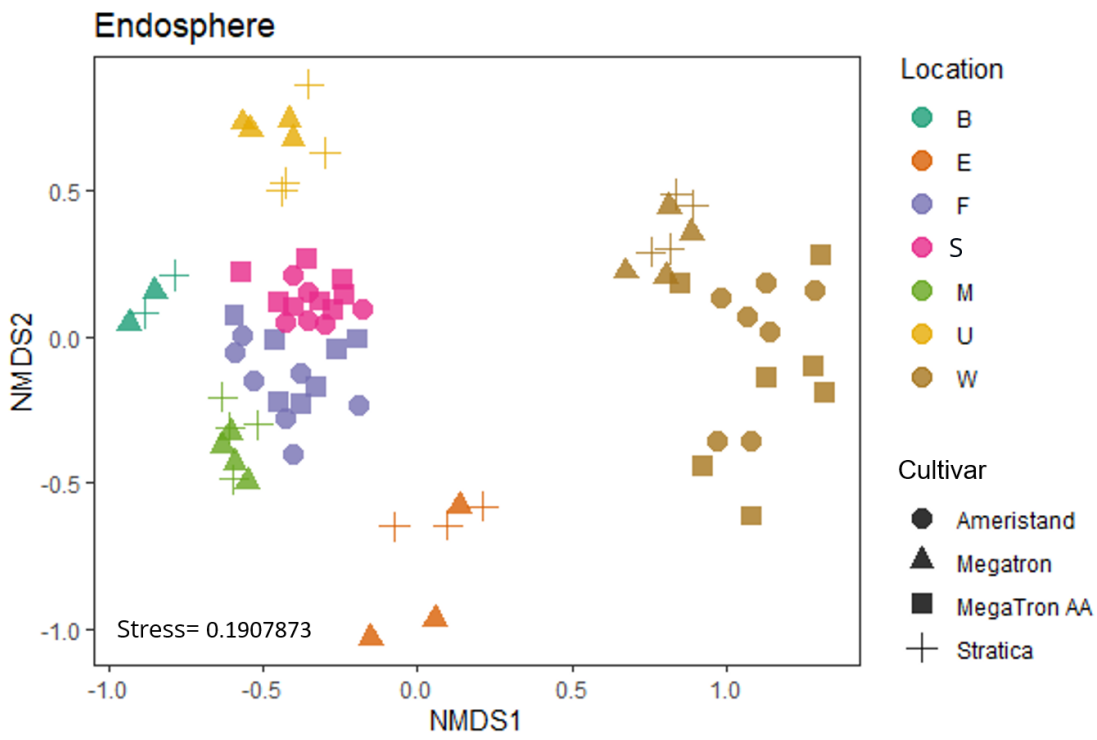


Figure 3.27. Fungal endosphere NMDS plot by cultivar and location for all eight locations. Abbreviations for locations are as follows: B: Beaver, WI; E: Emerald, WI; M: Marshfield, WI; U: Unity, WI; W: West Salem, WI (2020 and 2021); F: Frankfort, WI; S: Spencer, WI.

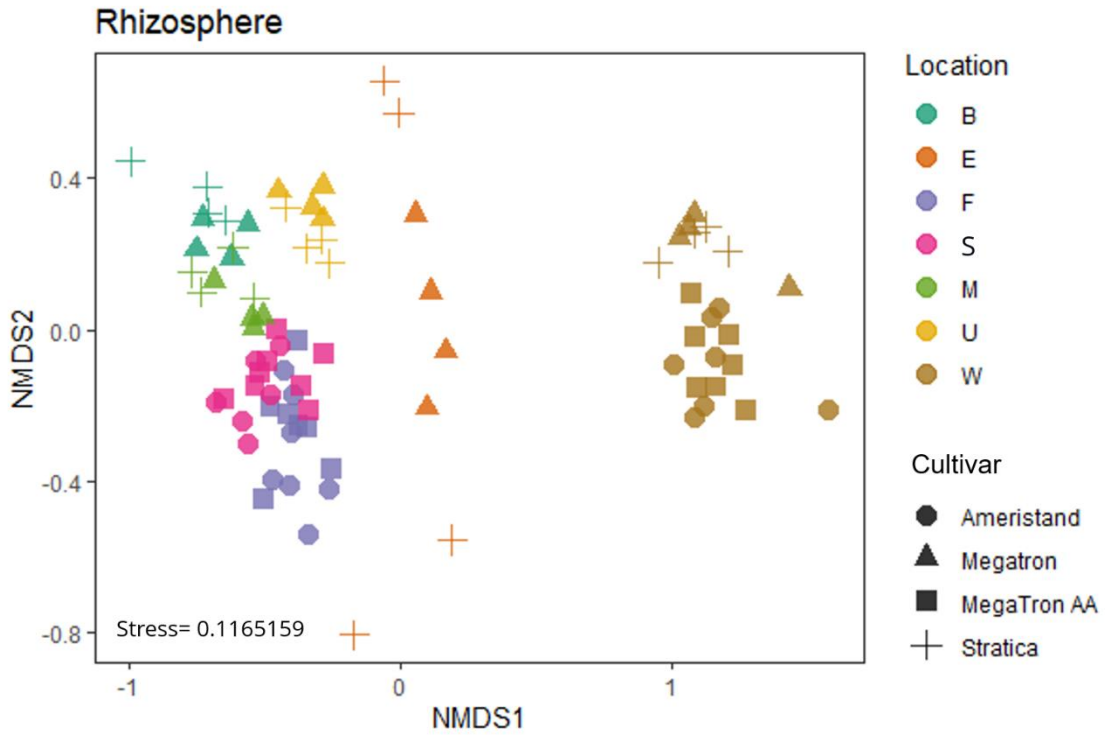


Figure 3.28. Fungal rhizosphere soil NMDS plot by cultivar and location for all eight locations. Abbreviations for locations are as follows: B: Beaver, WI; E: Emerald, WI; M: Marshfield, WI; U: Unity, WI; W: West Salem, WI (2020 and 2021); F: Frankfort, WI; S: Spencer, WI.

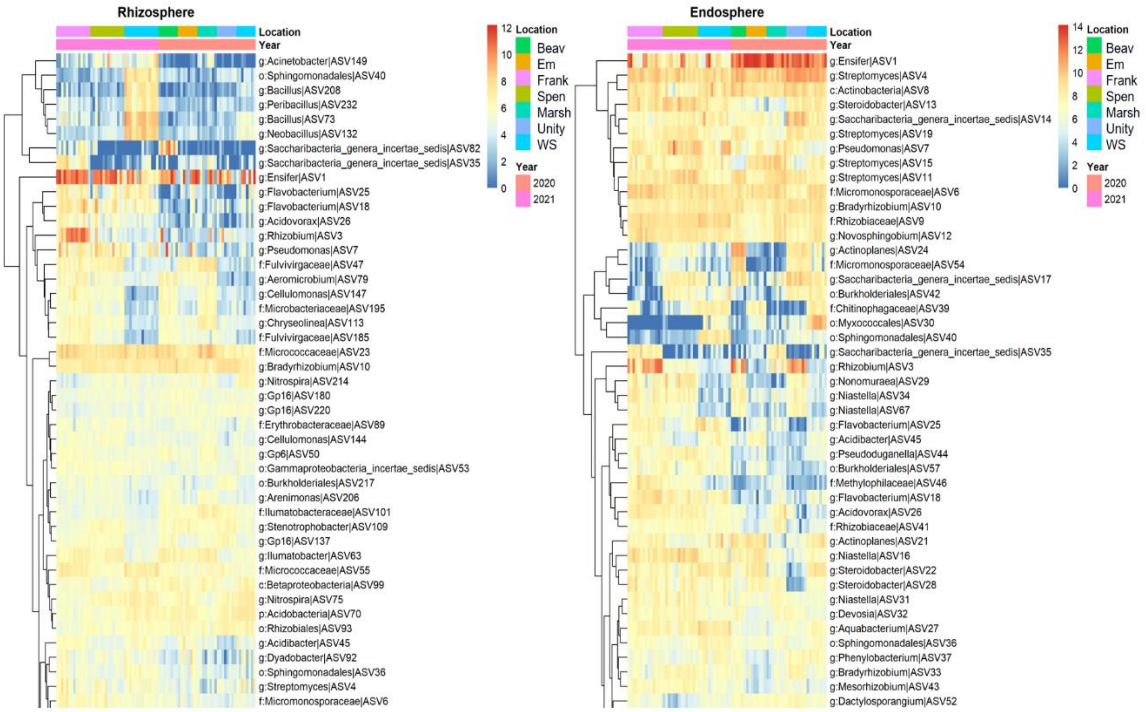


Figure 3.29. Heat map of most abundant bacterial species found in the endosphere and rhizosphere soil at the eight plot locations. Abbreviations for locations are as follows: Beav: Beaver, WI; Em: Emerald, WI; Marsh: Marshfield, WI; Unity: Unity, WI; WS: West Salem, WI (2020 and 2021); Frank: Frankfort, WI; Spen: Spencer, WI.

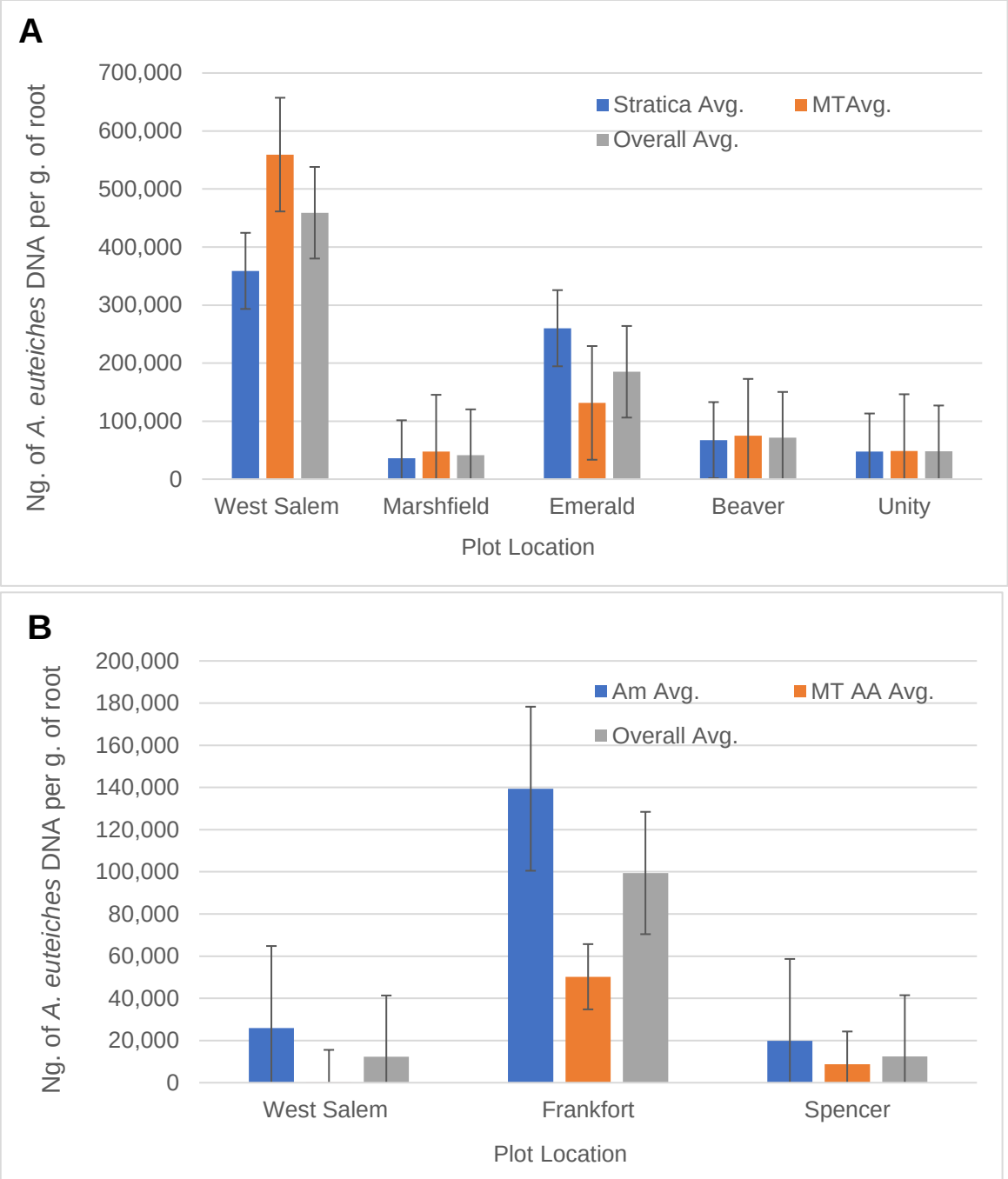


Figure 3.30. Quantification of *A. euteiches* in root samples. A) Average and standard deviation of nanograms (ng) of *Aphanomyces euteiches* DNA per gram (g) of root from each of the five 2020 plot locations; n=12. B) Nanograms (ng) of *Aphanomyces euteiches* DNA per gram (g) of root from each of the three 2021 plot locations; n=21.

