CHARACTERIZATION AND IDENTIFICATION OF *PYTHIUM* ON SOYBEAN IN NORTH DAKOTA

A Dissertation
Submitted to the Graduate Faculty
Of the
North Dakota State University
Of Agriculture and Applied Science

By

Kimberly Korthauer Zitnick-Anderson

In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY

Major Department:
Plant Pathology

November 2013

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Identification and characterization of *Pythium spp*. on *Glycine max* (soybean) in North Dakota

By

Kimberly Korthauer Zitnick-Anderson

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. Berlin Nelson
Chair

Dr. Steven Meinhardt

Dr. Jay Goos

Dr. Laura Aldrich-Wolfe

Approved:

11/08/2013
Date

Dr. Jack Rasmussen
Department Chair

ABSTRACT

The Oomycete *Pythium* comprises one of the most important groups of seedling pathogens affecting soybean, causing both pre- and post-emergence damping off. Numerous species of *Pythium* have been identified and found to be pathogenic on a wide range of hosts. Recent research on *Pythium sp*. infecting soybean has been limited to regions other than the Northern Great Plains and has not included North Dakota. In addition, little research has been conducted on the pathogenicity of various *Pythium* species on soybean or associations between *Pythium* communities and soil properties. Therefore, the objectives of this research were to isolate and identify the *Pythium sp*. infecting soybean in North Dakota, test their pathogenicity and assess if any associations between *Pythium sp*. and soil properties exist. Identification of the *Pythium sp.* was achieved using molecular techniques and morphological features. A total of 26 described *Pythium sp.* and several unknown species were recovered from soybean roots collected from 138 fields between 2011 and 2012. The majority of *Pythium* species (*P. attrantheridium, P. debaryanum, P. diclinum, P. dissotocum, P. heterothallicum, P. hypogynum, P. inflatum, P. intermedium, P. irregulare, P. kashmirense, P. lutarium, P. minus, P. oopapillum, P. perplexum, P. terrestris, P. viniferum, P. violae,*and an unknown *Pythium sp.*) caused pre-emergence damping off on soybean seedlings with less than 50% emergence and survival. In contrast, *P. orthogonon, P. nunn,* and *P. rostratifingens* had approximately 80% or greater emergence and survival of soybean seedlings. The negative and positive controls had 100% and 0% emergence and survival of soybean seedlings. Associations between soil properties and three *Pythium* groups were performed using logistic regression analysis. Logistic regression analysis determined that the presence of group one characterized by *P. ultimum* was correlated with zinc levels. Group two was characterized by *P. kashmirense* and an unknown *Pythium sp.* and was

correlated with cation exchange capacity (CEC) values. Group three was characterized by *P. irregulare* and *P. heterothallicum* and was correlated with calcium carbonate exchange and CEC.

ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to my advisor Dr. Berlin Nelson, for all the time and patience he has shown me through the entire process of getting my PhD. I would also like to thank Dr. Anne-Marie Fortuna, Dr. Luis del Rio-Mendoza, and Dr. Jack Norland, Kendra Rodel, Tracy Christianson, and Susilo for their help with sample preparations, analysis, and general guidance and assistance. In addition I would like to express my gratitude towards the members of my committee, Dr. Steven Mienhardt, Dr. Laura Aldrich-Wolfe, and Dr. Jay Goos. Thank you everyone for the encouragement and honesty.

I want to say thanks to Matt Anderson, my family, and friends. Your support and willingness to listen to me talk about my research regardless of how little you understood was greatly appreciated. I could not have asked for better support system and companionship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

LIST OF APPENDIX FIGURES

CHAPTER I: LITERATURE REVIEW

Taxonomy and Phylogeny

Taxonomy

The genus *Pythium* is classified in the family Pythiaceae, under the order Pythiales, which is in the class Oomycetes in the phylum Heterokontophyta in the kingdom Chromalveolata. *Pythium* was first established in 1858 by De Haan and Hoogkame. Since the establishment of the genus, *Pythium* has been systematically treated at least five times before Middleton in the early 1930's reviewed and attempted to assemble information on the various species (Plaats-Niterick, 1981). Middleton published The Taxonomy, Host Range, and Geographic Distribution of the Genus *Pythium* in 1943. The publication was the first in depth identification key that included detailed descriptions and pictures of *Pythium* species (Plaats-Niterick, 1981). Not only did Middleton present the first detailed identification key but was the first to include all the species of *Pythium* known at that time (Plaats-Niterick, 1981). In 1967 and 1968, Waterhouse revisited the genus and provided the diagnosis and descriptions of over 180 species of *Pythium*. However with more species being discovered and described, monographs are needed in order to continuously update and add new information to the old. The most widely used identification key to date is the Monograph of the Genus *Pythium*, which was compiled by Plaats-Niterick in 1981. However new species are continuously being discovered, described by such notable scientists as Levesque, de Cock, and Moorman.

Categorizing the species of *Pythium* has always been problematic due to various reasons, such as difficulty in isolating certain species and the lack of molecular identification data for species (Plaats-Niterick, 1981). Another problem many scientists have had since *Pythium* was first described has been the identification of the morphological features of the various species

(Bala et al. 2010). Many *Pythium* species can have pleomorphisms, multiple variations of a specific morphological feature within one species (Plaats-Niterick, 1981). An example of pleomorphism has been observed in *P. vexans,* in which the antheridia can be in the monoclinus, diclinus, intercalary, or terminal positions (Plaats-Niterick, 1981). Although rare, the shape of the oogonium can be smooth or ornamented in some *Pythium* species (Middleton, 1943). For these reasons identifying *Pythium* species based on morphological features has been a constant problem for even the most experienced mycologists (de Cock and Levesque, 2004). Although molecular techniques have significantly assisted in the identification of unknown *Pythium* species, morphological features are still essential in supporting the identifications defined by molecular techniques.

Morphology

Pythium species produce many unique distinguishing traits and features including both asexual and sexual structures. Asexual features predominantly observed are the mycelium, sporangia, and zoospores (Middleton, 1943). The mycelium is usually fine filamentous and ranges between five to ten microns in diameter. The cylindrical hyphae are typically coenocytic in early growth and can develop random septation as the mycelium ages (Middleton, 1943). When hyphae are young, protoplasm can be observed streaming through the coenocytic hyphae (Plaats-Niterick, 1981). Aerial mycelium can also be observed in terms of patterns formed or habit of growth (Middleton, 1943). Species can be grouped according to their habit of growth but is by no means a method of specific species identification, because multiple strains of a single species can vary and species with different morphologies can have identical habits of growth (Plaats-Niterick, 1981). The growth habits or patterns can be classified as chrysanthemum,

radiate, arachnoid, pulvinate, or cumulous (Plaats-Niterick, 1981). Aerial mycelium can vary in pigmentation from white, pale yellow, to even a greyish purple/lilac (Plaats-Niterick, 1981).

Sporangia are often found to be spherical or filamentous (Middleton, 1943). The filamentous sporangia can range between five to ten microns in length, and the spherical can be rather large in size, ranging from eight to 40 microns in diameter (Middleton, 1943). Direct germination of sporangia occurs when protoplasm accumulates in an evanescent apical vesicle where zoospores are formed (Middleton, 1943). Sporangia can be smooth, obvoid, globose, filamentous, limoniform and can be positioned intercalary, terminally, or proliferating (Middleton, 1943). Filamentous sporangia can appear as inflated lobed branches or be unbranched (Plaats-Niterick, 1981). Filamentous sporangia can be difficult to recognize because they appear indistinguishable from the vegetative thallus or only mildly inflated (Plaats-Niterick, 1981). Zoospores of *Pythium* species can differ in shape but have similar components (Middleton, 1943). The number of zoospores produced in a single vesicle can range from two to 128 (Plaats-Niterick, 1981). Zoospores are generally bean or pear shaped with two lateral flagella; one located at the anterior end comprised of two rows of hair, and the other whiplike flagellum located at the posterior (Middleton, 1943). The zoospores will gradually become immobile and will encyst and recess into a spherical form (Plaats-Niterick, 1981). Germination occurs when a germ tube develops from the spherical encyst form of the zoospore (Middleton, 1943).

The sexual features predominantly observed are the oogonium, antheridia, and oospores. The structures can prove to be difficult to procure sexual structures are only formed under very specific environmental conditions (Middleton, 1943). The ideal temperature for sex structure formation in many species is 30 ^{o}C under wet conditions (Plaats-Niterick, 1981). The sexual

structures are easier to identify due to very specific visible traits (Middleton, 1943). Oogonium, oospores, and antheridia are the structures classified as sex organs. The oogonium has less variation in shape than that observed for sporangia (Plaats-Niterick, 1981). Although both sporangia and oogonia can appear similar, the typical shapes of sporangia include either limoniform or spherical, whereas the oogonia walls can either be smooth or have ornamentation of various kinds (Plaats-Niterick, 1981). Oogonia can be positioned intercalary or terminally. Oogonia vary between six to 75 microns in diameter (Plaats-Niterick, 1981).

Antheridia are usually discernible after the oogonium has reached maturity (Middleton, 1943). Antheridia can be monoclinus, diclinus, hypogynous, helical or intercalary (Plaats-Niterick, 1981). The types of attachements seen can be apical, apical branched, campanulate, or broad. The antheridial stalk can be absent, short, or long (Middleton, 1943). One to four antheridia can be found per oogonium (Middleton, 1943). The three dominant types of antheridia are monoclinus, diclinus, and hypogynous (Middleton, 1943). Monoclinus is when the antheridium branches and attaches to the oogonium that has formed from the same hyphal strand (Plaats-Niterick, 1981). Diclinus is when an oogonium is being fertilized or attached by antheridia from separate hyphae (Plaats-Niterick, 1981). Hypogynous antheridia refer to the proximal part of the oogonial stalk becoming an antheridium (Plaats-Niterick, 1981). The morphology and origin of antheridia are very specific and are considered to be valuable for species identification (Middleton, 1943).

Once an antheridium attaches to an oogonium fertilization occurs and oospores form. The oospore is also termed a zygote. Gametangial meiosis occurs in *Pythium*, and the fertilized oogonium results in a diploid thallus (Plaats-Niterick, 1981). Usually only one oospore develops, however, although rare, multiple oospores have been known to develop within the oogonium for

some species (Middleton, 1943). Oospores can range between four to 48 microns in diameter (Plaats-Niterick, 1981). The oospore wall can be smooth or reticulate and the wall can be thin, but is usually thick (1.8 to 3.8 microns); (Plaats-Niterick, 1981). The contents of the oospore are also valuable in the assessment of identification (Middleton, 1943). The contents are comprised of protoplasm that appears granular and opaque (Middleton, 1943). If the oospore is filled with protoplasm, the oospore is described as plerotic, whereas if the cavity is not filled the description aplerotic is used (Middleton, 1943). The oospores will also have either one or multiple refringent bodies within the cavity (Plaats-Niterick, 1981). The previously stated morphological features are characteristics for species segregation.