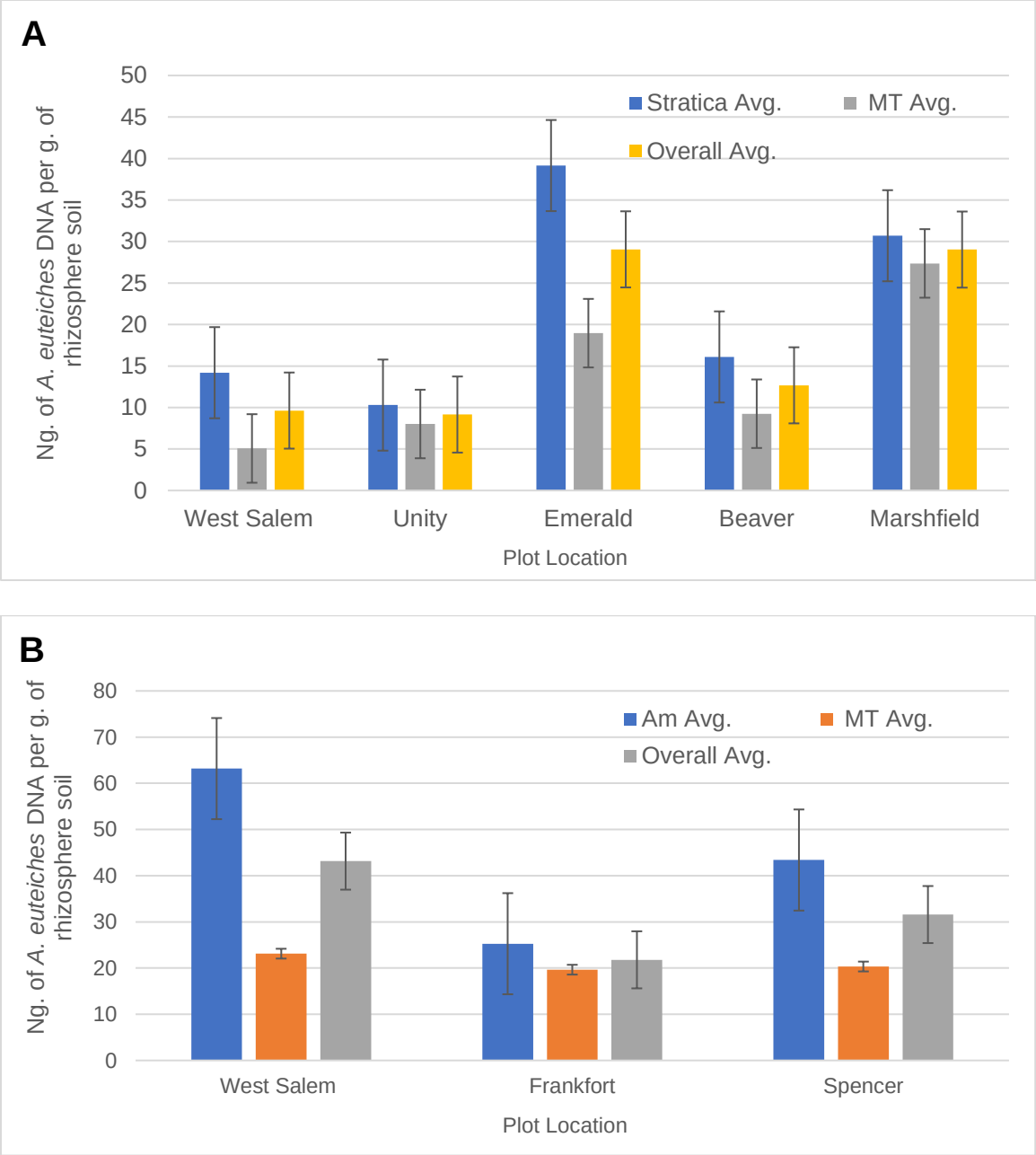


Figure 3.31. Quantification of *A. euteiches* in rhizosphere samples. A) Nanograms (ng) of *Aphanomyces euteiches* DNA per gram (g) of rhizosphere soil from each of the five 2020 plot locations; n=12. B) Nanograms (ng) of *Aphanomyces euteiches* DNA per gram (g) of rhizosphere soil from each of the three 2021 plot locations; n=21.

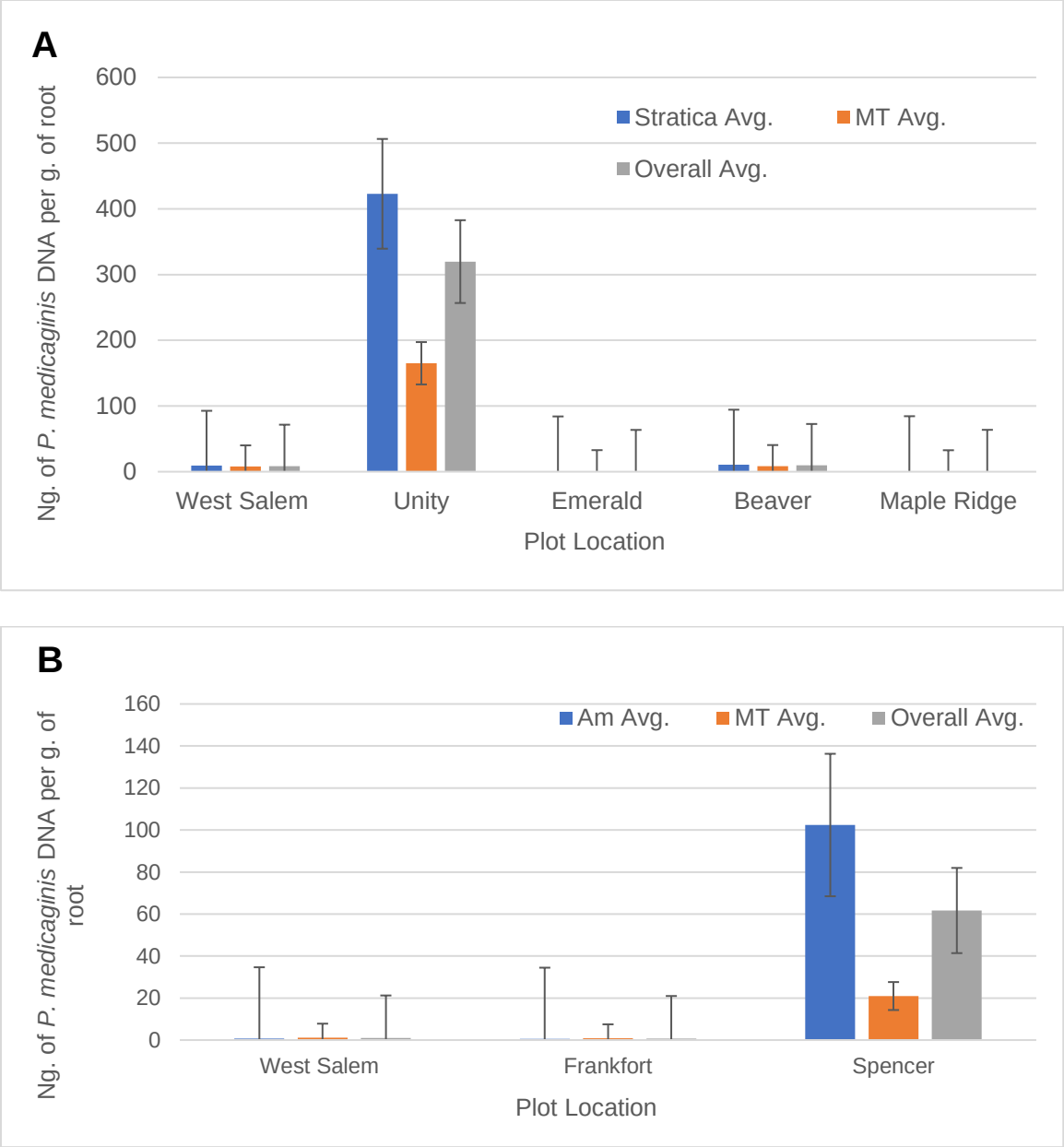


Figure 3.32. Quantification of *P. medicaginis* in root samples. A) Nanograms (ng) of *Phytophthora medicaginis* DNA per gram (g) of root from each of the five 2020 plot locations; n=12. B) Nanograms (ng) of *Phytophthora medicaginis* DNA per gram (g) of root from each of the three 2021 plot locations; n=21.

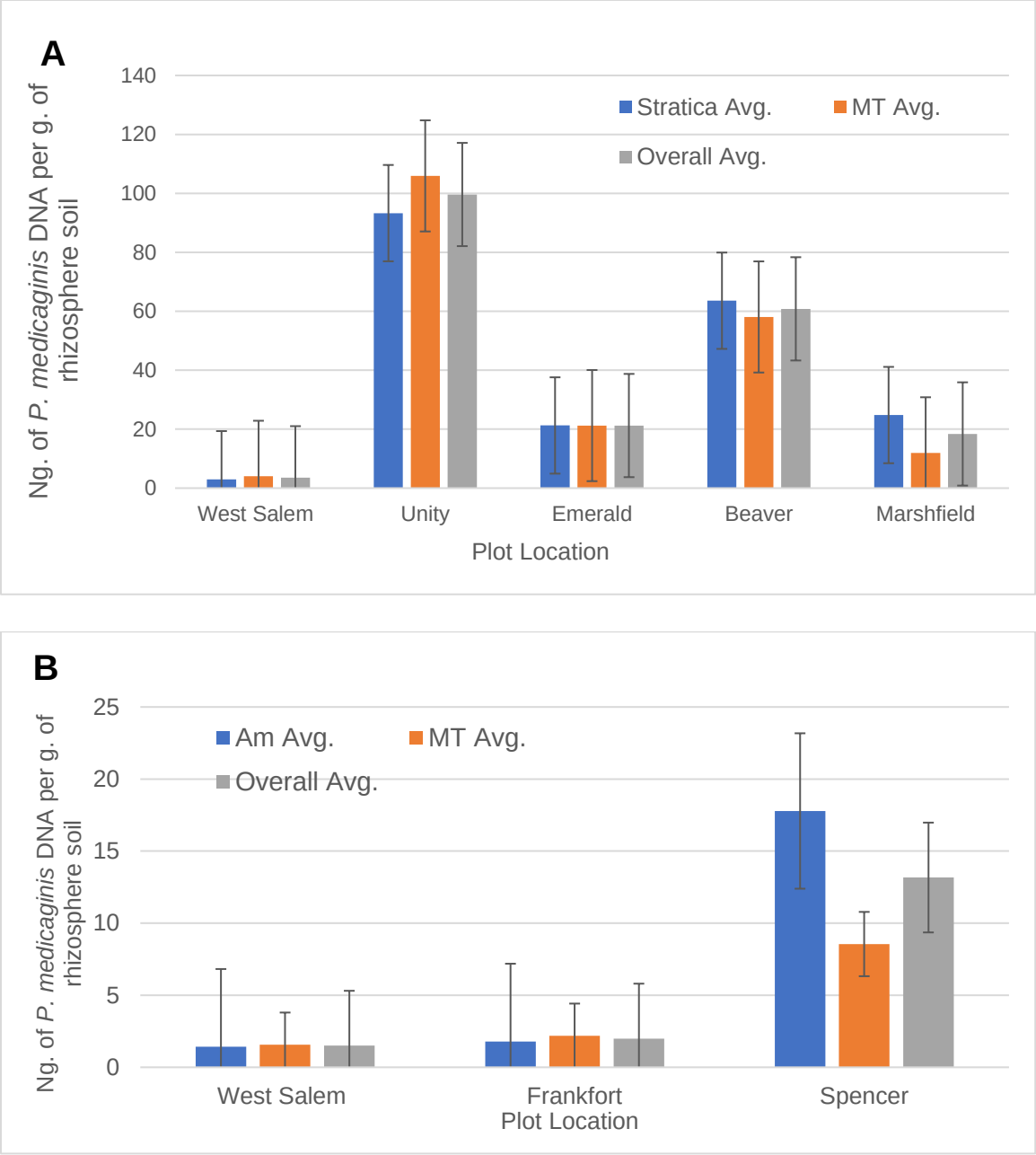


Figure 3.33. Quantification of *P. medicaginis* in rhizosphere samples. A) Nanograms (ng) of *Phytophthora medicaginis* DNA per gram (g) of rhizosphere soil from each of the five 2020 plot locations; n=12. B) Nanograms (ng) of *Phytophthora medicaginis* DNA per gram (g) of rhizosphere soil from each of the three 2021 plot locations; n=21.

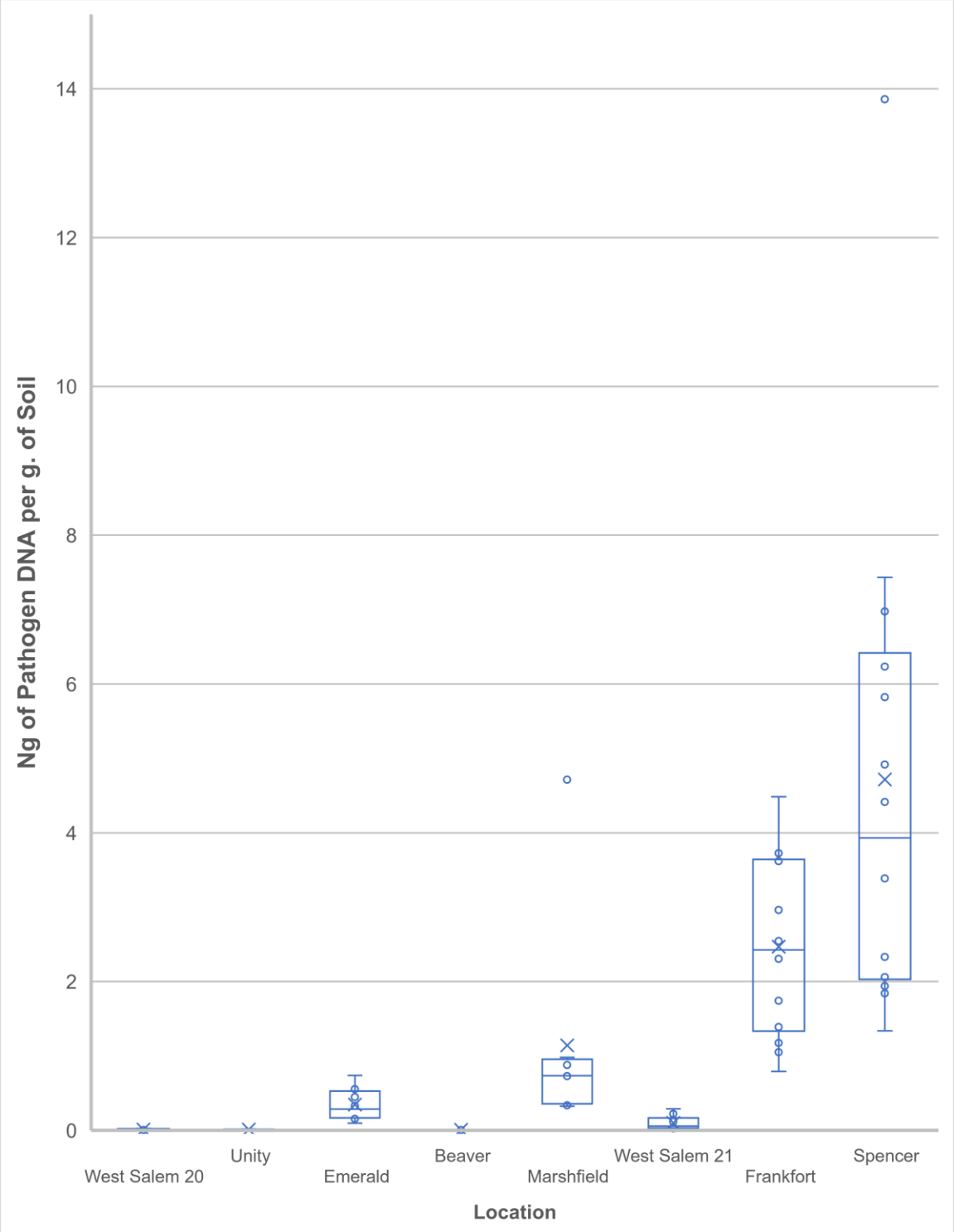


Figure 3.34. Quantification of *Pythium irregulare* in rhizosphere soil. Nanograms of *Pythium irregulare* DNA per gram of rhizosphere soil. Plot averages shown for 2020 (n=12) and 2021 (n=21) plots. X indicates mean of all samples.