Pythium were thought to be only homothallic until 1967, when Campbell and Hendrix found evidence that *Pythium* can also be heterothallic. Sparrow was one of the first to determine that *Pythium* was homothallic (Sparrow, 1931). Hyphal tipping was used to isolate single hyphae which were grown on water agar (Sparrow, 1931). The results showed that sexual structures were successfully produced despite the fact that the antheridia were diclinus (Sparrow, 1931). In contrast, similar studies of hyphal tipping and dual cultures on water agar resulted in the production of both sexual structures only in the zone of contact between two compatible partners, thereby concluding that *Pythium* can also be heterothallic (Campbell and Hendrix, 1967).

Despite the fact that much work has been conducted on the morphology of the various *Pythium* species, the lack of definitive structures has always been a major limitation in taxonomic identification of *Pythium* (Bala et al. 2010). An example of *Pythium* species that have similar features would be *P. aphanidermatum* and *P. deliense* (McLeod et al. 2009). Therefore

molecular techniques have proven to be critical to the identification of unknown and known *Pythium* species.

Molecular

The first molecular research performed on Pythium began in the early 1980's but it was not till 2004 that all the *Pythium* species recorded at the time were defined, characterized, and categorized into a comprehensive database (McLeod et al. 2009). Molecular identification is achieved through the use of the polymerase chain reaction (PCR) and molecular markers or primers. Primers include regions within the ribosomal DNA such as the internal transcribed spacer (ITS), the large subunit (LSU); (Fig. 1) and the cytochrome c oxidase subunit 1 (CO1), located in the mitochondria (Bala et al. 2010). The ITS is the most extensively used for identification due to the development of PCR primers that amplify a highly variable region across all taxa, including Oomycetes (Bala et al. 2010). The ITS has been successfully used for identification of *Pythium* species (Bala et al. 2010). However, a combination of similar ITS regions among *Pythium* species and the submission of erroneous DNA sequences into databases such as GenBank can lead to misidentifications of *Pythium* species (Martin and Tooley, 2003; Schroeder et al. 2013). Hence, the use of both morphological and molecular techniques for identification is advised (Schroeder et al. 2013). The CO1 and ITS regions within the rDNA have been used as barcodes for *Phytophora* species (one of the closest relatives to the genus *Pythium*) because of the high interspecific and low intraspecific variation (Martin and Tooley, 2003). The large subunit of the ribosomal DNA contains the highly divergent regions D1-D3, and has been successfully used as a molecular marker for *Pythium* species (de Cock and Levesque, 2004); however the largest sequence reference database, GenBank, predominantly accepts sequences of the ITS region (Schroeder et al. 2013). Most recent research using molecular markers has found

that the sequencing results constructed phylogenetic trees that gave a 95% confidence level, indicating that the molecular markers effectively classify and identify *Pythium* species (Bala et al. 2010; de Cock and Levesque, 2004; Schroeder et al. 2013). From the combined molecular and morphological characteristics, phylogenetic trees can be created depicting the relationship between *Pythium* species. Ultimately both morphology and the DNA barcoding are important in identification of Pythium species (de Cock and Levesque, 2004; Schroeder et al. 2013). Recent research has shown that although species have dissimilar morphological features can have similar DNA sequences suggesting that those species are actually related (de Cock and Levesque , 2004). An example of such would be *P. perplexans and P. mastophorum*. Both species are found to be in the same clade, meaning molecularly they are very similar but morphologically, *P. mastophorum* has spinal oogonium wall ornamentation whereas *P. perplexans* has an oogonium that are smooth (de Cock and Levesque, 2004). In contrast, the submission of a *Pythium* species into GenBank database for molecular identification can result in multiple species being a match (Schroeder et al. 2013). Reasons for multiple identifications include the submission of erroneous data entered into GenBank or the ITS regions being similar, exemplifying the importance of morphological identification in conjunction with the molecular identification (Schroeder et al. 2013).

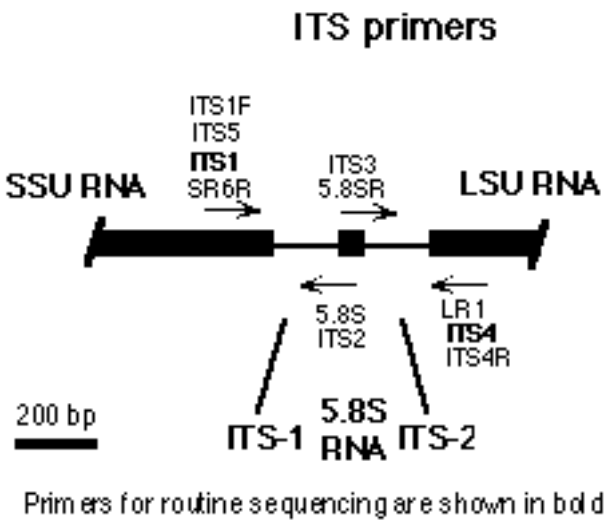

Figure 1. Displays the location of the ITS region within the ribosomal DNA (R. Vilgalys LAB, 2013).

Phylogeny

The phylogeny of *Pythium* has progressed with advances in molecular work. With the use of molecular markers, clades A-K have been developed within the genus based on ITS sequences and morphological features (Bala et al. 2010). Phylogeny based on ITS sequences shows that divergence occurs within *Pythium* when observing sporangia types (Bala et al. 2010). Research has shown that the globose type is likely to be ancestral because both outgroup species and the species in the outmost *Pythium* cluster develop globose sporangia (Bala et al. 2010). Clade D has similar ITS sequences but the sporangia are both globose and filamentous (Bala et al. 2010). Homothallism and heterothallism and oogonium ornamentation are taxonomic characteristics that also aid in the process of clade definition. For example oogonium ornamentations were found in six groups within the clades F and G (Bala et al. 2010). However many authors have mentioned that much of the previous phylogenetic analysis based on morphological features does not correlate with the evolutionary patterns (Bala et al. 2010; de Cock and Levesque, 2004; Martin and Tooley, 2003;Tambong et al. 2006). The combination of multiple primers for

different regions within the ribosomal DNA or combining both molecular and morphological identification techniques have been shown to be efficient in segregating *Pythium* species (Bala et al. 2010; Schroeder et al. 2013). Proper identification and characterization of *Pythium* is important in understanding the biology of and evolutionary relationships among the species.

Life Cycle

Taxonomy and phylogeny are important for identification but understanding the biology and ecology are important in determining proper disease management strategies. In order to develop effective management strategies one must understand the life cycle and ecology of *Pythium*. Oospores overwinter on plant debris (Agrios, 2005). Within plant debris coenocytic mycelium and sexual reproductive structures develop (Agrios, 2005). Once the antheridia attaches to oogonia, gametangial meiosis occurs followed by fertilization, resulting in the formation of an oospore (Agrios, 2005). At maturity the oospore germinates via a germ tube or a vesicle forms where zoospores develop (Agrios, 2005). The vesicle developed from the oospore is termed a zoosporangium (Agrios, 2005). Zoospores can then swim, encyst and develop a germ tube (Agrios, 2005). When an oospore germinates directly, the germ tube can infect the host just as the zoospores germ tubes do (Agrios, 2005). *Pythium* species infect the plant host through direct penetration of the plant cell wall (Agrios, 2005). The germ tube develops an appessorium and a penetration peg is inserted into the plant host (Agrios, 2005). Once inside the host mycelium begins to spread and develop throughout (Agrios, 2005). When the plant host has fully succumbed to the mycelium, asexual structures develop (sporangia) which either can re-infect the host through the production of zoospores or can overwinter on dead plant debris (Agrios, 2005). Consequently, *Pythium* are, in general, polycyclic in nature (Agrios, 2005). Environmental conditions have to be conducive for the pathogen to cause disease. Due to Soil

moisture is a huge factor in disease development, and is why *Pythium* is called a "water mold" (Agrios, 2005). Many studies have shown that the higher the soil matric potential the greater the disease severity. Other factors influence disease such as temperature (Bainbridge, 1970; Biedbrock and Hendrix, 1970; Schlub and Lockwood, 1981; Stanghellini and Hancock, 1971). The optimal temperature for many *Pythium* species to flourish is 30 ^{o}C (Leach, 1947).

Ecology

Pythium is a soil-borne pathogen and does not produce aerial spores for long distance dispersal. The area in which *Pythium* can infect plants only extends as far as the zoospores can travel (Stanghellini and Hancock, 1971). Zoospores need water for dissemination and, depending on the soil composition, can only travel as far as the capillaries and pore space within the soil allows (Stanghellini and Hancock, 1971). Long distance dispersal is predominantly due to humans transporting infected plant tissue and introducing the plant tissue to new surroundings. Research has shown that birds eating infected seed can also transport inoculum over long distances (Stanghellini and Hancock, 1971).

Vulnerability of the host to *Pythium* is greatest during seed germination. Germ tube and propagule formation in *Pythium* increases substantially when there are large quantities of exudates released by the seed (Stanghellini and Hancock, 1971). Environmental factors that increase seed exudates also increase the area around the seed that is high in nutrients. Such nutrients can stimulate microbial growth and development. Consequently these factors also increase the rate of seed infection (Stanghellini and Hancock, 1971). High soil moisture increases the distance that nutrients diffuse into the surrounding soil which can stimulate fungal propagules that could potentially infect the host. This is one reason why soil moisture is one of the major factors that affects *Pythium* germination (Stanghellini and Hancock, 1971). In addition

to environmental conditions influencing host susceptibility, time is also a factor (Chi and Hansn, 1962). Host susceptibility significantly decreases as the host ages (Chi and Hansn, 1962). In general, older plants are less likely to show signs and symptoms of *Pythium* infection (Chi and Hansn, 1962).