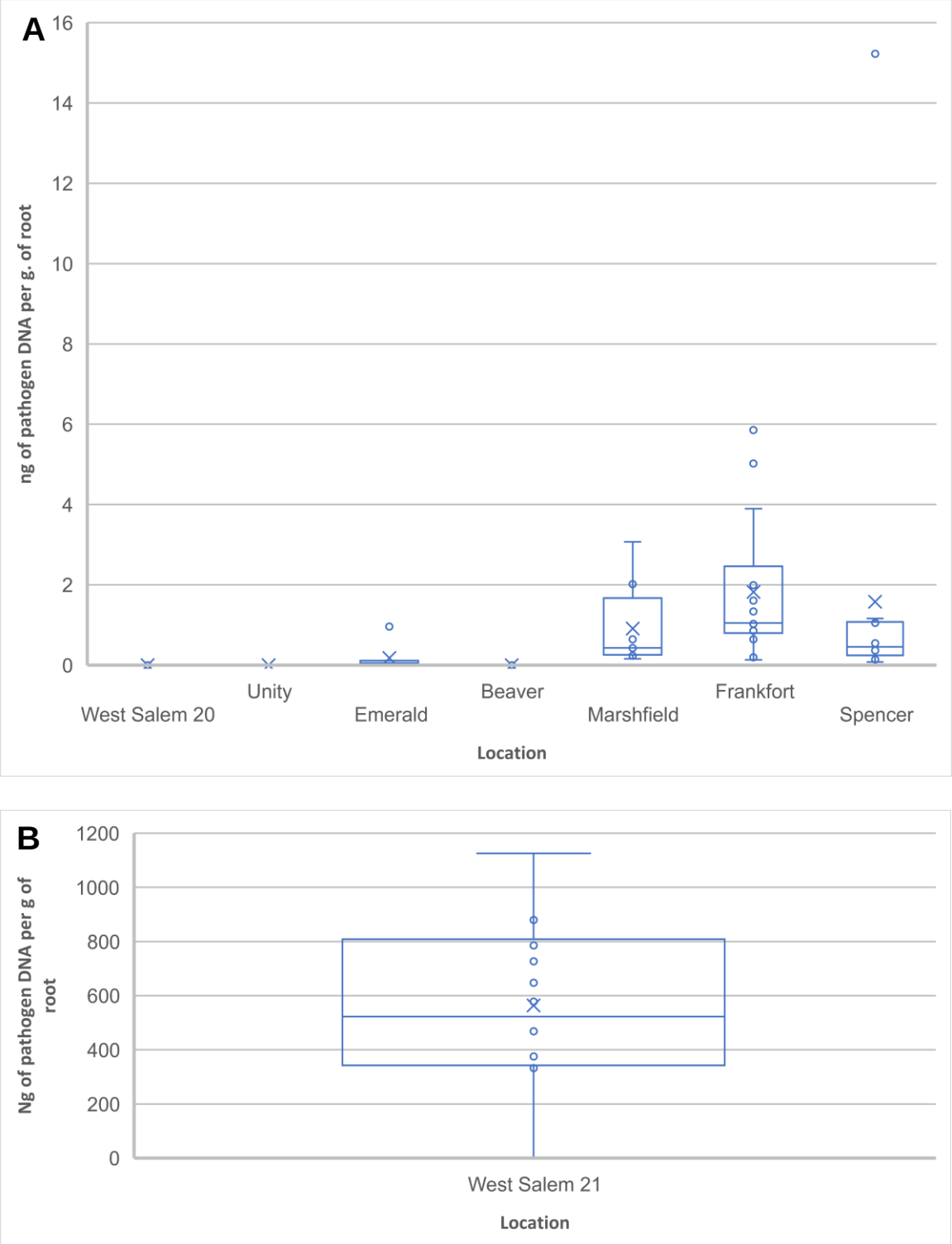


Figure 3.35. Nanograms of *Pythium irregulare* DNA per gram of root. A) Nanograms of *Pythium irregulare* DNA per gram of root. Plot averages shown for 2020 (n=24) and 2021 (n=42) plots. X indicates mean of all samples. B) Nanograms of *Pythium irregulare* DNA per gram of root at West Salem 2021 location.

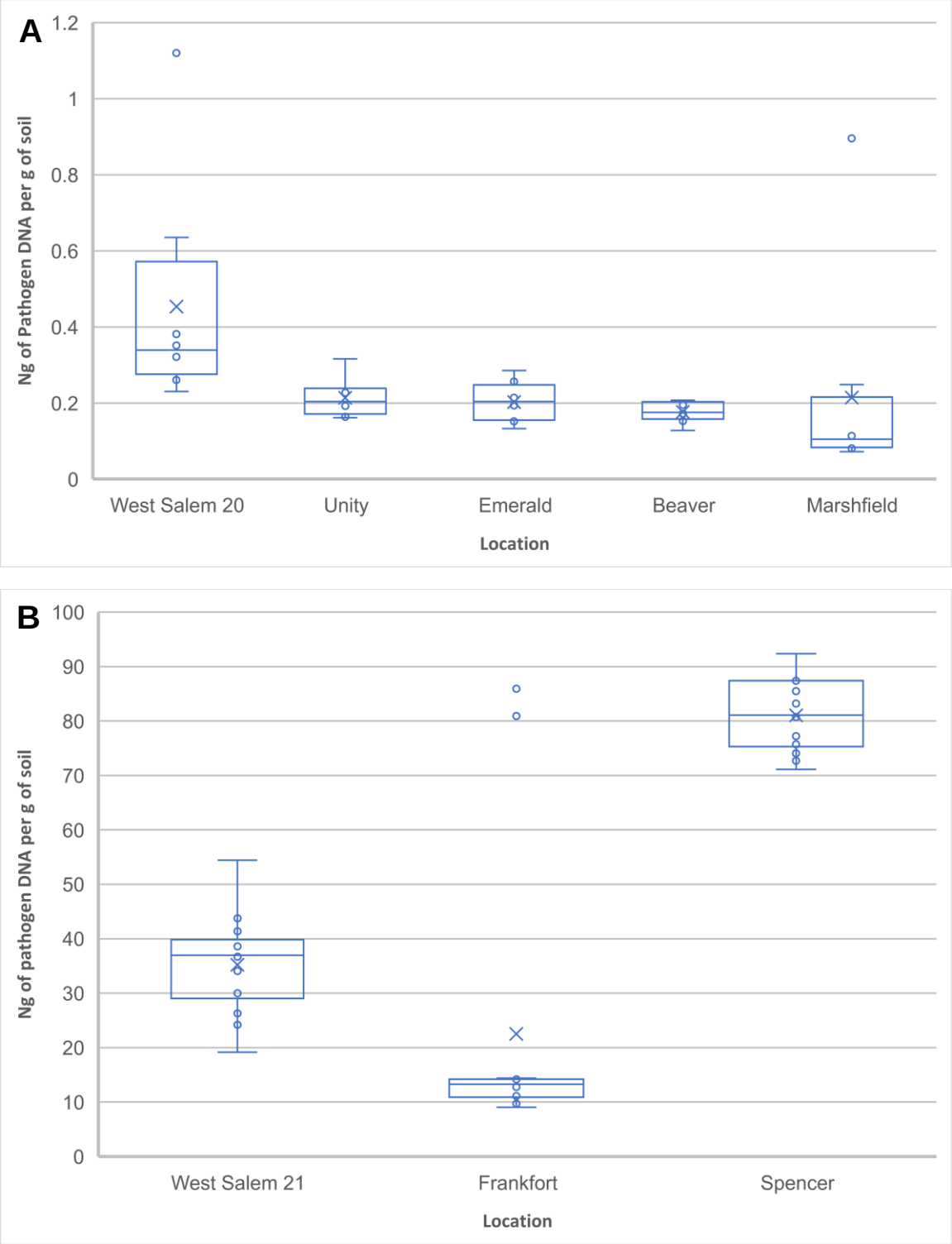
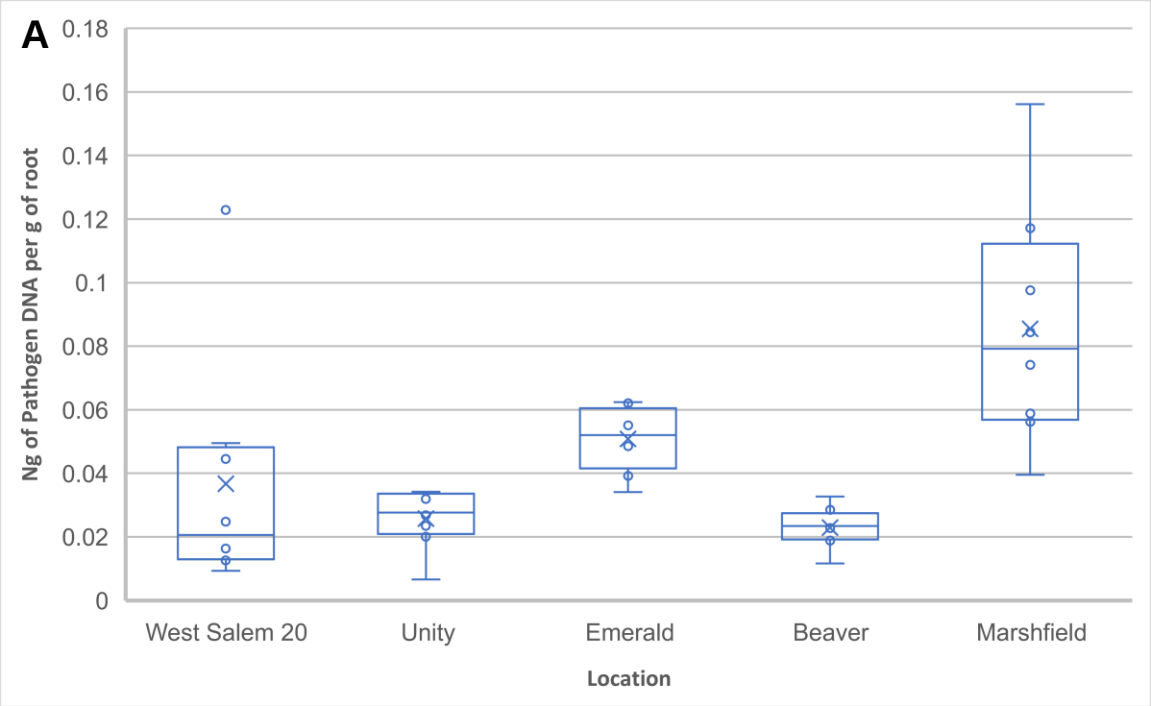


Figure 3.36. Quantification of *Pythium ultimum* in rhizosphere soil. Nanograms of *Pythium ultimum* DNA per gram of rhizosphere soil. A) Plot averages shown for 2020 (n=12). B) Plot averages shown for 2021 (n=21) plots. X indicates mean of all samples.



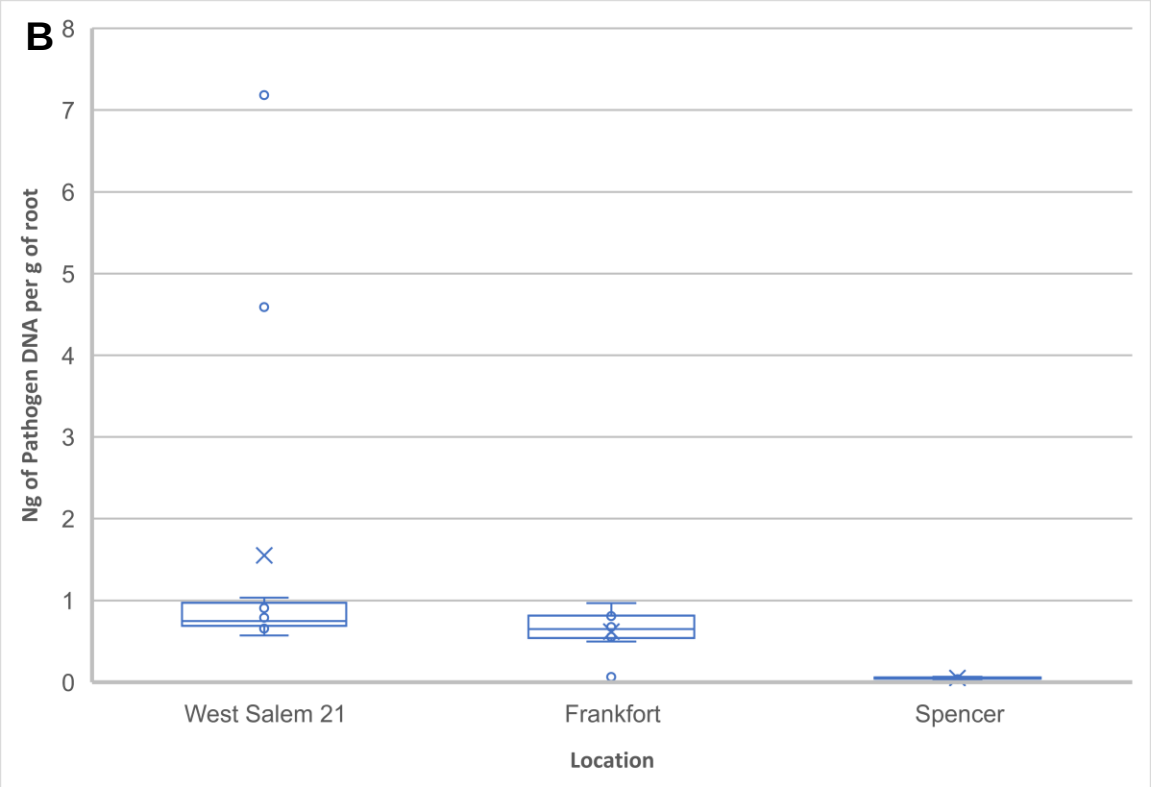


Figure 3.37. Quantification of *Pythium ultimum* in roots. Nanograms of *Pythium ultimum* DNA per gram of root. A) Plot averages shown for 2020 (n=12). B) Plot averages shown for 2021 (n=21) plots. X indicates mean of all samples.