Host Range

Pythium are very generalistic and non-specific in their host range and are found in habitats ranging from aquatic to terrestrial. The host range includes insects, mammals, algae, or fish but the majority *Pythium* species are plant pathogens found within the soil and are of great economic importance in regards to agriculture (Plaats-Niterick, 1981). *Pythium* harbors some of the most important seed and seedling pathogens (Plaats-Niterick, 1981). When *Pythium* infect seed or seedlings before emergence from the soil, the result often is pre-emergence damping-off (Hendrix and Campbell, 1973). *Pythium* is also capable of infecting roots and the hypocotyl of seedlings after germination resulting in post-emergence damping-off (Hendrix and Campbell, 1973). Although seedlings can survive infection, the productivity of the plant is substantially hindered (Hendrix and Campbell, 1973). In addition to seedlings, *Pythium* can also infect the roots of mature plants causing necrotic lesions and may stimulate excessive branching of adventitious roots (Larkin et al. 1995).

Environmental Factors

Soil texture

As previously stated, environmental factors affect *Pythium* in many ways. One of the most important factors is soil characteristics, e.g. texture, organic matter, metals, salinity, and pH. Plant diseases caused by *Pythium* are more likely to occur within wet soils than in areas with drier soils. The texture directly affects a soils ability to retain moisture. A soil with a sandy

texture will have more water permeability than a soil with a greater clay or silt content. Earlier studies have indicated that as soil moisture increased so did disease severity (Biesbrock and Hendrix, 1970). Soil field capacity is associated with a soik water potential of -0.033NPa, wilting point is associated with a water potential of -1.5 MPa, and saturation with a water potential of 0 (Hillel, 1998). In soybean for example, when soil water potential increased from -0.18 to -0.0018 MPa, a reduction in seedling emergence was observed (Schlub and Lockwood, 1981). Disease incidence positively correlates with the number of days the soil water potential was greater than -0.05 MPa (Schlub and Lockwood, 1981). When soil moisture decreased, the motility of zoospores was affected because they require free water to move (Stanghellini and Hancock, 1971). Soil texture and consequently moisture may also affect *Pythium* by increasing the microbial diversity and therefore competition (Lifshitz and Hancock, 1983). When the percent clay in a soil increases, moisture retention increases, and the level of oxygen decreases which allows for *Pythium* to become the primary saprophytic organism (Coleman et al. 2004; Schaetzl and Anderson, 2007). When the percent sand and silt in a soil increases, moisture decreases, and more organisms are able to thrive and therefore can outcompete for nutrients essential for *Pythium* germination (Lifshitz and Hancock, 1983).

Results from a recent study contrast with traditional knowledge and observed that as moisture levels increased in soils high in clay content, the level of disease incidence and *Pythium* presence decreased (Broders et al., 2009). The results suggest that although *Pythium* has historically always been associated with soils higher in clay content and therefore moisture, the genus rely on organic matter (OM) to survive in high moisture environments. (Broders et al. 2009). In an environment with high moisture content and low oxygen levels, *Pythium* can transition into a facultative saprophytic life cycle which requires organic matter for sustenance.

Soil organic matter

Multiple studies have attempted to use organic matter (crop residue, manure, etc) for control of damping-off caused by *Pythium* (Boehm et al., 1993; Gregorich et al., 2006; Stone et al., 2001; Stone et al., 2004). Soil organic matter is comprised of different particle sizes and densities depending on stage of decomposition. As organic matter progressively deteriorates, the suppressive qualities decline, resulting in more damping off caused by *Pythium* to occur. The particulate organic matter (POM) fraction is composed of coarser detritus and material at the beginning stages of decomposition. When POM is incorporated into sand mixtures in greenhouse settings suppression of damping off occurs for at least one year (Stone et al., 2001). The components of the POM, make the material suppressive (Gregorich et al., 2006; Stone et al., 2001). Studies have shown that as the rate of decomposition progresses, the C to N ratio and levels of O-alkyl and alkyl-carbohydrates decrease, which leads to an increase in disease incidence (Gregorich et al., 2006). Carbohydrates are believed to be directly responsible for suppressing *Pythium* growth. Research has suggested that the POM nutrient availability is not an immediate source of nutrients to *Pythium* (Gregorich et al., 2006; Stone et al., 2001). In contrast the more advanced the level of decomposition of organic matter, the more available the nutrients essential for *Pythium* growth (Gregorich et al., 2006).

Soil metals

Pythium require certain nutrients in order to produce sexual structures, zoospores, and hyphae. On the other hand, an excess of certain nutrients or metals can hinder *Pythium* growth. The metal nickel has been observed to increase a plants ability to directly inhibit *Pythium* prior to contact with the plant root system, although the exact mechanism of the inhibition is unknown (Ghaderian et al. 2000). Iron is another metal that indirectly suppresses *Pythium* by stimulating

siderophore formation within the commonly abundant soil organism *Pseudomonas fluorescens* (Matthijs et al., 2007). A siderophore is a small molecule that chelates or binds and transports metals like iron to or in microorganisms such as *Pythium*. Current research indicates that strains of *Ps. fluorescens* that produce siderophores display strong *in vitro* antagonism against *Pythium* (Matthijs et al. 2007). In contrast, zinc has been shown to be critical in the formation of the oogonia and the asexual vegetative growth of *Pythium* (Lenney and Klemmer, 1966). In addition, zinc has also been observed to inhibit the antagonistic activity of *Trichoderma spp*. on *Pythium* (Naar, 2006).

Soil salinity

Initially, salinity was thought to have little to no effect on vegetative growth or the formation of sexual structures (Rasmussen and Stanghellini, 1998). Plants grown in soils with high salinity levels were assumed to have become necrotic due to salinity and not the presence of *Pythium* (Rasmussen and Stanghellini, 1998). However, two studies conducted in Oman suggests that soil salinity may have an effect on oospore production (Al-Sadi et al. 2010a,b). In artificial soils used in the greenhouse with an electrical conductivity level of 20 dS m^{-1} *Pythium* produced no oospores (Al-Sadi et al. 2010b). However, when isolates of the same *Pythium* species were collected from various fields from various geographical locations, the species were all tolerant to a range of salinity with EC values between 20 and 62 dS m^{-1}in an artificial environment (Al-Sadi et al. 2010b). Ultimately, the researchers concluded that salt-tolerant *Pythium* species were able to infect vulnerable plants under the environmental stress of high salinity (Al-Sadi et al. 2010b).

Soil pH

Soil pH is another important factor that can influence *Pythium* (Barton, 1958). *Pythium* species are generally recovered from soils with pH of 6.8 to 7.2 (Johnson and Doyle, 1986), and

few species have been recovered in acidic soil with pH of 4.5 (Johnson and Doyle, 1986). Very rarely are *Pythium* even recovered from soils with pH of 5.3 to 5.5 (Johnson and Doyle, 1986). The pH can also influence aspects of the life cycle of *Pythium,* including susceptibility to lysis and formation of resting structures (Barton, 1958). *In vitro* investigations found that increases in pH and were positively correlated to the oospore production and germination (Adams, 1971). The bioavailability of nutrients, soil minerals and compounds are heavily regulated by pH levels (Lindsay, 1979). Consequently, pH can impact *Pythium* by changing the availability of nutrients or possible toxic compounds within soil (Lewis and Lumsden, 1984). An example of altering the pH to suppress *Pythium* can be observed when CaO is added to soils containing peas (Lewis and Lumsden, 1984). When CaO is added the inorganic ammonium salts are converted to NH_3 which inhibits many *Pythium* species (Lewis and Lumsden, 1984). Understanding the biology and ecology of *Pythium* is important for the purposes of developing methods of control.

Methods of Control

Methods of control for *Pythium* can include chemicals, cultural practices, and the use of biocontrol agents. A biocontrol agent is a bacterium or fungus that can suppress plant disease. One biocontrol agent that can be used against *Pythium* is saprophytic *Pythium* species. Like most *Pythium, P.oligandrum* and *P. nunn* are aggressive primary colonizers of organic material but are not pathogenic on plants (Martin and Hancock, 1987). These species are used as biocontrol agents because their association with other phytopathogenic species, they are associated with suppressive soils, and they are antagonistic to pathogenic species (Martin and Hancock, 1987). In one study a field was inoculated with the oospores of *P.oligandrum* and allowed to germinate (Martin and Hancock, 1987). The field had experienced damping-off of chickpea (Cicer arietinum) seeds and seedlings (Martin and Hancock, 1987). The results showed that

P.oligandrum was able to reduce the incidence of disease caused by the indigenous populations of phytopathogenic *Pythium* species (Martin and Hancock, 1987). To increase the effectiveness of the biocontrol agent, $CaCO_3$ was added to increase the pH of the soil (Martin and Hancock, 1987). The data revealed that the reductions in disease could have been due to a change in the ecological balance of the rhizosphere that favored the biocontrol agent over the pathogen (Martin and Hancock, 1987). In California, similar research concluded that with high chloride concentrations, non-pathogenic *P. oligandrum* had a competitive advantage over the chloride sensitive pathogenic *P. ultimum* (Martin and Hancock, 1986).

More common forms of control include fungicides and cultural practices (Tamm et al. 2010). Fungicides, especially seed treatments, continue to be the preferential method of controlling phytopathogenic *Pythium* (Tamm et al. 2010). The most common and effective fungicides on the market are Aliette, Subdue, and Terrazole (Tamm et al. 2010). Although effective, fungicides are also very expensive and may not be readily available for the average grower, hence most crop consultants will suggest a combination of fungicidal application and cultural practices be implemented (Tamm et al. 2010). Cultural practices include tillage, flame weeding, and crop rotation (Tamm et al. 2010). As previously stated, there is a stage within the *Pythium* life cycle that can overwinter or thrive on dead plant debris (Agrios, 2005). Tilling fields to overturn the soil and bury the plant debris reduces the amount of inoculum (Tamm et al. 2010).

When soil is tilled using mould-board plowing, the top portion of the soil is inverted and the material that was once used by *Pythium* in the upper horizons of the soil is now located at deeper depths (Tamm et al. 2010). Additionally zoospores are farther removed from the host, reducing infection (Stanghellini and Hancock, 1971). Inverting the soil would decrease zoospore

motility, because the distance increases between the zoospore and free water at greater depths (Stanghellini and Hancock, 1971). Flame-weeding refers to another method to dispose of plant material, which is essentially burning the plant debris. Crop rotation is also important and can be effective (Tamm et al. 2010). Implementing this practice can reduce the *Pythium* population due to the removal of the host (Tamm et al. 2010).

CHAPTER II: CHARACTERIZATION AND IDENTIFICATION OF *PYTHIUM* ON *GLYCINE MAX* (SOYBEAN) IN NORTH DAKOTA

Introduction

Pythium, a genus within the Oomycetes, comprises one of the most important groups of seedling pathogens affecting soybean, causing both pre- and post-emergence damping-off. Oomycetes are fungal-like, but are a completely different group of microorganisms compared to fungi, and are often called water molds and in the kingdom Chromalveolata (Agrios, 2005). Numerous *Pythium* species are known to be pathogenic on soybean (Broders et al. 2007, Matthiesen and Robertson 2013; Jiang et al. 2012). Proper identification of the species causing infection is important for developing effective management strategies. *Pythium* species can react differently to different fungicidal applications (Broders et al. 2007) therefore one cannot assume that all *Pythium* species can be managed using the same methods. Infection by *Pythium sp*. typically results in pre- and post-emergence damping-off that affects soybean seeds, seedlings, and to a lesser extent, adult plants. Symptoms may also include discoloration of the hypocotyl and roots (Agrios, 2005). Total disintegration of soybean seed is also possible under appropriate conditions (Agrios, 2005).

The United States is a major soybean producing country (Ash, 2012). Total planted area for soybean in 2012 was estimated at 30.8 million hectares for the United States, with 1.8 million hectares located in North Dakota (Ash, 2012). Damping-off can be devastating, especially during

wet years, and is of great economic importance in the soybean industry (Ash, 2012). An important question is what role *Pythium* plays in damping-off and seedling disease of soybean.

There has been little research on the species of *Pythium* present in the Northern Plains region and no research conducted in North Dakota (Chase and Bartlett, 2013; Matthiesen and Robertson, 2013; Jiang et al., 2012; Rojas et al., 2013). We do not know which species are soybean pathogens in North Dakota or how important they are in causing disease in the field. With funding from the North Dakota Soybean Council, research was initiated on the identification and characterization of *Pythium* species in this northern production area. The results of the research could be used to develop management tools for diseases of soybean caused by *Pythium*.

Materials and Methods

Collection of plants

In June of 2011 and 2012 soybean seedlings were collected from 88 and 50 soybean fields, respectively, in 20 counties in the eastern half of North Dakota, the primary soybean production area of the state (Fig. 2). Areas sampled had high amounts of soybean fields and had been in soybean production for many years. Soybean fields were chosen at approximately 6 to 8 km intervals between fields or until a field was observed. Ten seedlings with roots at the first trifoliolate leaf stage were collected at random from each field with approximately 2.5 m between plants. GPS coordinates were recorded for each field (Appendix C). Seedlings were transported to the laboratory in coolers and were gently rinsed with lukewarm tap water to remove soil particles and lightly patted dry with paper towels. Seedlings were usually processed within 24 h of collection or stored at 4 °C until used.

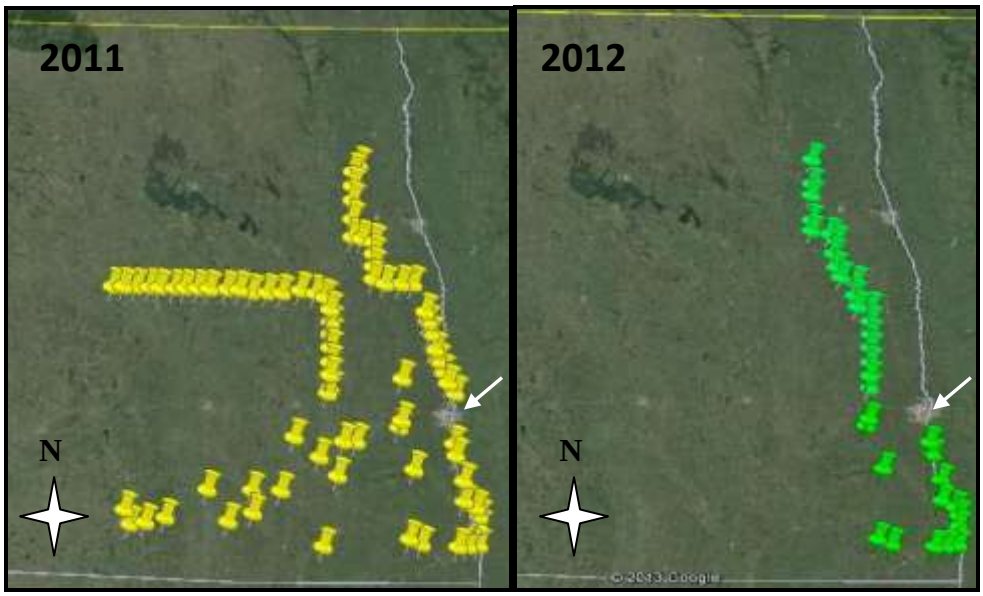

Figure 2. Map of North Dakota showing the location of the soybean fields sampled for *Pythium* in 2011 and 2012. Fargo in indicated by arrows (Google Earth).

Isolation and identification of isolates

Nine (2 cm long) cuttings were made at random from the roots of each plant and plated onto selective medium PARP+B (primaricin, sodium ampillicin, rifampicin, pentachloronitrobenzene, and benomyl) water agar (WA) using the under the block technique (Broders et al. 2007). *Pythium* can be present on seedlings regardless of the presence of disease symptoms, therefore justifying the random root cuttings of every plant collected. Root samples were incubated at 23 ± 2 °C in an incubator for 72 h then examined at 10X to 20X with a BX43 clinical microscope (Olympus, Center Valley, PA) under phase one lighting for presence of *Pythium*-like growth. Defining characteristics, such as coenocytic hyaline hyphae with flowing protoplasm, were used as criteria to select cultures for transfer. Sub-cultures were taken from the tips of hyphae and plated onto another selective medium, P_{10}VP V8 agar, containing pentachloronitrobenzene, primaricin, and vancomycin (Tsao and Ocana, 1969). Subcultures were grown for three days and then transferred to potato-dextrose agar (PDA Difco Laboratories).

Cultures colonized PDA for four to five days in the incubator at 23° C prior to attempts at identification.

Wet mounts of each isolate were made for morphological descriptions. Isolates that did not readily produce sexual structures on PDA were cultured using a grass leaf culture technique that was modified from Abad et al. (1994) by Zitnick and Nelson (2012b). Briefly, tap water was used instead of deionized water to boil a combination of grass clippings (tall fescue, cv. Grande II and Kentucky Bentgrass), the water was allowed to cool and was decanted off and saved. Agar plugs with mycelium were placed in the water used to boil the grass clippings in 100 x 20 mm petri dishes and incubated at room temperature for three to five days. Sexual structure production for heterothallic species (*P. diclinus, P. intermedium, P. kashmirense, P. attrantheridium, P. sylvaticum, and P. heterothallicum, P. inflatum*) was accomplished by combining multiple isolates of the same species into one petri dish. All morphological structures for each isolate were examined as described previously. All morphological features were photographed and recorded using an Infinity 2 digital camera and, Infinity analysis computer program (Lumenera Corp., Ottawa, Canada.).

Morphological features were compared to descriptions of species listed in the identification keys by Plaats-Niterick (1981) and Dick (1990). These keys do not include a number of newly described species. When an isolate could not be identified using either key, DNA sequence analysis was used as described below to obtain a putative identification. From those potential species identities the original publications describing the species were consulted and morphological features of the unknown were compared to those described in the literature.

In addition to morphological features, DNA sequences were also used to identify isolates to species. The internal transcribed spacer (ITS) sequence is a widely used DNA region that has

good resolution and is method accepted by the mycology community for species identification (Robideau et al. 2011). The primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used to amplify a section of the 18S region, ITS1, the 5.8S region, ITS2, and a section of 28S region of ribosomal DNA (Broders et al. 2007). The DNA extraction and PCR methods were as stated in Broders et al (2007). The DNA extraction and PCR were performed on all isolates three times to confirm the molecular identification. The DNA sequence data were compared to known sequences that had been deposited in the National Center for Biotechnology Information (NCBI) nonredundant database to confirm morphological identification or to assist in the identification of isolates where using morphological identification was not attainable. The BLAST parameters for the sequences were sequence lengths, e-values, maximum identity match, and query coverage. The sequence lengths were approximately 800 bp or greater. Identities were selected based on e-values of 0.0, maximum identity match of 95% or greater, and a query coverage of 98% or greater (Appendix C).

Pathogenicity trials

Three isolates of each species were randomly selected and tested for pathogenicity on soybean. Although pathogenicity has been previously recorded for certain *Pythium* species on soybean, this study yielded species for which pathogenicity on soybean was unknown. Pathogenicity was defined as a pathogens ability to cause disease (Agrios, 2005). The focus of the pathogenicity trials was to determine if the *Pythium* species were pathogens and not to compare degree of pathogenicity between species.

Inoculum was prepared using the methods stated in Broders et al (2007) with slight modifications. A soil plus cornmeal substrate was prepared from 237.5 g of sandy loam soil (La Prairie sandy loam), 12.5 g of cornmeal, and 80 ml of deionized H_2O in a 1000 ml beaker, then

autoclaved for 30 minutes. Sub-cultures of each isolate on PARP+B medium were plated onto PDA and incubated at 23° C for 6 d. An isolate was then diced, mixed into the autoclaved soil plus cornmeal substrate, covered with tin foil and allowed to colonize the substrate for nine days at room temperature. The substrate was occasionally shaken to enhance inoculum production.

Plastic cups (500 mL; Solo cups, Dart Container Corp., Mason MI) with drainage holes drilled in the bottom were used to grow plants in the presence of the *Pythium* species. Approximately 283 g of autoclaved non-infested La Prairie sandy-loam soil was placed into a plastic cup, followed by 83 g of inoculum, and an additional layer of 114 g of non-infested autoclaved soil. Ten Barnes soybean seeds were then planted 3 cm deep into each cup. Plants were incubated at 23 ± 2 °C in a growth chamber (19 cu ft; Percival 35LL, Boone IA) with 12 h of fluorescent light daily for 14 days. Pathogenicity was assessed using the following criteria: number of emerged seedlings, and number of living seedlings. Results for each species were compared to the positive and the negative controls. The positive control consisted of *P. ultimum* infested soil. *Pythium ultimum* was selected for a positive control due to the extensive documentation citing *P. ultimum* as a highly aggressive pathogen on soybean. The negative control consisted of non-infested soil. Emergence was defined as a plant that broke the soil surface and the cotyledons were visible above the soil surface. Surviving plants were extracted from the cups and examined for evidence of discoloration or lesions on the root system or base of the stems. For isolates that caused disease, the seeds, roots, and shoots were sampled and pieces were placed onto PARP+B WA for re-isolation and identification of *Pythium* as previously described.