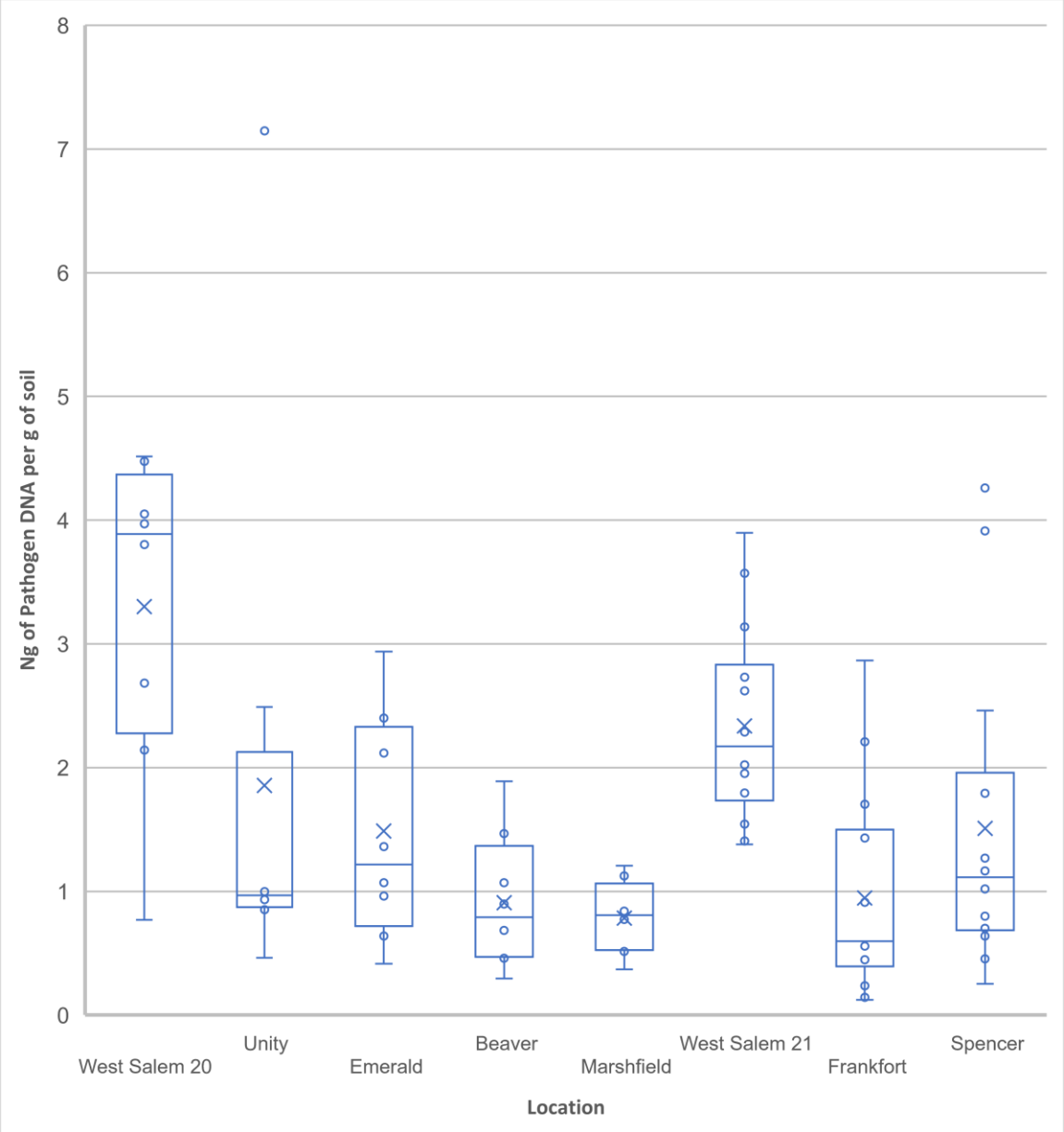


Figure 3.38. Quantification of *Pythium sylvaticum* in rhizosphere soil. Nanograms (ng) of *Pythium sylvaticum* DNA per gram (g) of rhizosphere soil. Plot averages shown for 2020 (n=12) and for 2021 (n=21) plots. X indicates mean of all samples.

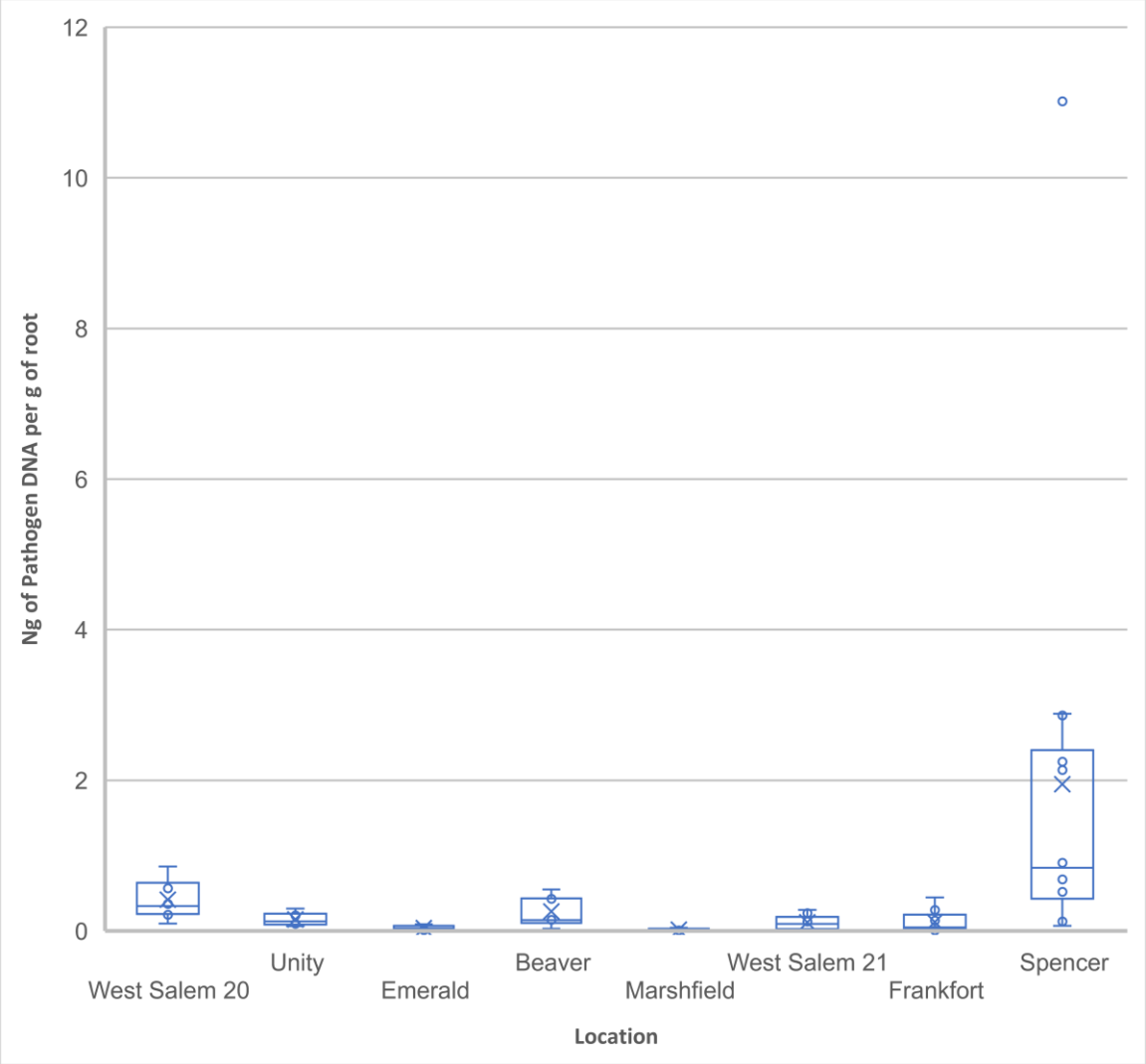


Figure 3.39. Quantification of *Pythium sylvaticum* in roots. Nanograms (ng) of *Pythium sylvaticum* DNA per gram (g) of root. Plot averages shown for 2020 (n=12) and for 2021 (n=21) plots. X indicates mean of all samples.

Chapter 4: Discussion

Wet soil syndrome in alfalfa, caused by soilborne pathogens in cold, wet, poorly drained soils, continues to plague alfalfa stands across the United States. These pathogens contribute to stand failures even though cultivars are commercially available that have high genetic resistance to some pathogens and are treated with fungicide seed treatments offering protection to a number of alfalfa seedling diseases. Recent studies have identified aggressive *Pythium* strains not well controlled by Apron (metalaxyl)/Apron XL (mefenoxam) fungicide seed treatments (Berg et al., 2017) indicating that through overreliance on this mode of action for many years in a number of crops, *Pythium* spp. are becoming resistant. In addition to *Pythium* spp., aggressive isolates of *Aphanomyces euteiches* have been identified that may challenge the genetic resistance currently available in commercial varieties. While Apron and ApronXL have activity against *Pythium* spp. and *P. medicaginis*, it is not active against *A. euteiches*. Recently, Stamina (pyraclostrobin) was labeled for use on alfalfa seed for protection against *A. euteiches* and fungal pathogens. Preliminary *in vitro* tests showed that Evergol Energy and Intego Solo had good activity against oomycete pathogens of alfalfa (Samac, unpublished). Furthermore, it is possible that not all members of the wet soil syndrome have been identified. Current identification methods of pathogens causing seedling diseases rely on culture-based methods of seedling baiting and isolation techniques which are not highly quantitative and can overlook pathogens that are not easily cultured.

This study measured the efficacy of new and improved fungicide seed treatments through use of field trials, standard pathogen tests, and growth chamber bioassays using soils from fields where stand failures due to disease occurred in the past. This study also identified the entire microbial community (oomycetes, fungi, and bacteria) associated with field soils from previously failed alfalfa seedings and quantified the abundance of oomycete pathogen in those soils. Overall, the results of this work will assist both growers and seed marketers by evaluating new fungicide seed treatments that can potentially be used commercially. Identifying which pathogens are causing the most pressure in contributing to wet soil syndrome disease complex in alfalfa will help to target the appropriate fungicide seed treatment to the field and provide a foundation for improving genetic resistance to damaging pathogens. This study also provides the information and resources needed for disease clinics or companies to develop qPCR

tests to quantify known alfalfa pathogens in soil and help growers in identifying which pathogen(s) may be causing disease in their alfalfa stands.

4.1. Efficacy of fungicides when used under field settings

Stand Counts and Dry Matter Yield

Differences were observed at second stand count across all locations and varieties as well as at the Spencer, WI location for the second stand count. Rainfall initially after planting at all plot locations was limited, so oomycete zoospore formation was likely limited and therefore pathogen infection was not a factor early in the season, as the pathogens that typically infect alfalfa seedlings do so in wet soil conditions, and therefore could be the reason we did not see variation between the five different treatments at the first stand count (first trifoliate stage) across all locations. However, we cannot rule out that the seed treatments were ineffective in reducing seed rot and damping-off so that no differences in stand counts were observed.

Statistical differences between treatment 1 and treatment 5 were observed across all locations for the second stand count. At the second count stage (4 to 6 trifoliate stage) more differences may have been observed due to two factors: increased rainfall as compared to early in the season as well as *A. euteiches* infection at the plot locations. Typically, *Pythium* spp. and *P. medicaginis* will infect early in the season in cold/cool soils, whereas *A. euteiches* will infect in warmer soils around the same time our plots reached the 4 to 6 trifoliate stage.

When analyzed by location, second stand count differences were only observed at the Spencer, WI location. When separated by cultivar, differences were observed between Ameristand treatments but not MegaTron AA. This could indicate that we observed more of a cultivar effect in Ameristand than MegaTron AA and that Ameristand resistance genetics to the pathogens present in the soil may not be as effective as MegaTron AA. As sequencing analyses revealed, both *P. medicaginis* and *A. euteiches* pressure was high at the Spencer, WI location. Differences in the effectiveness of treatments at the first stand count could have been due to *P. medicaginis* pressure while variation across the treatments at second stand count could have been due to both *P. medicaginis* infection early coupled with *A. euteiches* pressure once soils began to warm later in May.

Differences in dry matter yield were observed at the first harvest at the West Salem location. Across all locations differences among treatments were only observed in the second cutting. At the West Salem location, differences in treatments may have been observed due to high *A. euteiches* pressure in the endosphere, as revealed by amplicon sequencing and qPCR results. This plot received both irrigation and more rainfall as compared to the other plot locations indicating that environment may have been more suitable for *A. euteiches* oospores to germinate and infect the alfalfa roots.

A number of studies have shown that while positive effects of fungicide seed treatments can be seen under controlled conditions with single pathogens, the effect is not always observed under field conditions (Wu et al., 2018; You, Lamichhane, Barbetti, & Aubertot, 2020). This is most likely because most seed and seedling diseases are disease complexes, caused by multiple pathogens that may have synergistic interactions (Lamichhane, You, Barbetti, & Laudinot, 2020). For example, You et al. (2020) evaluated nine fungicides against *Pythium irregulare, A. trifolii, P. clandestina*, and *R. solani* infecting seeds and seedling of subterranean clover. They found that in controlled environment studies, fungicide seed treatments were effective in controlling seedling disease from infecting the clover plants but under field conditions, where complexes are present that are comprised of several pathogens, there was rarely any benefit from using fungicide seed treatments. Similarly, Wu et al. (2018) evaluated five seed treatments in controlled conditions and in the field for control of *A. euteiches* on pea. They also found that under controlled environment studies, fungicide seed treatments provided varying degrees of protection but did not show any benefit in the field settings. Nonetheless, several studies have shown effectiveness of seed treatments under field conditions. Wang et al. (2021) showed that metalaxyl-resistant *P. ultimum* seed rot and damping-off of chickpea seed was controlled in field conditions by ethaboxam (Intego Solo) under moderate but not severe disease pressure. Ethaboxam has also been shown to have efficacy in field situations with *Phytophthora, Phytopythium,* and *Pythium* species that cause early season seed decay and pre-emergence and post-emergence damping-off of soybean (Scott, Eyre, Mcduffee, & Dorrance, 2020). Several of these studies have shown the benefit of seed treatments to augment genetic resistance (Wu et al. 2019, Scott et al. 2020). In the case of seed and seedling diseases of alfalfa, the most important pathogen to control appears to be *A. euteiches*.