Isolates were tested in groups of three at a time due to the limited space in the growth chamber. The experimental design was a random design with three replications (each cup as a

replicate). Each of the three isolates was tested once, then the data from the three isolates of a species were combined to represent the pathogenicity data for that species. The data from the three isolates of a species were combined only if isolates had similar effects on plants. Positive and negative controls were used in all trials. A pathogenicity test was considered successful only if the results from the positive and negative controls were as expected (+ control had 0% seedling emergence and survival; - control had 100% seedling emergence and survival). Data from all species tested were combined and analyzed using PROC UNIVARIATE in Statistical Analysis System (SAS version 9.1; SAS Institute, Cary, NC). Confidence intervals were obtained for each *Pythium* species and graphed.

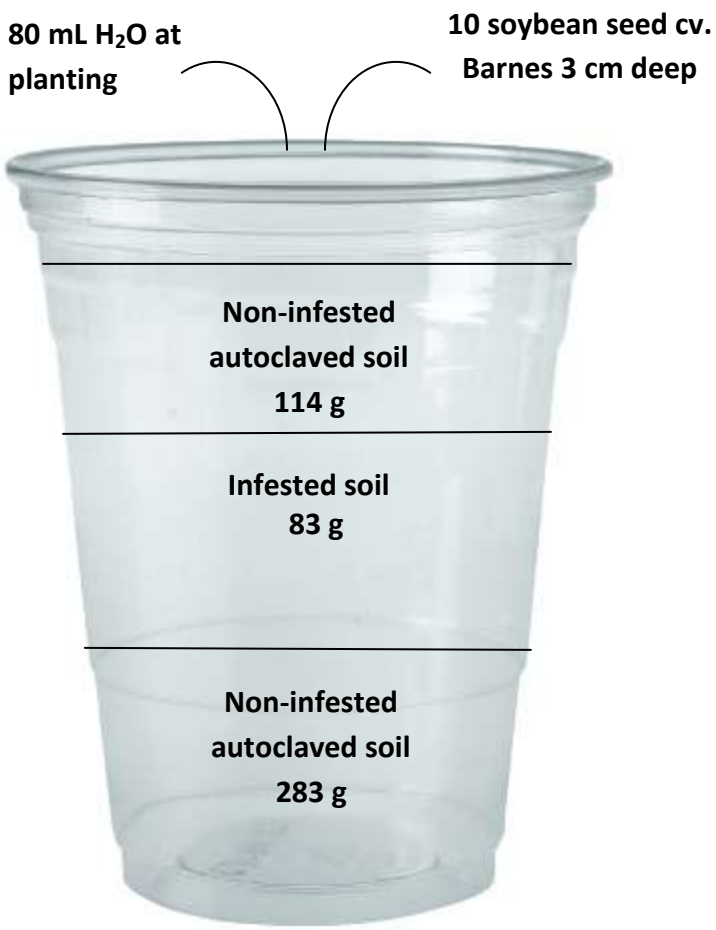

Figure 3. Design of the experimental unit used to test pathogenicity of *Pythium sp*. Soil was infested by growing *Pythium sp.* in a sandy loam soil and cornmeal mixture for nine days.

Results

Isolation and identification

A total of 2657 isolates of *Pythium* were recovered from 88 fields in 20 counties during 2011 (Fig. 3). In 2012 only 270 isolates of *Pythium* were recovered from 50 fields in 10 counties, even though 1024 isolates of *Pythium* like organisms were initially isolated on the selective medium (Fig. 4). Overall, 26 species (*P. attrantheridium, P. aristosporum, P. arrhenomanes, P. coloratum, P. debaryanum, P. diclinum, P. dissoctum, P. heterothallicum, P. hypogynum, P. inflatum, P. intermedium, P. irregulare, P. kashmirense, P. lutarium, P. minus, P. nunn, P. oopapillum, P. orthogonon, P. periilum, P. perplexan, P. rostratifingens, P. sylvaticum, P. terrestris, P. ultimum, P. viniferum, and P. viola*) of *Pythium* were identified using both DNA sequence analysis and morphological features (Fig. 3-5). A substantial portion of the total number of isolates from the 2011 survey was unknown *Pythium spp*. (Fig. 3). The 16% that were not able to be identified to species had three accession numbers from the NCBI database. There were 219 isolates that were identified as HQ643829.1, and HQ643823.1but based on the BLASTn parameters, the isolates could not be differentiated between the two GenBank accession numbers. There were 252 isolates that were identified as HQ643777.1, the most frequently occurring of the three unknown *Pythium species*. Twenty-four and five species identified from the 2011 and 2012 root samples, respectively.

The results of the *Pythium* identification using DNA sequence analysis, on occasion, yielded multiple identities of a single isolate according to the NCBI database. Therefore, species identification in these cases was based primarily on morphological features and comparing those of the unknowns to the various species identified by sequence analysis. However, the most recent *Pythium* identification key (Dick, 1990) did not include six species (*P. attrantheridium, P.*

kashmirense, P. oopapillum, P. rostratifingens, P. terrestris, and *P. viniferum,*) identified by sequence analysis. Original publications (Allain-Boulé et al. 2004; Bala et al., 2010; de Cock and Lévesque, 2004; Paul, 2002; Paul and Bala, 2008; Paul et al. 2008) first describing those six species were used to match the morphological features (Table 1) with the unknowns to confirm the identification by sequence analysis. Examples of the unique features used for identification of the six species include structure and ornamentation of the sporangia and oogonia, and number/placement of antheridia on oogonia (Fig. 6). Isolates with accession numbers HQ643829.1, HQ643823.1, and HQ643777.1 have yet to be described as a species. All isolates identified to species by sequence analysis had e-values of 0.0, maximum identity match of 95% or greater (only two were less than 99%) and query coverages of 96% or greater to reference (only five were less than 100%) GenBank accessions.

In 2011, the three most abundant species isolated were *P. ultimum, Pythium sp.* (unknown; GenBank HQ643777.1), and *P. heterothallicum,* representing 21, 16, and 12% of the total isolates respectively (Fig. 4). Four species, *P. irregular, P. attrantheridium, P. sylvaticum,* and *P. perplexum* represented 9, 8, 7, and 6% of the isolates, and the remaining 20 species and *Mortierella* each represented 3% or fewer of the isolates. The three most abundant species isolated during 2012 were *P. rostratifingens, P. inflatum,* and *P. heterothallicum,* representing 9, 6 and 6% of the total isolates, respectively (Fig. 5). In addition to *Pythium,* the Zygomycete *Mortierella* was isolated on the selective medium, identified morphologically and molecularly, and was the most prevalent organism isolated in 2012.

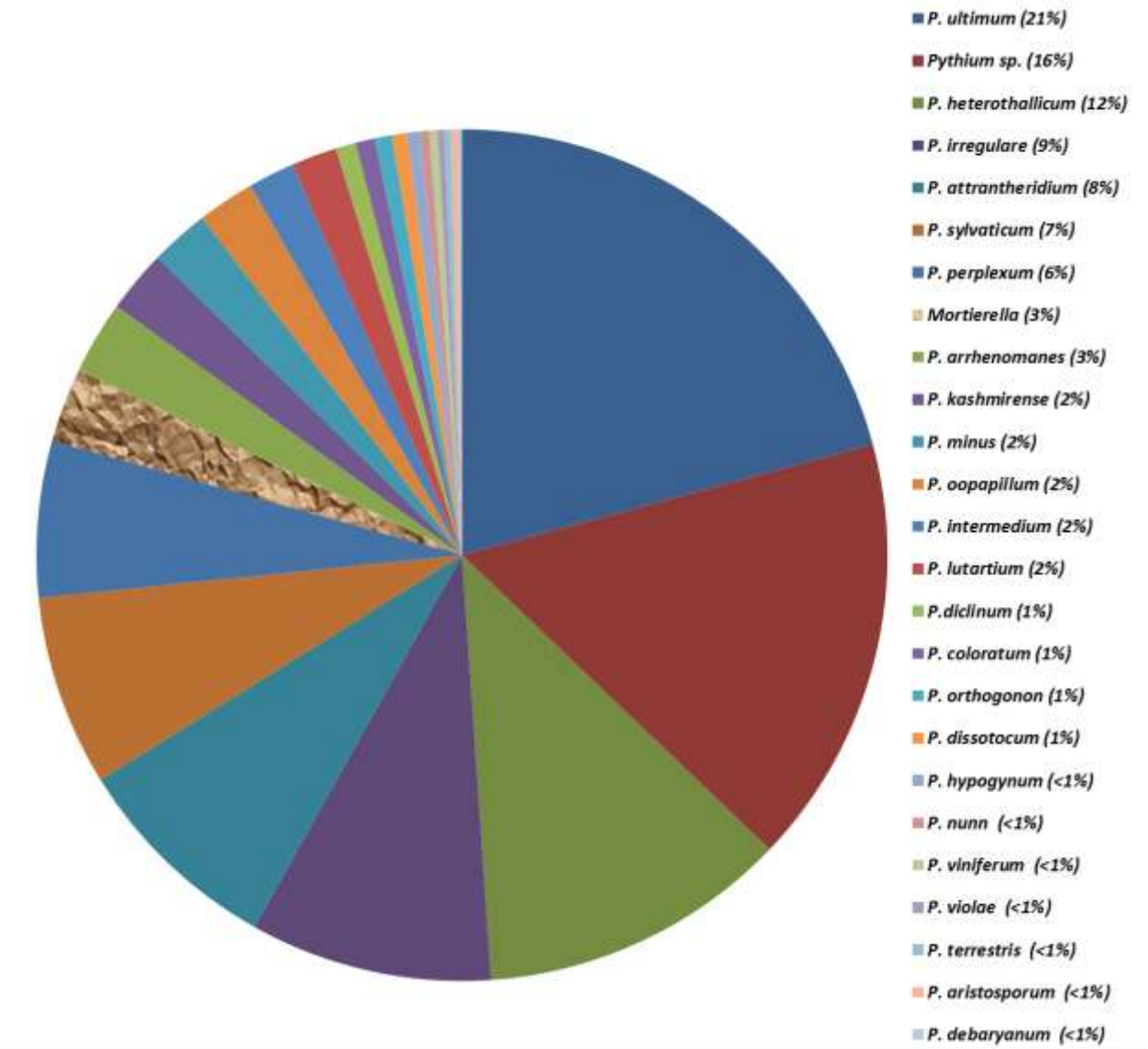

Figure 4. Frequency distribution of *Pythium* species for 2657 isolates recovered from soybean roots collected from 87 fields in North Dakota in 2011. The frequency of each species is indicated after the name. *Mortierella* is a zygomycete.

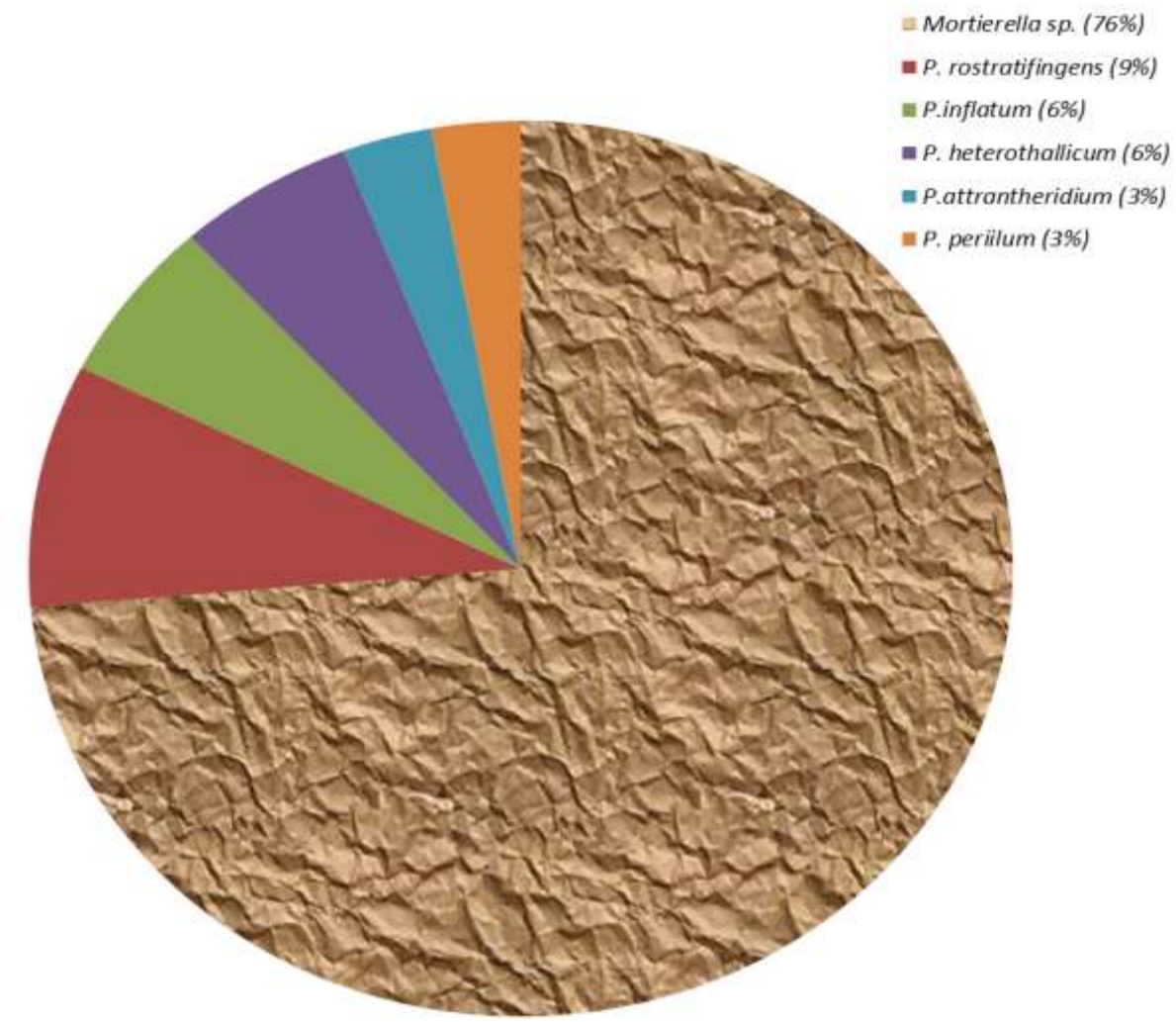

Figure 5. Frequency distribution of *Pythium* species and *Mortierella* out of the 1024 isolates recovered from soybean roots collected from 38 fields in North Dakota in 2012. The percent frequency of each organism is indicated after the name. *Mortierella* is a zygomycete.

Table 1. Defining morphological characteristics used to identify six *Pythium* species not found in the monographs by Plaats-Niterick (1981) and M.W. Dick (1990).

Pythium species	Asexual	Oogonia	Antheridia	Oospore	Growth Pattern
P. viniferum[a]	Sporangia sickle shaped appressoria bearing sexual structure	Intercalary, can be elongated or dumbbell shaped	Hypogynous, monoclinous sessile, or monoclinous on short branches	Mainly plerotic, can be elongated and peanut shaped.	On PDA: colonies are submerged and radial/arachnoid patterned
P. oopapillum[b]	Sporangia filamentous inflated	Mostly intercalary, smooth, globose	1-2 per oogonium, monoclinous, diclinous on branched stalks club-shaped, making apical or lateral contact	Thick-walled and with a papilla	On PDA: chrysanthemum patterned
P. rostratifingens[c]	Sporangia intercalary, occasionally terminal/oval, discharge tubes up to 30µm long, many sporangia do not develop zoospores	Intercalary, globose, and smooth	1-4, mainly 2 per oogonium, monoclinous, occasionally diclinous, on short stalk or hypogynous	Wall thickness up to 1.5µm	On PDA: colonies are submerged and chrysanthemum patterned
P. terrestris[d]	Sporangia globose and can be elongated, mainly intercalary, has short discharge tubes	Smooth walled, intercalary, and densely filled with protoplasm	Hypogynous or monoclinous which can coil around oogonial stalk and form a knot	Aplerotic, wall thickness between 2-4µm	On PDA: colonies are submerged narrow chrysanthemum patterned
P. attrantheridium[e]	Sporangia only produced by + mating type, terminal/intercalary, globose, discharge tube 27µm in length	Terminal, .5µm wall thickness	Diclinous, vanishes after fertilization, inflated and broad apical attachment	Plerotic or aplerotic, 1.5µm wall thickness	On PDA: + mating type vague radiate patterned, - mating type chrysanthemum patterned
P. kashmirense[f]	Numerous, filamentous, inflated, contiguous	Mainly intercalary, chain-like formation, densely filled with granular protoplasm	Diclinous, tight/loose coiling around oogonial stalk, 1-6 antheridia attached to the oogonia	Both aplerotic/plerotic, only one ooplast per oogonium, spherical, very thin wall .75-2µm wide	On PDA: colonies submerged, broad chrysanthemum patterned

[a] Paul et al. 2008
[b] Bala et al. 2010
[c] de Cock and Levesque, 2004
[d] Paul 2002
[e] Allain-Boulé et al 2004
[f] Paul and Bala 2008

Figure 6. Examples of unique features of six *Pythium* species. A. hypogynous antheridia, as indicated by arrows, *P. rostratifingens*; B. wavy exterior oospore wall, *P. attrantheridium*; C. thick walled oospore with papillae, as indicated by arrow, *P. oopapillum*; D. papillated sporangia indicated by arrows, *P. terrestris*; E. six antheridia attached to one oogonium, *P. kashmirense*; F. sickle shaped sporangia bearing elongated oogonium, as indicated by arrow, *P. viniferum*.

Pathogenicity trials

Confidence intervals, generated at the 95% confidence level, indicated that there were no differences in pre-emergence damping-off and survival of plants among 20 of the *Pythium* species. *Pythium orthogonon, P. nunn,* and *P. rostratifingens* were noticeably different from the other *Pythium* species. The majority of *Pythium* species (*P. attrantheridium, P. debaryanum, P. diclinum, P. dissotocum, P. heterothallicum, P. hypogynum, P. inflatum, P. intermedium, P. irregulare, P. kashmirense, P. lutarium, P. minus, P. oopapillum, P. perplexum, P. terrestris, P. viniferum, P. violae, Pythium sp.* (unknown; GenBank HQ643777.1)) caused pre-emergence damping-off on soybean with less than 50% seedling emergence compared to the negative

control which had 100% seedling emergence and the positive control which had 0% seedling emergence (Fig. 7); (Appendix C).

Two *Pythium* species, *P. periilum and P. heterothallicum*, had approximately 70 to 75% emergence but less than 20% of the plants survived two weeks after planting (Fig. 7 and 8); (Appendix C). In contrast, P. *orthogonon, P. nunn,* and *P. rostratifingens* had approximately 80% or greater seedling emergence (Fig. 7) and at the end of the two week period all seedlings appeared healthy based on above ground appearance (Fig.8). The other *Pythium* species had zero to 45% seedling survival after the two weeks (Fig. 8). However, most surviving plants in all species except P. *orthogonon, P. nunn,* and *P. rostratifingens* were stunted, discolored, and had numerous lesions on the roots/shoots similar to those shown in Fig. 9-10 (Appendix A). Although the surviving plants of *P. orthogonon, P. nunn*, and *P. rostratifingens* appeared healthy, the roots of those plants had small (4 cm long), brown lesions (Fig. 9-10). *Pythium coloratum*, *P. aristosporum*, and *P. arrhenomanes* were not included in the pathogenicity trials, because they could not be recovered from storage. Also two of the unknown species were not tested for pathogenicity. All *Pythium* species were re-isolated from lesions on infected plants and re-identified using the methods previously described.

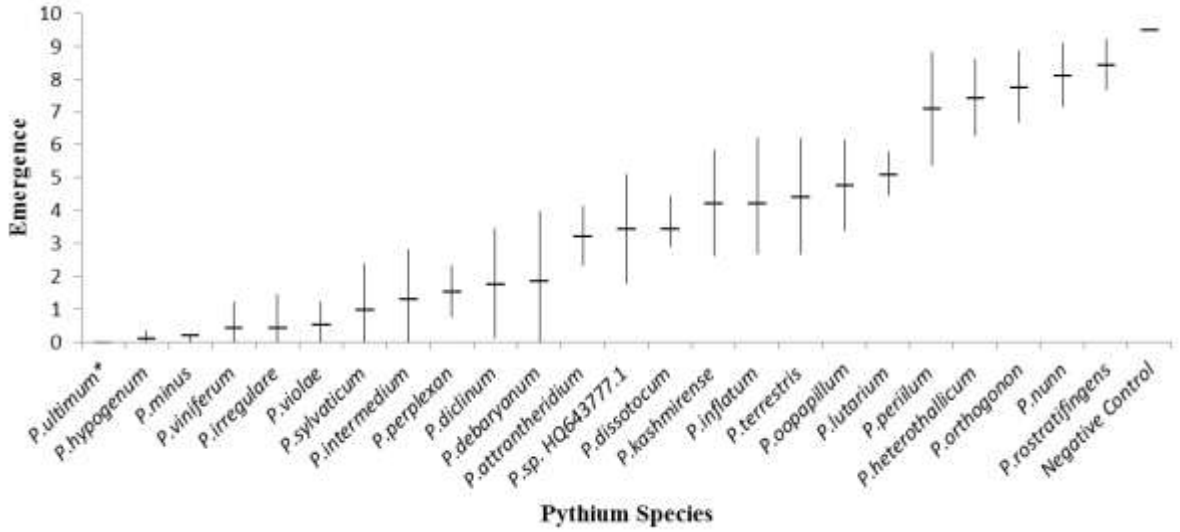

Figure 7. Confidence intervals for emergence of soybean plants in the presence of *Pythium sp*. Data are the average number of plants per replicate combined over three experiments. The asterisk indicates the positive control. Plants were grown for two weeks in cups containing a mixture previously described in the materials and methods. Emergence was defined as any plant that broke the soil surface.