Bioassays and Standard Pathogen Tests

The bioassays were done to test the seed treatments with field soil under controlled conditions since few differences were observed among treatments in field conditions. In the bioassay results from 2020 and 2021 plot locations, statistical differences were observed between treatments 4 and 5 compared to treatments 1 and 2 across all locations and pooled across both cultivars, although the percentage of resistant plants was low (35%). When split by location, differences were observed at the Marshfield, Unity, and West Salem 2021 locations. Trends showed that treatment 4 and treatment 5 offered the most control to the pathogens that were present in these plot soils. This could indicate that the Intego Solo used in treatment 4 along with Apron XL and Stamina as well as the Intego Solo and Maxim combination used in treatment 5, along with Apron XL and Stamina, are offering improved control to the aggressive *A. euteiches* and *P. medicaginis* isolates that may be present in those soils. This could also indicate that the Intego Solo component of treatment 4 and treatment 5 may be adding control to *Pythium* strains in those soils that are potentially not well controlled by Apron/Apron XL. Finally, the Maxim component in treatment 5 might be adding additional control to the true fungal pathogens that could be found at those locations that the other treatments do not provide any control to. Overall, the seed treatments did not increase plant health in field soils under conducive disease conditions. This may explain the lack of differences in stand counts and forage yields compared to control untreated seeds.

The standard pathogen test assays revealed varying results based on the pathogen used. In the *A. euteiches* race 1 and race 2 test results, no statistical differences were observed among treatments. This was unexpected, since Stamina has been shown to be effective against *A. euteiches* in previous experiments (Samac et al., 2017; Smith & Watson, 2014) The results of the *P. medicaginis* standard test indicate that treatments do increase protection against *P. medicaginis* in addition the disease resistance genetics. The results also indicate that seed treated with Apron XL and Stamina would offer the same amount of control as those treated with an Apron XL, Stamina, and Intego Solo combination or those treated with an Apron XL, Stamina, Intego Solo, and Maxim combination or in other words, that treatment 3, 4, and 5 would all offer similar control. The *Pythium* spp. standard plate assays revealed similar results to *P. medicaginis* where treatments 3, 4, and 5 all provided control of *Pythium irregulare*, *P. ultimum*, and *P. paroecandrum*. The lack of control of Aphanomyces root rot by the seed treatments and high levels of *A. euteiches* in field soils, explains the low

percentage of protected plants in the field soil bioassays. Because Evergol Energy and Intego Solo have excellent activity against *A. euteiches* in vitro, and Stamina has activity in vitro and as the single fungicide on seed, it is possible that the concentrations of active ingredients were not sufficient for Aphanomyces root rot control under the conditions in the bioassay. The seed treatments included application of zinc and manganese micronutrients, Ascend® plant growth regulator, and Nitragin Gold rhizobium as well as the test fungicides, which may have diluted the fungicide active ingredients. Higher rates of fungicide could be tested for activity as well as possible negative effects on plant germination and growth to achieve effective field control of *A. euteiches*.

Pests and pathogens develop resistance to pesticides through natural mutation. Resistant populations develop when the mutation does not decrease fitness and is selected by continued use of the pesticide in the environment. Apron and ApronXL are considered to be at high risk for development of resistant oomycete populations and a number of resistant strains have been isolated from nature and in some cases been shown to have an impact in the field (Wang et al., 2021). Although ApronXL resistant *Pythium* species were isolated that infect alfalfa, the extent of Apron/ApronXL resistance in alfalfa fields is unknown. Since alternative seed treatment fungicides are available for controlling *Pythium* species and *P. medicaginis* on alfalfa, it would be prudent to rotate seed treatments or utilize more than one fungicide on seeds to reduce the potential for developing resistant populations.

4.2. Identify the microbial community (oomycetes, bacterial, and fungal communities) associated with alfalfa roots

Currently identified pathogens that cause seed rot, damping-off, and root rot in alfalfa that were identified in high abundance in sequencing results were *A. euteiches*, *P. medicaginis*, *Pythium ultimum*, and *P. sylvaticum*. The alfalfa root rot pathogens, *A. euteiches* and *P. medicaginis*, were identified more abundantly than *Pythium irregulare*, *P. sylvaticum*, and *P. ultimum* var. *ultimum* that are the most pathogenic in causing seed rot and damping-off in alfalfa. These data indicate that the root rot pathogens caused more disease pressure in the 2020 and 2021 plot locations (Fig. 3.16, 3.17, 3.18, 3.19; Table 3.4 and 3.5.)

Previous surveys from several alfalfa growing states revealed Aphanomyces root rot was more prevalent than Phytophthora root rot (Munkvold & Carlton, 1995; Vincelli et al., 1994). Those studies used baiting techniques to isolate the pathogens which can favor isolation of *A. euteiches* over *P. medicaginis*. However, this study supports those results as *A. euteiches* was the most prevalent root rot pathogen and bioassays indicated that race 2 was present. However, at Unity and Spencer plot locations we identified *P. medicaginis* as the main root rot pathogen. The abundance of *P. medicaginis* was higher in the endosphere in those samples as compared to *A. euteiches* (Fig. 3.16; Table 3.4), indicating that *P. medicaginis* continues to play a major role in contributing to wet soil syndrome, causing seedling and mature plant root disease in alfalfa.

In addition, this study also identified *Phytophthora sansomeana* as a potential contributor to seedling disease of alfalfa. At locations where *P. sansomeana* was identified in highest abundance the previous crop in the rotation was soybeans. This is not surprising as *P. sansomeana* is known as a soybean pathogen (Hansen et al., 2017). It was identified in relatively high abundance at the Beaver, Spencer, Marshfield, and Frankfort locations in endosphere samples and was identified in relatively high abundance at the Unity, West Salem 2020, and Frankfort sites in rhizosphere soil samples (Fig. 3.18; Table 3.5). Due to the high abundance in the alfalfa endosphere and rhizosphere and from results of our first pathogen assay using *P. sansomeana*, this indicates we may be seeing the pathogen infect both soybeans and alfalfa and therefore it is not specific to only soybeans.

Phytophthora sansomeana is closely related to *P. medicaginis* (Cooke, Drenth, Duncan, Wagels, & Brasier, 2000). Previously, *P. sansomeana* had been identified in alfalfa fields in New York but was recovered from weed species (Hansen et al., 2017). *P. sansomeana* is most commonly known as a pathogen of soybeans. A metagenomic analysis of the communities of oomycetes from soybean fields with high levels of disease identified *P. sansomeana, P. sojae*, several *Phytopythium* species and numerous *Pythium* species (Navarro et al. 2021). In disease assays, *P. sojae, P. sansomeana, Pythium irregulare, P. perplexum,* and *P. ultimum* caused the most damage to soybean roots. Our assay results inoculating alfalfa seedlings with *P. sansomeana* indicated that it may cause more damping-off in alfalfa seedlings as opposed to root rot. Symptoms on alfalfa seedlings occurred primarily as a rot of the

hypocotyl and tap root. We saw greater infection from *P. sansomeana* in younger seedlings as opposed to older. More testing is recommended to see if *P. sansomeana* causes root rot in mature stands.

Peronospora species were identified in all plots in both endosphere and rhizosphere samples at relatively high abundance. The downy mildew pathogen of alfalfa, *P. trifoliorum,* is an obligate pathogen that grows systemically in alfalfa. Symptoms appear in spring and fall during periods of high humidity and can lead to significant yield losses through defoliation and plant stunting. It is not typically associated with stand establishment problems but the possible presence of the pathogen in young plants warrants further investigation of its role in seedling diseases and wider development of resistance to downy mildew in alfalfa cultivars.

Metagenomic analysis of oomycete communities from pea fields in Canada with pea root rot identified primarily *Pythium* species, particularly *P. herterothallicum* (Taheri et al. 2017a). In 57% of samples, they detected *A. euteiches* but at a relatively low abundance, although abundance increased by use of more specific primers (Taheri et al. 2017b). *Pythium* spp. were most abundant in the rhizosphere soil samples as opposed to the endosphere and *Pythium* diversity was higher in the rhizosphere as opposed to endosphere. This could indicate that *Pythium* was not a major player causing seedling disease and root disease at the time we sampled (first bloom during first harvest) but may have contributed to seed rot and damping-off earlier in the season if the environmental conditions favored infection. Of the other *Pythium* spp. identified, there were a few species where pathogenicity toward alfalfa is unknown. Those species are *Pythium aritosporum, P. selbyi, P. monospermum,* and *P. radicola. P. aritosporum* has been reported in causing corn stalk rot in China (Gao et al., 2016) and has also been reported as causing damping-off in both field pea (Alcala et al., 2007) and soybean (Zitnick-Anderson & Nelson, 2015). Therefore, more testing would be warranted to see if it also causes seed rot and damping-off in alfalfa. In future studies, sampling earlier in the season when soils are cold/cool is recommended.

Berg et al. (2017) studied the pathogenicity of *P. irregulare*, *P. sylvaticum*, *P. ultimum* var*. ultimum, P. attrantheridium*, *P. heterothallicum*, *P. pleroticum*, *P. perplexum*, *and P. inflatum* toward alfalfa, which were all species identified in relatively high abundance by amplicon sequencing. The most pathogenic toward alfalfa seedlings were *P. irregulare, P. sylvaticum, and P. ultimum* var*. ultimum* and strains of these

species were identified that were not well controlled by metalaxyl/mefenoxam fungicides. Overall, *Pythium* spp. contributed to 26%of the abundance of oomycete species in the endosphere and 43% in the rhizosphere. However, those species that have previously been identified in contributing the most to disease in alfalfa seedlings (*P. ultimum, P. sylvaticum*, and *P. irregulare*) were only identified at 5% abundance in the endosphere and 6% abundance in the rhizosphere. Previous studies have shown that amplicon sequencing can underestimate the abundance of a *Pythium* spp., as a few *Pythium* spp. of certain clades have identical ITS sequences (Navarro et al., 2021). Further studying is needed to identify if *Pythium aritosporum, P. selbyi, P. monospermum,* and *P. radicola* cause disease in alfalfa seedlings.

Relative abundance of *A. euteiches* was lowest in MegaTron AA. This cultivar is rated as highly resistant to race 1 and race 2 of Aphanomyces root rot. In an Ohio soybean amplicon sequencing study, *Phytophthora sojae* abundance was higher in susceptible cultivars as compared to resistant cultivars (Navarro et al., 2021), and this study therefore supports the conclusion that relative abundance of pathogens is typically higher in susceptible cultivars as compared to resistant cultivars. Previous research showed that resistance is a result of a hypersensitive reaction that results in very low or no colonization of plants by *A. euteiches* (Samac, Yu, and Missaoui 2021). However, highly resistant cultivars contain susceptible plants and the cores sampled could have contained susceptible plants that typically have very high numbers of oospores in roots. Ameristand had the highest abundance of *A. euteiches*. This is consistent with our original hypothesis that Ameristand would have the highest amount of *A. euteiches* pressure among all cultivars because Ameristand only has resistance to race 1 Aphanomyces root rot. This indicates that race 2 was very abundant at the 2021 plot locations. In addition, in soil bioassays the race 1 resistant check, WAPH-1, had very low numbers of resistant plants but the race 2 resistant check, WAPH-5 had high resistance in the Beaver, Emerald, Marshfield, West Salem, and Frankfort soils indicating that race 2 was present. However, WAPH-5 and MegaTronAA had < 50% resistant plants in the Unity and Spencer soils suggesting the presence of additional aggressive pathogens such as *Pythium*, *Phytophthora sansomeana,* or true fungal pathogens, *Fusarium*, *Paraphoma radicina*, *Plectosphaerella cucumerina*, or even more aggressive isolates of *A. euteiches* which could overcome race 2 resistance. Isolation of *P. sansomeana*, *Paraphoma radicina*, and *Plectosphaerella cucumerina* are required to fully evaluate

how pathogenic and aggressive they might be on alfalfa and if they contribute significantly to alfalfa seedling disease.

Similar to the Unity and Spencer locations, West Salem 2021 bioassay results revealed low percent resistance in the MegaTron AA cultivar across all treatments, however, WAPH-5 cultivar was still at 66% resistance to the pathogens in the soil (Fig. 3.15). This could indicate that there are aggressive *A. euteiches* isolates present at the West Salem location that WAPH-5 offers protection to but the genetics in MegaTron AA do not offer protection to. In addition, added pressure from the true fungal pathogens or other oomycete pathogens may also be at play, however, the treatments did not offer any added protection to the pathogens present at this location, as WAPH-5, which is untreated, offered the most protection across all cultivars.

True fungal species that have been identified as contributing to the root rot complex of alfalfa root diseases in China were identified in high abundance at all plot locations (Table 3.6). In Inner Mongolia, China in 2016, *Paraphoma radicina* was isolated from infected alfalfa plants (Cao et al., 2020). Greenhouse experiments with the pathogen revealed that two months after inoculation the pathogen reduced above and belowground biomass of alfalfa plants. This pathogen was identified in high abundance at our plot locations (ASV14, ASV57) (Fig. 3.29) and could be contributing to the root rot complex of diseases in alfalfa seedlings as well as mature stands and has potentially been overlooked in the United States in the past.

In addition to *Paraphoma radicina*, the *Plectosphaerella* genus was identified in relatively high abundance at all plot locations (Table 3.6 and 3.7; Fig. 3.29). *Plectosphaerella cucumerina* was reported as causing alfalfa root rot in 2020 in the Inner Mongolia Autonomous Region of China (Zhao et al., 2021). We were not able to identify the genus to the species level in sequencing results, but *Plectosphaerella*-ASV4 could potentially be *Plectosphaerella cucumerina* and could be causing additional root rot and has gone potentially overlooked in the past.

This highlights the benefits of using amplicon sequencing-based techniques to identify all pathogens present in the soil as previously used baiting techniques and direct isolation for diseased plants could have overlooked the fungal pathogens as well as *Phytophthora sansomeana* in the past. Further sequencing from alfalfa soils from other

states and regions, and isolation of these pathogens, is required to identify whether or not they contribute to significant damage in alfalfa seedlings.

Fusarium species that cause the most disease in alfalfa seedlings, as well as *Rhizoctonia solani* were not confirmed to a species level in our fungal sequencing results. Berg et al. (2017) identified *F. oxysporum* and *F. incarnatum-equiseti* as the most pathogenic on alfalfa seedlings. ASVs of confirmed *Fusarium* species that were identified at relatively high abundances were *F. waltergamsii* (ASV3) and *F. acutatum* (ASV5) (Fig. 3.29; Table 3.6 and 3.7). ASV8 and ASV32 could not be identified past the genus level, which indicates that they both or one of them could potentially be either *F. oxysporum* or *F. incarnatum-equiseti*. Ceratobasidiaceae is the family of *Rhizoctonia solani*, which has also been shown to contribute to seed rot and damping-off in alfalfa and could not be identified past the family level in sequencing, this family was not identified in large abundances but was still among the top 50 ASVs.

Bacterial sequencing revealed an abundance of rhizobia in both the endosphere and rhizosphere (Table 3.8 and 3.9). Few pathogenic OTUs were identified. The endosphere was dominated by actinomycetes, particularly Streptomycetes. These gram-positive filamentous bacteria are ubiquitous in soil and are well known for producing secondary metabolites, including antimicrobials (Vurukonda, Giovanardi, & Stefani, 2018; Worsley et al., 2020). They are frequently found as endophytes in plant roots and in some cases have been shown to have plant growth promoting effects and provide protection from biotic and abiotic stresses. Their role in alfalfa roots has not been investigated previously although some strains have shown to protect alfalfa seedlings from disease including Phytophthora root rot (Xiao, Kinkel, & Samac, 2002).

4.3 Quantify the abundance of known seedling disease pathogens

Real-time qPCR assays have previously been developed for five alfalfa pathogens, *A. euteiches* (Gangneux et al., 2014), *P. medicaginis* (Vandemark & Barker, 2003), *Pythium ultimum* (Schroeder et al., 2006)*, P. irregulare* (Kernaghan et al., 2008)*, and P. sylvaticum* (Schroeder et al., 2006). These assays are valuable tools for determining not only the presence of each pathogen in plants and soil, but also their abundance. However, qPCR assays present several challenges. Isolation of total DNA from soil can result in contamination by PCR inhibitors, and pathogen density in soil may be low and unevenly distributed in a field (Gilbert, Edel-Hermann, Moussa et al. 2021;

Ophel-Keller et al. 2008). Additionally, although high quality reproducible standard curves are generated from purified DNA of each pathogen, it may not be easy to relate DNA concentration to pathogen propagules in soil. The qPCR assays target a portion of the rDNA ITS sequence which can be present in variable copy numbers within each species. Multiple copies of the target sequence in the genome increases the sensitivity of the assay but complicates quantifying pathogen propagules. Gilbert et al. (2021) found that the ITS copy number per haploid genome of two *A. euteiches* isolates pathogenic on pea varied from 8.5 ± 0.3 copies to 239 ± 45 copies. The ITS copy number in the *A. euteiches* pathogenic on alfalfa is unknown, as are the copy numbers of ITS in *P. medicaginis* and the *Pythium* species assayed. If the ITS copy number varies as widely in alfalfa pathogens as in the pea pathogens, the abundance measured by sequencing and qPCR assays may inflate the true density of *A. euteiches* in soil and plant samples. Nonetheless, *A. euteiches and P. medicaginis* was detected in most rhizosphere soils and root samples (except for four plots out of 82 total from plots in all seven locations) suggesting that distribution was homogeneous in these locations.

In 2021, *A. euteiches* nanograms of DNA per gram of root was highest in the Ameristand cultivar roots as compared to MegaTron AA roots (Fig. 3.30) This indicates the race 2 *A. euteiches* was most likely present at the Spencer, Frankfort, and West Salem locations. The highest *A. euteiches* DNA concentrations were found in the roots as compared to rhizosphere soil or bulk soil DNA. This indicates that this pathogen rapidly infects and colonizes the roots of alfalfa plants, especially those that are susceptible to the pathogen (Samac et al., 2018).

Of the five pathogens tested in qPCR, *A. euteiches* was the most commonly detected in high levels across all plot locations indicating that distribution was relatively homogeneous. Distribution of *P. medicaginis* was evenly distributed at all locations but not always found in high concentrations. This indicates that *P. medicaginis* levels are still potentially damaging at the Spencer and Unity plots and could have contributed to seed rot and damping-off earlier in the cooler soils if rainfall was sufficient to encourage pathogen infection, as *P. medicaginis* infection occurs earlier in the season when causing seed rot and damping-off in cold soils, as compared to *A. euteiches* infection occurring in warmer soils. Cultivars with resistance to Phytophthora root rot were first released in the 1970s and the majority of modern cultivars have high resistance. Our

results show that this pathogen remains at high levels in alfalfa producing areas and development of highly resistant cultivars needs to be continued.

Pythium quantities varied by sample type and location. *Pythium irregulare* was not detected in high abundance by sequencing but was quantified in qPCR assays in plots; however, these quantities were relatively low (Fig. 3.34 and 3.35), and outliers drove increased quantities in certain plots. Berg et al. (2017) identified *P. irregulare* as one of the abundant and widespread *Pythium* spp. that caused significant disease in alfalfa seedlings. This study revealed that *P. irregulare* abundance was not high at our plot locations.

Pythium ultimum was a *Pythium* species that Berg et al. (2017) identified as a contributor to alfalfa seed rot and damping-off and one that was identified in high abundance in Minnesota soils. Our qPCR results found that *P. ultimum* was the most homogeneously distributed *Pythium* sp. at all the plot locations as compared to *P. irregulare* and *P. sylvaticum*. The *P. ultimum* quantities were highest in soil indicating that the pathogen does not cause significant root rot but may contribute seed rot and damping-off earlier in the season (Fig. 3.36 and 3.37).

P. sylvaticum was detected in roots in higher quantities than *P. irregulare* and *P. ultimum* across all locations indicating that *P. sylvaticum* does cause more root rot as compared to *P. irregulare* and *P. ultimum* (Fig. 3.38 and 3.39). In results from ITS sequencing, *P. sylvaticum* relative abundance was also highest in roots as compared to *P. ultimum* and *P. irregulare* across all locations but some plots had rhizosphere abundances higher than endosphere abundance and others had endosphere abundances higher than rhizosphere abundance (Table 3.4 and 3.5).

Because all *Pythium* species quantities varied by sample type and samples had outliers, this could indicate that *Pythium* inoculum is clustered in field settings as opposed to being uniform across an entire field. Oospore amounts could have varied by each sample type. Therefore, when collecting samples from field locations, it is important to sample many locations throughout the field for an accurate view of all pathogens that could be present. In addition, we may have seen lower *Pythium* abundance in sequencing and lower quantities in qPCR as compared to the root rot pathogens because we sampled at first harvest (07/12/2021) and not earlier in the season in cold/cool soils when *Pythium* would have caused the most severe disease to alfalfa

seedlings. Sampling multiple times during a growing season may provide a more accurate view of all pathogens that could be contributing to alfalfa seedling diseases caused by wet soil pathogens.

In general, the relative abundance of the oomycete pathogens detected by sequencing was similar to that obtained by qPCR assays. The advantage of ITS sequencing is obtaining data on the entire community, which can uncover organisms for which qPCR assays have not been developed or that have not been recognized as pathogen problems in the past. Obtaining data from sequencing can be slower than a qPCR assay, depending on the sequencing service used, and more expensive. However, as sequencing methods continue to evolve and analysis pipelines become more standardized, this technology may become a routine method for pathogen detection and identification. For large surveys with many soil or plant samples, community sequencing would be preferable in terms of time and labor costs for determining pathogen presence and abundance compared to qPCR assays.

Although sensitive qPCR assays have been developed for many plant pathogens, they have not been widely used for indexing field soils or to develop risk assessments for diseases. DNA-based soil testing services operate in Australia to assist grain growers in predicting the likely extent of losses from several soil-borne pathogens before planting so that appropriate cropping systems, cultivar selection, fungicide treatments and other management decisions can be made before planting (Ophel-Keller et al. 2008). Risk categories were developed based on data from pathogen population levels, bioassays or field data on economic damage, and local environmental data. Because there are few options for managing alfalfa diseases once plants are infected and showing symptoms, prior knowledge of disease risk would be of particular benefit to alfalfa farmers. The qPCR assay and bioassay data from the field plot locations as well as 31 soil samples from seven (Illinois, Michigan, Minnesota, New York, Ohio, South Dakota, Wisconsin) states determined in this study (Appendix 1) can be used to initiate risk assessments for oomycete pathogens of alfalfa.

Chapter 5: Conclusions and future directions

Post-planting risks due to soilborne pathogens are the most important challenge for famers. The wet soil syndrome causes significant damage to alfalfa seedlings limiting the ability to reach their full genetic potential and become healthy, high yielding, and persistent stands. Seeds and other establishment costs are the largest, most important input for alfalfa farmers. Those planting early into cool and wet soils and fields with a history of establishment problems from crusting, flooding, soil compaction or poorly drained soil are the most likely to benefit from fungicide seed treatment. This study revealed that seed treatments provide added control to resistance genetics in controlling *P. medicaginis* and that seed treatments provide adequate control to preventing seed rot and seedling damping-off from *Pythium*. This study also revealed that microbial communities of alfalfa plants are very diverse but *A. euteiches* is the dominating pathogen. However, we suspect added pressure from oomycete and fungal pathogens that have previously gone unrecognized in the United States as contributing to alfalfa seedling disease. Quantitative PCR assays allowed for quantification of known alfalfa pathogens and can provide insights in the future to alfalfa growers on which pathogens may be causing disease in their soils.

Further evaluation of the efficacy of the fungicide seed treatments when used in field conditions is needed due to below normal amounts of rainfall in spring 2021. Testing the fungicides at locations where the plots can be irrigated to ensure conducive disease conditions of oomycete pathogens is recommended to fully evaluate the effectiveness of each fungicide. Treating seeds with just one fungicide versus combining all fungicides together and eliminating other additive such as minerals and growth regulators, is recommended to see if Intego Solo provides adequate control of *Pythium* spp. and *A. euteiches* as compared to Stamina.

Using plot locations where aggressive *Pythium* spp. have been identified that are not controlled by Apron/Apron XL is also recommended to fully evaluate Intego Solo control of the *Pythium* spp. that are resistant to Apron/Apron XL. Further *in vitro* testing of fungicides against *P. sansomeana* to see if fungicides offer control to this pathogen is also needed with follow up tests of treated seeds. This can be done in lab settings similar to the *P. medicaginis* standard test or *Pythium* infested soil test. Individual testing of just Maxim treated seed against the true fungal pathogens identified in this study are needed to fully evaluate the efficacy of Maxim in controlling fungal pathogens.

Further sequencing of the alfalfa microbial community is needed from other states where alfalfa is grown to draw conclusions on the most prevalent pathogens in the alfalfa microbial community. This will allow for identification of the most prevalent oomycetes, fungi, and bacteria in the alfalfa microbiome that could be contributing to alfalfa seedling diseases from more regions than just Wisconsin. Identification of *P. sansomeana*, *Plectosphaerella*, or *Paraphoma radicina* would help confirm whether these pathogens play a significant role in contributing to disease in alfalfa across a widespread geography. In addition, isolation and testing of *Pythium aritosporum, P. selbyi, P. monospermum,* and *P. radicola* is needed to see if these *Pythium* spp. cause disease in alfalfa. Direct isolation of these pathogens that could be contributing to alfalfa seedling disease and have previously gone unrecognized is needed to study the pathogenicity of these organisms. Selecting for genetic resistance to these pathogens is warranted if they are found to be widespread across alfalfa growing regions. Significant progress to multiple *Pythium* species was obtained in one or two cycles of selection using a single moderately aggressive strain of *Pythium* (Samac, Dornbusch, & Ao, 2019). Improving resistance to *Pythium* species in alfalfa would likely improve seedling establishment and stand life.

Sampling early in the season in addition to sampling after first cutting, as we did in this study, could provide a more accurate view of all pathogens that could make up the wet soil syndrome complex. Sampling earlier in the season when soils are colder could reveal more *Pythium* pressure and *P. medicaginis* pressure leading to seed rot and damping-off caused by both pathogens as this could have gone overlooked when we sampled later in the season.

University disease clinics could begin to offer qPCR assays for *A. euteiches*, *P. medicaginis*, *Pythium* spp., and *Fusarium* spp. to help alfalfa growers identify which pathogens could be contributing to the largest amount of disease pressure in their alfalfa seedlings and mature stands. If *P. sansomeana* proves to be a contributor to alfalfa seedling diseases and stand failures, development of *P. sansomeana* primers for qPCR and standard curves would be warranted and the pathogen should be added to those already being tested for in qPCR assays. Potentially, the addition of true fungal pathogen qPCR assays that were identified in this study (*Paraphoma radicina* and/or the potentially identified *Plectosphaerella cucumerina*), as well as *Mycoleptodiscus terrestris*, *Fusarium oxysporum* and F. *incarnatum-equiseti* may also be needed if those

pathogens continue to be found in sequencing results from soils across the Midwest and Eastern alfalfa growing regions.

Bibliography

Agrios, G. N. (2005). *Plant Pathology* (Fifth Ed.). San Diego: Elsevier Academic Press.

Alcala, A. V. C., Paulitz, T. C., Schroeder, K. L, ., Porter, L. D., Derie, M. L., & du Toit, L. J. (2016). *Pythium* species associated with damping-off of pea in certified organic fields in the Columbia Basin of Central Washington. *Plant Disease*, *100*(5), 916–925. Retrieved from http://dx.doi.org/10.1094/PDIS-07-15-0774-RE

Altier, N. A., Barnes, D. K., Thies, J. A., & Samac, D. A. (1995). Pythium seed rot and damping-off resistance. *Plant Disease*, *79*, 341–346.

Alva, A. K., Lanyon, L. E., & Leath, K. T. (1985). Excess soil water and Phytophthora root rot stresses of Phytophthora Root Rot sensitive and resistant alfalfa cultivars. *Agronomy Journal*, *77*, 437–442. https://doi.org/10.2134/agronj1985.00021962007700030019x

Bakker, M. G., Moorman, T. B., & Kaspar, T. C. (2017). Isolation of cultivation-resistant oomycetes, first detected as amplicon sequences, from roots of herbicide-terminated winter rye. *Phytobiomes Journal*, *1*, 24–35.