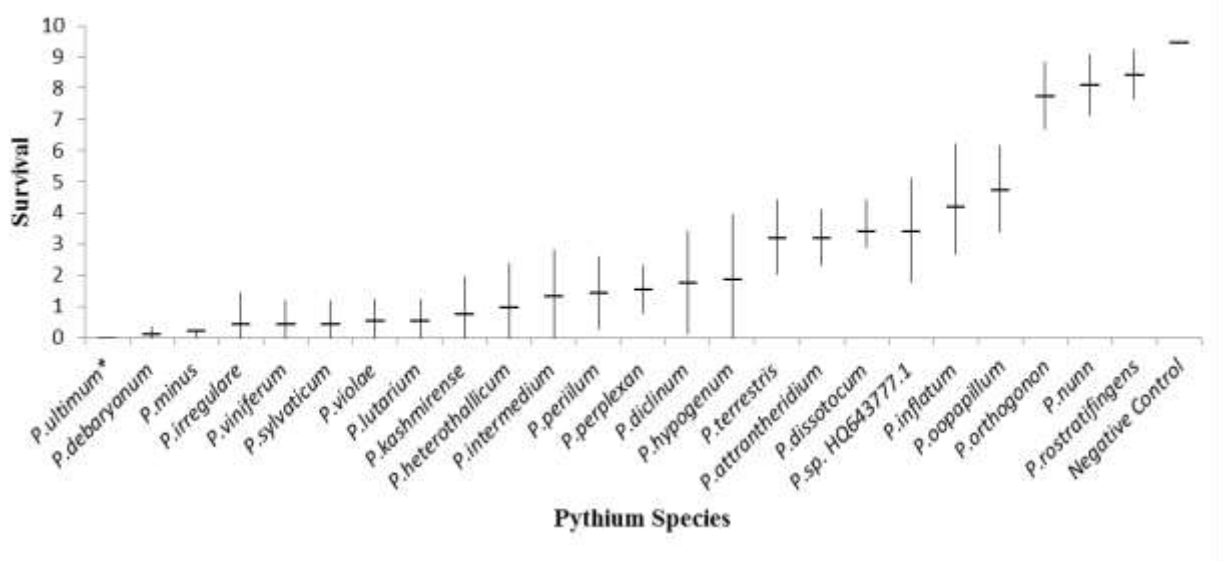

Figure 8. Confidence intervals for survival of soybean plants in the presence of *Pythium sp*. Data are the average number of plants per replicate combined over three experiments. The asterisk indicates the positive control. Plants were grown for two weeks in cups containing a mixture previously described in the materials and methods. Survival was defined as any plant that did not damp-off or die.

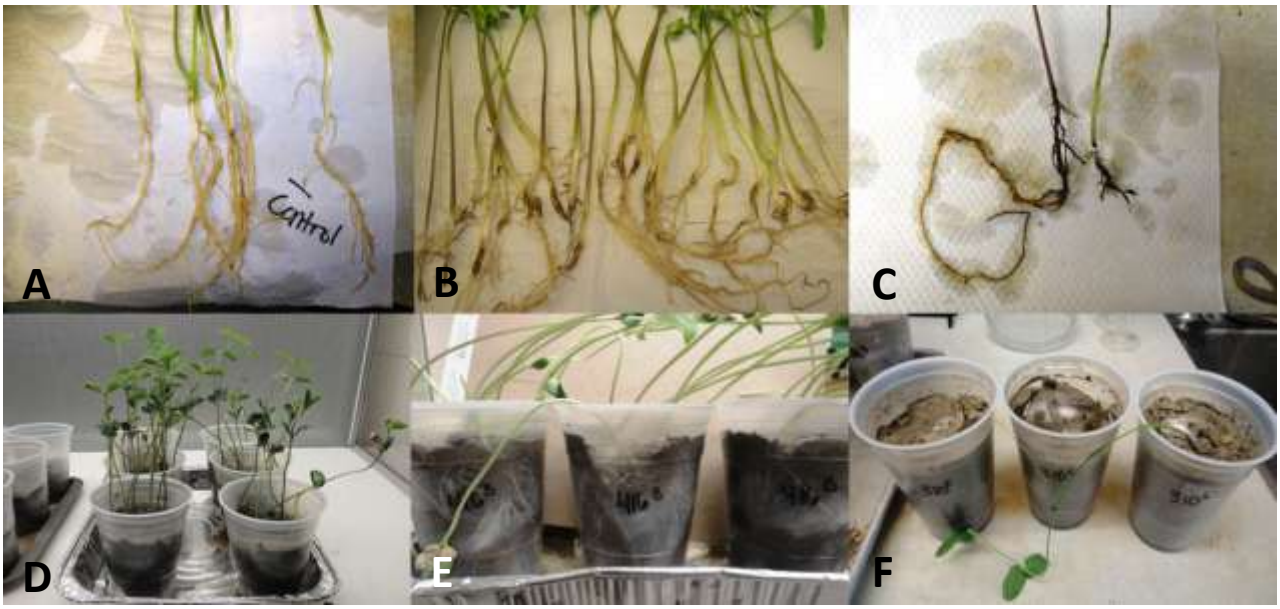

Figure 9. Pathogenicity of *P. rostratifingens* on soybean cultivar Barnes. A. (-0 control B. *P. rostratifingens* C. (+) control D. (-) control E. *P. rostratifingens* F. (+) control.

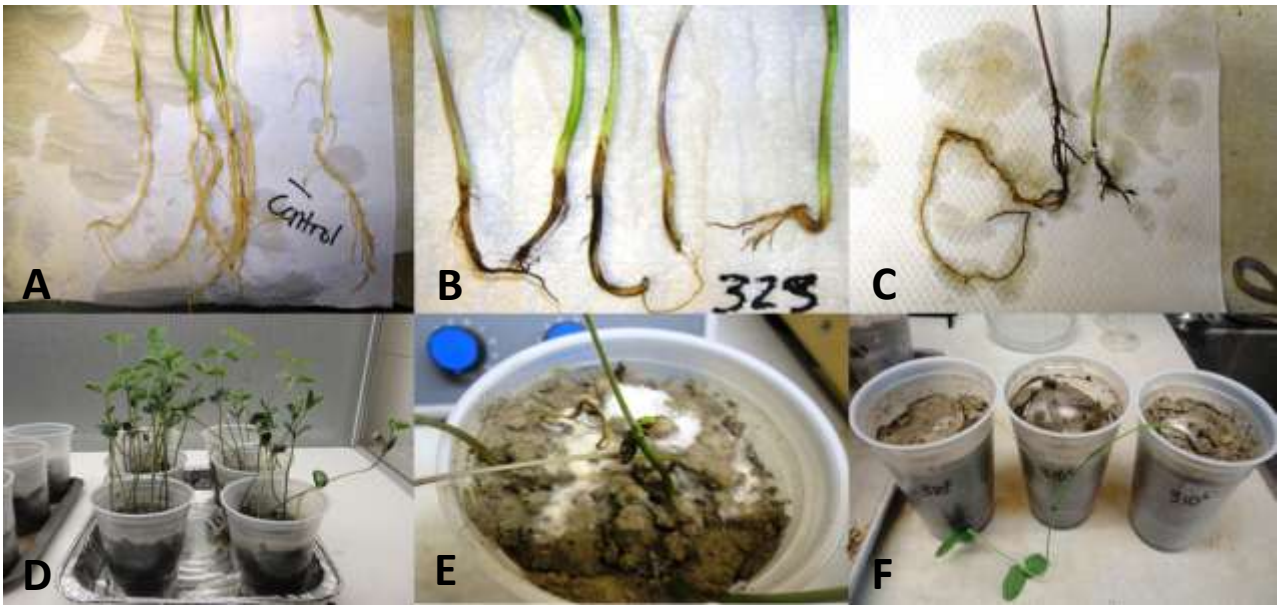

Figure 10. Pathogenicity of *P. oopapillum* on soybean cultivar Barnes. A. (-) control B. *P. oopapillum* C. (+) control D. (-) control E. *P. oopapillum* F. (+) control.

Discussion

Knowledge of *Pythium* diversity on soybean in the northern Great Plains, specifically in North Dakota and Minnesota is limited. Only recently have a number of studies in the northern Great Plains begun to describe the species associated with soybean and/or characterize their pathogenicity (Chase and Bartlett, 2013; Matthiesen and Robertson, 2013; Rojas et al. 2013; Jiang et al., 2012). Probably the earliest study on *Pythium* associated with soybean in the region was by Brown and Kennedy (1965) who reported *P. ultimum* and *P. debaryanum* as the two species found in roots in Minnesota. This current study indicates that there is a highly diverse community of pathogenic *Pythium* associated with soybean roots in the northern soybean production area.

All isolates obtained in this study were identified to species using both morphological and DNA sequence analysis. The ITS region was used for the molecular identification and is widely used for species identification (Robideau et al. 2011). Sexual and asexual structures for all isolates were obtained allowing comparisons to species descriptions in keys and the original species descriptions. Not all isolates readily produced sexual structures on PDA. A modified grass-leaf culture technique (Zitnick-Anderson and Nelson, 2012b) was essential to induce reproductive structure formation. For the six *Pythium* species that were not included in the Plaats-Niterick or Dick keys, once an identify was made with the DNA sequence analysis using the NCBI database, the original species descriptions were used (Allain-Boulé et al. 2004; Bala et al., 2010; de Cock and Lévesque, 2004; Paul, 2002; Paul and Bala, 2008; Paul et al., 2008) to verify identification based on morphological features.