Berg, L. E., Miller, S. S., Dornbusch, M. R., & Samac, D. A. (2017). Seed rot and damping-off of alfalfa in Minnesota caused by *Pythium* and *Fusarium* Species. *Plant Disease*, *101*(11), 1860–1867. https://doi.org/10.1094/PDIS-02-17-0185-RE

Bullied, W. J., Entz, M. H., Smith, S. R., & Bamford, K. C. (2002). Grain yield and N benefits to sequential wheat and barley crops from single-year alfalfa, berseem and red clover, chickling vetch and lentil. *Canadian Journal of Plant Science, 82*(1), 53-65.

Cao, S., Liang, Q. W., Nzabanita, C., & Li, Y. Z. (2020). Paraphoma root rot of alfalfa (*Medicago sativa*) in Inner Mongolia, China. *British Society for Plant Pathology*, *69*, 231–239. https://doi.org/10.1111/ppa.13131

Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G., & Brasier, C. M. (2000). A molecular phylogeny of *Phytophthora* and related Oomycetes. *Fungal Genetics and Biology 30*(1), 17–32. https://doi.org/10.1006/fgbi.2000.1202

Delwiche, P. A., Grau, C. R., Holub, E. B., & Perry, J. B. (1987). Characterization of *Aphanomyces euteiches* isolates recovered from alfalfa in Wisconsin. *Plant Disease*, *71,* 155–161.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, 10, 996–998.

Edgar, R. C. (2016a). SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. bioRxiv 074161. https://doi.org/10.1101/074161

Edgar, R. C. (2016b). UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. bioRxiv 081257. doi: https://doi.org/10.1101/081257

Edwards, E. E., Russell, H. H., History, S. M., & Mar, N. (1938). Wendlin Grimm and alfalfa. *Minnesota Historical Society Press*, *19*(1), 21–33.

Erwin, D. C. (1954). Root rot of alfalfa caused by *Phytophthora cryptogea*. *Phytopathology*, *44*(12), 700-704.

Fawke, S., Doumane, M., & Schornack, S. (2015). Oomycete interactions with plants : Infection strategies and resistance principles. *Microbiology and Molecular Biology Review*, *79*(3), 263–280. https://doi.org/10.1128/MMBR.00010-15

Fernandez, A., Sheaffer, C., Tautges, N., Putnam, D., & Hunter, M. (2019). *Alfalfa, Wildlife & the Environment* (Second Ed.). National Alfalfa and Forage Alliance. St. Paul, MN.

Fitzpatrick, S., Brummer, J., Hudelson, B., Malvick, D., & Grau, C. (1998). Aphanomyces Root Rot Resistance (Races 1 and 2). Retrieved from https://www.naaic.org/resource/stdtests.php

Frosheiser, F. I. (1980). Conquering Phytophthora Root Rot with resistant alfalfa cultivars. *Plant Disease*, *64*(10), 909–912.

Frosheiser, F. I., & Barnes, D. K. (1973). Field and greenhouse selection for Phytophthora Root Rot Resistance in Alfalfa. *Crop Science*, *13*, 1970–1973. https://doi.org/10.2135/cropsci1973.0011183X001300060044x

Gangneux, C., Cannesan, M., Bressan, M., Castel, L., Moussart, A., Vicré-gibouin, M., … Laval, K. (2014). A sensitive assay for rapid detection and quantification of *Aphanomyces euteiches* in Soil. *Phytopathology*, *104*(15), 1138–1147. https://doi.org/http://dx.doi.org/10.1094 /PHYTO-09-13-0265-R

Gao, X. D., Zhang, M. M., & Li, Y. G. (2016). First report of *Pythium aristosporum* causing corn stalk rot in China. *Plant Disease*, *105*(9), 2736.

Gibert, S., Edel-hermann, V., & Mcolo, R. M. (2021). Risk assessment of *Aphanomyces euteiches* root rot disease:Quantification of low inoculum densities in field soils using droplet digital PCR. *European Journal of Plant Pathology*, *161*, 503–528.

Grau, C. R. (1992). Registration of WAPH-1 alfalfa germplasm with resistance to Aphanomyces root rot. *Crop Science*, *32*, 287–288. https://doi.org/10.2135/cropsci1992.0011183X003200010074x

Hancock, J. G. (1983). Seedling diseases of alfalfa in California. *Plant Disease*, *67*, 1203–1208.

Hansen, E. M., Wilcox, W. F., Reeser, P. W., Sutton, W., Hansen, E. M., Wilcox, W. F., … Wilcox, W. F. (2017). *Phytophthora rosacearum* and *P. sansomeana*, new species segregated from the *Phytophthora megasperma* “complex.” *Mycologia*, *101*(1), 129–135. https://doi.org/10.3852/07-203

Jeffers, S. N., & Martin, S. B. (1986). Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease*, *70*, 1038–1043.

Judelson, H. S. (2012). Dynamics and innovations within Oomycete genomes:Insights into biology, pathology, and evolution. *Eukaryotic Cell*, *11*(11), 1304–1312. https://doi.org/10.1128/EC.00155-12

Kernaghan, G., Reeleder, R. D., & Hoke, S. M. T. (2008). Quantification of *Pythium* populations in ginseng soils by culture dependent and real-time PCR methods. *Applied Soil Ecology*, *40*, 447–455. https://doi.org/10.1016/j.apsoil.2008.06.011

Kim, D., Chun, S., Jeon, J., Lee, S., & Joe, G. (2004). Synthesis and fungicidal activity of ethaboxam against Oomycetes. *Pest Management Science*, *60* (10), 1007–1012.

https://doi.org/10.1002/ps.873

Lacefield, G. D., Henning, J. C., Rasnake, M., & Collins, M. (1997). *The Queen of Forage Crops*. http://www2.ca.uky.edu/agcomm/pubs/agr/agr76/agr76.pdf

Lamichhane, J. R., You, M. P., Barbetti, M. J., & Laudinot, V. (2020). Benefits of fungicide seed treatment rate and volume of fungicides used for seed treatment. *Plant Disease*, *104*(3), 610–623. https://doi.org/10.1094/PDIS-06-19-1157-FE

Lehman, W. F., Erwin, D. C., & Stanford, E. H. (1969). Registration of *Phytophthora*-tolerant alfalfa germplasm, UC 38 and UC 47 (Reg. Nos. GP 8 and GP 9). *Crop Science*, *9*(4), 527-527.

Lueschen, W. E., Barnes, D. K., Rabas, D. L., Frosheiser, F. I., & Smith, D. M. (1976). Field performance of alfalfa cultivars resistant and susceptible to Phytophthora root rot. *Agronomy Journal, 68*(2), 281-285.

Malvick, D. K., & Grau, C. R. (2001). Characteristics and frequency of *Aphanomyces euteiches* races 1 and 2 associated with alfalfa in the Midwestern United States. *Plant Disease*, *85*(7), 740–744.

Malvick, D. K., Grünwald, N. J., & Dyer, A. T. (2009). Population structure, races, and host range of *Aphanomyces euteiches* from alfalfa production fields in the central USA. *European Journal of Plant Pathology*, *123*, 171–182. https://doi.org/10.1007/s10658-008-9354-6

Marks, G. C., & Mitchell, J. E. (1971). Factors involved with the reaction of alfalfa to root rot caused by *Phytophthora megasperma*. *Phytopathology*, *61*, 510–514.

Miller, S. A., & Maxwell, D. P. (1984). Light microscope observations of susceptible, host resistant, and nonhost resistant interactions of alfalfa with *Phytophthora megasperma*. *Canadian Journal of Botany*, *62*(1), 109–116.

Munkvold, G. P., & Carlton, W. M. (1995). Prevalence and distribution of *Aphanomyces euteiches* and *Phytophthora medicaginis* in Iowa alfalfa fields. *Plant Disease*, *79*, 1251–1253.

Munkvold, G. P., Carlton, W. M., State, I., Benton, E., Brummer, E. C., … Undersander, D. J. (2001). Virulence of *Aphanomyces euteiches* isolates from Iowa and Wisconsin and benefits of resistance to *A. euteiches* in alfalfa cultivars. *Plant Disease*, *85*(3), 328–333.

National Alfalfa and Forage Alliance. (2020). Alfalfa Variety Ratings 2020. National Alfalfa and Forage Alliance. Retrieved from https://www.alfalfa.org/

Navarro, K. A., Wijeratne, S., Culman, S., Benitez, M., & Dorrance, A. E. (2021). Comparison of the species communities of *Phytophthora*, *Pythium* and *Phytopythium* associated with soybean genotypes in high disease environments in Ohio. *Phytobiomes Journal*, *5*(3), 1–17. https://doi.org/10.1094/PBIOMES-12-20-0089-R

Nygaard, S., Tofte, J., & Barnes, D. (1995). Phytophthora Root Rot--Seedling Resistance. Retrieved from https://www.naaic.org/resource/stdtests.php

Ophel-Keller, K., Mckay, A., Hartley, D., Herdina, & Curran, J. (2008). Development of a routine DNA-based testing service for soilborne diseases in Australia. *Australasian*

Plant Pathology, *37*, 243–253.

Radmer, L., Anderson, G., Malvick, D. M., Kurle, J. E.,., & Paul, S. (2017). *Pythium*, *Phytophthora*, and *Phytopythium* spp. associated with soybean in Minnesota, their relative aggressiveness on soybean and corn , and their sensitivity to seed treatment fungicides. *Plant Disease*, *101*, 62–72.

Samac, D., Dornbusch, M., & Ao, S. (2017). Enhancing alfalfa yields and stand life by improving management of seed rot and seedling damping off. https://alfalfa.org/pdf/USAFRI/Final%20Reports/2017/17Samac.pdf

Samac, D.A., Dornbusch, M. R., & Ao, S. (2019). Strategies for reducing seed rot and seedling damping-off of alfalfa (*Medicago sativa*). *Phytopathology, 109*, S2.60.

Samac, D.A., Dornbusch, M. R., Bucciarelli, B., Miller, S. S., & Yu, L.-X. (2018). Genetic mapping of resistance to Aphanomyces root rot in alfalfa. In *Second World Alfalfa Congress* (pp. 81–83). Cordoba, Argentina: Instituto Nacional de Tecnología Agropecuaria (INTA). Retrieved from http://www.worldalfalfacongress.org/%0AInstituto

Samac, D. A., Rhodes, L. H., & Lamp, W. O. (2015). *Compendium of Alfalfa Diseases and Pests* (3rd Edition). APS Press, St. Paul.

Samac, D. A., Yu, L. X., & Missaoui, A. M. (2021). Identification and Characterization of Disease Resistance Genes in Alfalfa and *Medicago truncatula* for Breeding Improved Cultivars. In *The Alfalfa Genome* (pp. 211-233). Springer, Cham. https://doi.org/10.1007/978-3-030-74466-3

Schroeder, K. L., Okubara, P. A., Tambong, J. T., Lévesque, C. A., & Paulitz, T. C. (2006). Identification and quantification of pathogenic *Pythium* spp. from soils in Eastern Washington using real-time polymerase chain reaction. *Phytopathology*, *96*(6), 637–647. https://doi.org/10.1094 /PHYTO-96-0637

Schroeder, K. L., Martin, F. N., Cock, A. W. A. M. De, Okubara, P. A., & Paulitz, T. C. (2013). Molecular detection and quantification of *Pythium* species: Evolving taxonomy, new tools, and challenges. *Plant Disease*, *97*(1), 4–20.

Scott, K., Eyre, M., Mcduffee, D., & Dorrance, A. E. (2020). The efficacy of ethaboxam as a soybean seed treatment toward *Phytophthora*, *Phytopythium*, and *Pythium* in Ohio. *Plant Disease*, *104*(5)1421–1432. https://doi.org/10.1094/PDIS-09-19-1818-RE

Smith, D. L., & Watson, Q. (2014). Aphanomyces Root Rot management in alfalfa. In *2014 Wisconsin Crop Management Conference* (Vol. 53, pp. 86–92).

Smith, D., & Watson, Q. (2014). Stamina seed treatment for Aphanomyces Root Rot control on alfalfa. Retrieved from https://badgercropdoc.com/2014/05/14/stamina-seed-treatment-for-aphanomyces-root-rot-control-on-alfalfa/

Stanger, T. F., & Lauer, J. G. (2008). Corn grain yield response to crop rotation and nitrogen over 35 years. *Agronomy Journal*, *100*, 643–650. https://doi.org/10.2134/agronj2007.0280

Syngenta. (2022).Maxim 4FS. Retrieved from https://www.syngenta-us.com/current-label/maxim_4fs.

Taheri, A. E., Chatterton, S., Gossen, B. D., & Mclaren, D. L. (2017a). Degenerate ITS7 primer enhances oomycete community coverage and PCR sensitivity to *Aphanomyces* species, economically important plant pathogens. *Canadian Journal of Microbiology*, *63*, 769–779.

Taheri, A. E., Chatterton, S., Gossen, B. D., & Mclaren, D. L. (2017b). Metagenomic analysis of oomycete communities from the rhizosphere of field pea on the Canadian prairies. *Canadian Journal of Microbiology*, *63,*758–768.https://doi.org/10.1139/cjm-2017-0099

Team, R. C. (2021). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.r-project.org/

Uchida, M., Roberson, R. W., Chun, S., & Kim, D. (2005). In vivo effects of the fungicide ethaboxam on microtubule integrity in *Phytophthora infestans*. *Pest Management Science*, *792*, 787–792. https://doi.org/10.1002/ps.1045

Undersander, D., Cosgrove, D., Cullen, E., Grau, C., Rice, M., Renz, M., … Sulc, M. (2011). *Alfalfa Management Guide*. American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc.

Uzuhashi, S., Tojo, M., & Kakishima, M. (2010). Phylogeny of the genus *Pythium* and description of new genera. *Mycoscience*, *51*, 337–365.

Vandemark, G J, & Barker, B. M. (2003). Quantifying *Phytophthora medicaginis* in susceptible and resistant alfalfa with a real-time fluorescent PCR assay. *Phytopathology*, *151*, 577–583.

Vandemark, G J, Barker, B. M., & Gritsenko, M. A. (2002). Quantifying *Aphanomyces euteiches* in alfalfa with a fluorescent polymerase chain reaction assay. *Phytopathology*, *92*, 265–272.

Vandemark, G. J., Ariss, J. J., & Hughes, T. J. (2010a). Real-time PCR suggests that *Aphanomyces euteiches* is associated with reduced amounts of *Phytophthora medicaginis* in alfalfa that is co-inoculated with both pathogens. *Journal of Phytopathology*, *158*, 117–124. https://doi.org/10.1111/j.1439-0434.2009.01583.x

Vaziri, A., Keen, N. T., & Erwin, D. C. (1981). Correlation of medicarpin production with resistance to *Phytophthora megasperma* f. sp. *medicaginis* in alfalfa seedlings. *Phytopathology*, *71*, 1235–1238.

Vincelli, P., Nesmith, W. C., & Eshenaur, B. C. (1994). Incidence of *Aphanomyces euteiches* and *Phytophthora medicaginis* in Kentucky alfalfa fields. *Plant Disease*, *78*, 645–647.

Vurukonda, K. S. S. P., Giovanardi, D., & Stefani, E. (2018). Plant growth promoting and biocontrol activity of *Streptomyces* spp. as endophytes. *International Journal of Molecular Sciences*, *19*(952), 1–26. https://doi.org/10.3390/ijms19040952

Wang, M., Vleet, S. Van, Mcgee, R., Paulitz, T., Porter, L., Schroeder, K., … Chen, W. (2021). Chickpea seed rot and damping-off caused by metalaxyl-resistant *Pythium ultimum* and its management with ethaboxam. *Plant Disease*, *105*(6), 1728–1737. https://doi.org/10.1094/PDIS-08-20-1659-RE

Wiersma, D. W., Grau, C. R., & Undersander, D. J. (2013). Alfalfa cultivar performance

with differing levels of resistance to Phytophthora and Aphanomyces Root Rots. *Journal of Production Agriculture*, *8*(2), 159–263. https://doi.org/10.2134/jpa1995.0259

Worsley, S. F., Newitt, J., Rassbach, J., Batey, S. F. D., Holmes, N. A., Murrell, J. C., … Hutchings, I. (2020). *Streptomyces* endophytes promote host health and enhance growth across plant species. *Applied and Environmental Microbiology*, *86*(16), 1–17.

Wu, L., Chang, K., Hwang, S., Conner, R., Fredua-agyeman, R., Feindel, D., & Strelkov, S. E. (2018). Evaluation of host resistance and fungicide application as tools for the management of root rot of field pea caused by *Aphanomyces euteiches*. *The Crop Journal*, *7*(1), 38–48. https://doi.org/10.1016/j.cj.2018.07.005

Xiao, K., Kinkel, L. L., & Samac, D. A. (2002). Biological control of Phytophthora Root Rots on alfalfa and soybean with *Streptomyces*. *Biological Control*, *295*(23), 285–295. https://doi.org/10.1006/bcon.2001.1015

Yost, M. A., Morris, T. F., Russelle, M. P., & Coulter, J. A. (2014). Second-year corn after alfalfa often requires no fertilizer nitrogen. *Agronomy Journal*, *106*(2), 659–669. https://doi.org/10.2134/agronj2013.0362

You, M. P., Lamichhane, J. R., Barbetti, M. J., & Aubertot, J.-N. (2020). Understanding why effective fungicides against individual soilborne pathogens are ineffective with soilborne pathogen complexes. *Plant Disease*, *104*, 904–920. https://doi.org/10.1094/PDIS-06-19-1252-RE

Zhao, Y. Q., K. Shi, X., Yu, Y., & Zhang, L. J. (2021). First report of alfalfa root rot caused by *Plectosphaerella cucumerina* in Inner Mongolia autonomous region of China. *Plant Disease*, *105*(9), 782828.

Zitnick-Anderson, K. K., & Nelson, B. D. J. (2015). Identification and pathogenicity of *Pythium* on soybean in North Dakota. *Plant Disease*, *99*(1), 31–38. https://doi.org/10.1094/PDIS-02-14-0161-RE

Zivanov, D., Tancic Zivanov, S., & Samac, D. (2021). First report of *Mycoleptodiscus terrestris* causing crown and root rot of alfalfa (*Medicago sativa*) in Minnesota. *Plant Disease*, *105*(1), 214.

Appendix

Supplementary Table 1. Field names with state and county information on soils requested from fields from other states in Illinois, Michigan, Minnesota, New York, Ohio, South Dakota, and Wisconsin.

State	County	Field Name
IL	Iroquois	Terry Fanning
MI	Mason	Saya's
MI	Mason	Gmas South
MI	Mason	Town East
MI	Mason	Weinerts
MI	Mason	Courtland West
MN	Dakota	Rosemount
MN	Ramsey	Campus Corn
MN	Ramsey	Campus Alfalfa
MN	Rice	Kelm Rebound AA
MN	Rice	Saemrow Turkey
MN	Le Sueur	Samerow Townsend
MN	Rice	Kuball East
MN	Rice	Kuball West
MN	Nicollett	Cross Country Harvatron
MN	Nicollett	Wenner Jaster West
MN	Nicollett	Amnexstand
NY	Wyoming	Hubert 2
NY	Wyoming	Leta 1
OH	Columbus	Swamp
OH	Columbus	E-3
SD	Lake	Lake County 1
SD	Lake	Lake County 2
SD	Lake	Lake County 3
WI	Marathon	4-5 MW
WI	Marathon	Joe's East MR
WI	Marathon	LA-34 MR
WI	Fond du Lac	Bruce Peterson
WI	Dane	Statz
WI	Marathon	Maple Ridge 2021
WI	Pierce	Hager City
WI	Clark	Beaver
WI	St. Croix	Emerald
WI	Marathon	Frankfort
WI	Marathon	Marshfield

WI	Clark	Spencer
WI	Marathon	Unity
WI	LaCrosse	West Salem

Supplementary Table 2. Bioassay percent resistant plants for all plot locations. Abbreviations for cultivars/treatments are as follows: MT: MegaTron AA treatment 1; MT2: Megatron AA treatment 2; MT3: MegaTron AA treatment 3; MT4: MegaTron AA treatment 4; MT5: MegaTron AA treatment 5; Am1: Ameristand treatment 1: Am2: Ameristand treatment 2; Am3: Ameristand treatment 3; Am4: Ameristand treatment 4; Am5: Ameristand treatment 5; S: Saranac; A: Agate; W1: WAPH-1; W5: WAPH-5.

Field Name	State	MT 1	MT 2	MT 3	MT 4	MT 5	Am 1	Am 2	Am 3	Am 4	Am 5	S	A	W1	W5
Gmas South	**MI**	4	46	81	72	77	33	34	76	78	60	48	46	30	37
Sayas	**MI**	36	91	82	86	86	31	-----	-----	-----	87	60	52	76	57
Rosemount	**MN**	80	77	72	94	94	81	92	84	84	89	77	57	83	86
Campus Corn	**MN**	77	94	95	92	90	98	-----	-----	-----	-----	68	61	94	81
Campus Alfalfa	**MN**	78	93	90	86	91	71	-----	-----	-----	-----	62	65	78	43
Kelm Rebound AA	**MN**	64	72	83	82	83	82	-----	-----	-----	-----	7	74	75	85
Samerow Turkey	**MN**	64	54	67	66	58	22	-----	-----	-----	-----	10	22	35	71
Samerow Townsend	**MN**	29	39	27	40	62	0	-----	-----	-----	-----	0	0	0	59
Kuball East	**MN**	77	89	71	87	87	63	-----	-----	-----	-----	28	71	71	78
Kuball West	**MN**	84	88	92	89	85	87	-----	-----	-----	-----	71	84	88	85
Cross Country HarvaTron	**MN**	43	59	33	54	52	3	-----	-----	-----	-----	0	0	0	48
Wenner Jaster West	**MN**	36	-----	49	31	56	-----	-----	-----	-----	-----	0	15	17	43
Annexstand	**MN**	91	82	93	92	89	67	-----	-----	-----	-----	70	56	76	88
Hubert 2	**NY**	44	51	50	46	40	0	-----	-----	-----	-----	0	0	0	58
Leta 1	**NY**	83	68	81	86	84	62	-----	-----	-----	-----	88	78	48	94
Swamp	**OH**	45	38	53	55	41	0	-----	-----	-----	-----	0	0	0	50
E3	**OH**	75	84	90	91	95	94	-----	-----	-----	-----	82	68	92	97
Lake County 1	**SD**	82	97	87	87	93	90	-----	-----	-----	-----	84	87	85	97
Lake County 2	**SD**	88	80	94	92	90	86	-----	-----	-----	-----	71	72	83	96
Lake County 3	**SD**	29	92	90	92	91	62	-----	-----	-----	-----	57	33	92	71
4-5 MW	**WI**	14	14	26	30	43	0	0	0	0	2	0	0	0	6
Joes East MR	**WI**	62	37	47	60	58	0	15	2	32	20	28	17	21	69
LA-34 MR	**WI**	44	64	26	39	56	0	5	9	0	0	0	0	0	40

Bruce Peterson	**WI**	11	6	31	30	35	2	-----	-----	-----	-----	0	0	0	13
Statz	**WI**	22	34	41	37	29	8	-----	-----	-----	-----	0	0	0	47
Maple Ridge 2021	**WI**	21	59	62	50	50	0	-----	-----	-----	-----	0	0	0	57
Hager City	**WI**	24	33	76	82	51	47	-----	-----	-----	-----	9	17	10	52
Beaver	**WI**	63	36	43	58	42	0	0	6	6	0	0	0	0	77
Emerald	**WI**	42	49	52	61	49	0	0	7	0	13	0	0	4	78
Frankfort	**WI**	64	67	71	76	76	18	11	34	53	37	0	0	19	62
Marshfield	**WI**	54	47	45	60	68	2	6	3	4	14	0	0	0	69
Spencer	**WI**	28	41	52	39	54	0	0	17	9	8	0	0	0	38
Unity	**WI**	36	35	37	53	60	0	6	8	12	17	0	0	0	15
West Salem 2021	**WI**	18	48	27	35	43	0	0	3	23	13	0	0	0	66
Overall Avg		**52**	**63**	**66**	**68**	**69**	**42**	**29**	**34**	**39**	**43**	**34**	**36**	**43**	**63**

Supplementary Table 3. Average quantities of nanograms of pathogen DNA per gram of bulk soil for soils requested from Illinois, Michigan, Minnesota, New York, Ohio, South Dakota, and Wisconsin.

		Avg. Quantities of Pathogen in ng of Pathogen DNA per g of Bulk Soil				
State	**Field Name**	***Aphanomyces euteiches***	***Phytophthora medicaginis***	***Pythium irregulare***	***Pythium ultimum***	***Pythium sylvaticum***
IL	Terry Fanning	0.00004114	0.00000000	0.00000000	0.00001942	0.01431347
MI	Saya's	0.00090834	0.11093963	0.89457777	18.32519472	0.71818908
MI	Gmas South	0.00000000	0.00760447	798.21523428	0.30925593	185.56809701
MI	Town East	0.00066156	0.04724438	0.07159911	4.17074176	0.66576151
MI	Weinerts	0.00013646	0.02292734	0.03102732	3.29956611	0.50148848
MI	Courtland West	0.00000000	0.00605595	239.56118220	1.57762322	0.12421201
MN	Rosemount	0.00000000	0.00032375	0.00000000	0.00000000	327.21951684
MN	Campus Corn	0.00237386	0.01425464	92.49238426	10.46189144	0.01795651
MN	Campus Alfalfa	0.00233408	0.00788112	0.00955305	14.87264087	0.01473570
MN	Kelm Rebound AA	0.00020100	0.02290000	0.00000000	0.00200068	0.00000000
MN	Samerow Turkey	5288.53512550	0.06460000	0.00051333	0.00905144	0.00000000
MN	Samerow Townsend	0.00152300	0.00000000	2.62522555	0.00166143	0.00000000
MN	Kuball East	2343.02908268	0.00000000	0.00018646	0.00141598	0.00000000
MN	Kuball West	7543.90635083	0.14960000	0.03998515	0.00293118	0.00000000
MN	Cross Country Harvatron	0.00271750	0.00000000	0.00000000	0.00000000	0.01133660
MN	Wenner Jaster West	0.00038200	0.00000000	0.00000000	0.00000000	0.00000000
MN	Amnexstand	3978.77300000	0.00187333	0.00000000	0.00004327	0.00195768
NY	Hubert 2	0.04765852	0.00968028	0.00624914	2.52770009	0.00107868
NY	Leta 1	50.04935707	0.01579114	0.00000000	2.88557166	0.00069484
OH	Swamp	0.00097890	0.00000000	0.00678482	0.01000780	0.00000000
OH	E-3	1083.37203035	26903.13000000	0.00050829	0.01391762	0.00000000
SD	Lake County 1	853.70173407	0.00814758	0.00274366	2.97567130	0.00431065
SD	Lake County 2	0.01046636	0.00878567	0.05420738	3.71234355	0.00116039
SD	Lake County 3	0.00019300	0.01210000	0.00092063	0.00238418	0.00000000
WI	4-5 MW	0.02391558	0.03750707	0.25946749	0.59504048	2.72367767
WI	Joe's East MR	0.00988138	0.03798672	0.33370609	0.84696271	5.10995798
WI	LA-34 MR	0.03713277	0.02327708	0.31184920	0.87065594	3.71537165
WI	Bruce Peterson	73.15628136	0.00515005	499.81164932	10.67924493	0.03457050
WI	Statz	0.00891714	0.01991715	0.40745570	4.32312911	0.06739902

WI	Maple Ridge 2021	0.01695625	46070.13833333	7620.48500000	455.24109715	0.27075672
WI	Hager City	0.00421333	41721.36000000	0.37428000	0.00012813	0.87132682
WI	Beaver	1.85719174	0.08516327	25.42584232	0.47463491	0.00795569
WI	Emerald	25.40477750	0.12923812	9.23136847	0.02113407	0.10818268
WI	Frankfort	0.05969467	838.57757378	0.00289005	0.00008651	0.02800431
WI	Marshfield	3352.94400670	0.00000000	161.47736895	0.05813604	193.72139028
WI	Spencer	0.00000000	1540.42835781	47.95539641	10.18916187	35.60195201
WI	Unity	0.24268288	276884512.68939	117967.536	1.66017397	1.25843102
WI	West Salem 2020	0.00137630	0.32138125	130.18720582	0.01045008	10.59846603
WI	West Salem 2021	0.355622095	0.028958294	0.00000000	0.288369879	3.058496031