The problems and concerns researchers can have when only using sequence based identification of Oomycetes, have been addressed in several recent publications (Kang et al.

2010; Robideau et al., 2011; Schroeder et al. 2013). The reader is referred to the excellent review by Schroeder et al. (2013) on taxonomy and current and future methods of identification of *Pythium*. An example of how problematic using only sequence data for identification would best be described when select isolates from 2011 were morphologically identified as *P. coloratum* but according to the NCBI database the isolate had a 100% sequence match to *P. dissotocum, P. diclinum,* and *P. coloratum*. Through the process of elimination *P. coloratum* was determined to be the identity based on the fact that the isolate had branched antheridia eliminating *P. dissotocum,* and the presence of both mono- and diclinous antheridia eliminating *P. diclinum*. This research demonstrates the benefit of using both morphological and DNA sequence based identification when identifying species of *Pythium*. In addition to clarifying conflicting results, employing both techniques can also help confirm unusual results such as the identification of *P. kashmirense, P. viniferum,* and *P. terrestris* from soybean roots in 2011. These three *Pythium* species were reported to occur in India, Turkey, and France, respectively, but not in the U.S.

In 2011, 24 *Pythium* species were isolated from soybean roots, while in 2012 there were only five species isolated. In addition, there was approximately eight times the number of *Pythium* isolates obtained from soybean roots in 2011 compared to 2012. The three dominant species from 2011 were *P. ultimum, Pythium sp.* (unknown; GenBank HQ643777.1), and *P. heterothallicum* making up 21, 16 and 12% of the total isolates respectively and the three dominant species from 2012 were *P. rostratifingens, P. inflatum,* and *P. heterothallicum* comprising 9, 6, and 6% of the total isolates respectively. Pythium species were obtained from 87 fields in 2011 and only seven fields in 2012 (Appendix C). This large difference in species diversity and number of *Pythium i*solates between the two years is probably due to differences in

soil moisture and temperature. Oomycetes are generally more active in a cool/wet climate (Agrios, 2005) and there was a difference in climate between the two years with 2012 a far drier year than 2011. Soybeans are typically planted in May in North Dakota and the root samples in this study were obtained in June of each year. A comparison of the average soil temperatures at 4 in depth from ten weather stations in the eastern half of North Dakota revealed that the average soil temperature for the combined months of May and June was 15.2° C in 2011 and 18.1° C in 2012 (all data from the North Dakota Agricultural Weather Network, http://ndawn.ndsu.nodak.edu/) . The average air temperature for May and June was 14.6^{o}C in 2011 and 16.6^{o} C in 2012. The rainfall during April to June at those same ten weather stations was 20.6 cm in 2011 compared to 16.0 cm in 2012. Thus, soil and air temperatures were cooler and soil moisture was likely greater for soybean during the seedling stage in 2011 compared to 2012. The noticeable reduction in moisture and warmer temperatures during the seedling stage in 2012 possibly reduced or inhibited the germination/growth of many *Pythium* species thus reducing colonization of soybean roots and isolation of *Pythium* species (Matthiesen and Robertson, 2013).

The Zygomycete *Mortierella* was included in the data on *Pythium* because *Mortierella* was isolated along with *Pythium* on the selective medium and was the dominant organism isolated from soybean roots in 2012. Although there are many different fungal and fungal-like organisms in soybean roots (Killebrew et al. 1993), this high frequency of recovery of *Mortierella* we considered unusual and thus is reported here. A similar report of such high frequency of *Mortierella* isolated from soybean roots has not been found. Furthermore, this high frequency of *Mortierella* also points out the stark difference in isolation of *Pythium* from soybean roots in a wetter verses a drier period during the early part of the growing season. The

morphology of *Mortierella* on the selective medium in the first two to five days following isolation is similar to *Pythium*. The hyphae are coenocytic and the growth patterns are similar to *Pythium*, i.e. chrysanthemum, pulvinate, etc. *Mortierella* is a soil fungus that is known as an early colonizer of roots and is reported to be associated with soybean roots (Ivarson and Mack 1972; Bienapfl et al. 2010), but is usually non-pathogenic on plants and animals with the exception of *M. wolfii* which is an animal pathogen (Davies, 2010). Recent research found a *Mortierella sp.* in conjunction with arbuscular mycorrhizal fungi, was shown to significantly increase shoot/root dry weight of the herbaceous halophyte *Kostelelzkya virginica* when grown under saline conditions (Zhang et al. 2011). This fast growing genus thrives best in drier soil conditions, which would explain why it was commonly isolated from roots in 2012 but not in 2011. The role that *Mortierella* plays in the microbial colonization of soybean roots should be investigated.

The three species *P. coloratum*, *P. aristosporum*, and *P. arrhenomanes*, that were not evaluated for pathogenicity had been in storage for 16 months, and all attempts to revive the cultures were unsuccessful. A number of *Pythium* species, such as *P. oopapillum*, have been reported to be difficult to maintain in storage (Bala et al. 2010). Bacterial contamination of the storage medium is critical to avoid, as many isolates were difficult to recover if there was such contamination. Fortunately, 95% of the total number of isolates obtained in this study were successfully stored for 26 months.

All *Pythium* species identified, with the exception of *P. orthogonon* and *P. nunn*, have been documented pathogens on a wide host range (*Citrus, Pinus*, turfgrass, etc.). *Pythium attrantheridium, P. debaryanum, P. diclinum, P. dissotocum, P. heterothallicum, P. hypogynum, P. inflatum, P. intermedium, P. irregular, P. lutarium, P. oopapillum, P. orthogonon, P.*

perplexum, P. sylvaticum, and P. ultimum have previously been documented as pathogens on soybeans (Broders, 2007; Jiang et al. 2012; Plaats-Niterink 1981; Matthiesen and Robertson, 2013). This is the first report of *P. kashmirense, P. minus, P. periilum, P. perplexum, P. rostratifingens, P. terrestris, P. viniferum, and P. violae* as pathogens of soybean seedlings in the United States. The pathogenicity trials corroborate with previously recorded research and indicate that the majority of the species collected were pathogenic on soybean (Broders et al. 2007; Jiang et al. 2012). The results also indicate that all but three *Pythium* species (*P. orthogonon, P. nunn, and P. rostratifingens*) cause pre-emergence damping off. All *Pythium* species were able to cause lesions on the roots of soybean seedlings suggesting that all species including previously recorded beneficial species are capable of causing infection on soybean. All but three species (*P. orthogonon, P. nunn, and P. rostratifingens*) resulted in fewer than 50% of the seedlings surviving the two weeks period. However, if the pathogenicity study had continued over a longer period, post-emergence damping off may have occurred with the surviving plants due to the presence of numerous black/brown necrotic lesions on the roots. Although *P. orthogonon, P. nunn,* and *P. rostratifingens* had approximately 80% of the seedlings surviving the two weeks and remained visibly healthy, small (4 cm long) brown lesions were present on the tap roots. *Pythium nunn* has been documented as antagonistic to *P. ultimum* and suggested for use as a biocontrol agent (Kobayashi, 2010). Contrary to previous studies, the presence of lesions on the roots indicates that *P. nunn* is capable of causing some disease on soybean (Kobayashi et al. 2010; Lifshitz et al., 1984a,b). There is evidence that species may differ in pathogenicity at different temperatures (Matthiesen and Robertson, 2013; Wei et al. 2011). Only one temperature was used in this study, thus the results may be different at other temperatures. Future studies on which species are pathogenic at certain temperatures more

commonly found during planting may provide useful data toward understanding the importance of individual species.

Conclusion

In conclusion, there is a diverse population of *Pythium* species associated with soybean roots in the northern soybean growing area of the Great Plains of the U.S. Twenty-six known species and three unknown species were associated with soybean roots in this study. One of the most prevalent species isolated was a pathogenic unknown, emphasizing the importance of further research on the isolate for proper identification. Many of these species are pathogenic on soybean seedlings and may play a role in seed rot and damping-off of soybeans in commercial soybean planting. Understanding the biology of these various species will be important for developing strategies for control of seedling disease caused by *Pythium*. This is the first report of *P. kashmirense, P. minus, P. periilum, P. rostratifingens, P. terrestris, P. viniferum*, and *P. violae* as pathogens of soybean seedlings. In addition this is the first report of *P. kashmirense, P. viniferum*, and *P. terrestris* in the United States.

CHAPTER III: ASSOCIATIONS BETWEEN SOIL PROPERTIES AND PRESENCE OR ABSENCE OF *PYTHIUM SPP*. IN SOYBEAN FIELDS OF NORTH DAKOTA

Introduction

The genus *Pythium* contains numerous species that are economically important to a wide range of crops. *Pythium spp.* typically cause pre- and post-damping-off, a devastating agricultural and horticultural disease. *Pythium* as a soil-borne pathogen does not produce aerial spores for long distance dispersal, and much of the life cycle occurs within the soil (Agrios, 2005). The pathogen infects plants primarily through the root system (Agrios, 2005). The primary inoculum is generally zoospores, and the activity of these flagellated spores are limited by the amount of moisture within the soil (Agrios, 2005). Zoospores need water for dissemination and depending on the soil composition, can only travel as far as the capillaries and pore space within the soil allows.

There has been limited research on the effects of soil properties on *Pythium* growth, occurrence, and disease (caused by *Pythium*). Texture, organic matter, and certain metals have been observed to have positive to adverse effects on *Pythium* (Lifshitz and Hancock, 183; Schaetzl and Anderson, 2007). The characteristics of soil texture indirectly describe the amount of moisture a soil can retain. Soils higher in clay content retain more moisture than soils with more sand or silt (Hillel,1998). When soil moisture decreases, the motility of the zoospores is negatively affected because the spores require free water to move (Coyne, 1999). However, a recent study suggested that the opposite was true for a diverse *Pythium* population found on soybean in the Ohio Valley (Broders et al. 2009). A decrease in disease incidence as clay

increased could be due to the presence of organic matter, which was also observed to have a positive relationship with disease incidence. Multiple studies have attempted to use organic matter for control of damping-off caused by *Pythium* (Boehm et al., 1993; Gregorich et al., 2006; Stone et al., 2001; Stone et al., 2004). Research has suggested that the nutrients in fresh or less decomposed organic matter are not readily accessible to *Pythium* (Gregorich et al., 2006; Stone et al., 2001). The organic matter provides more nutrients to *Pythium* when the decomposition is more advanced (Gregorich et al., 2006). Ultimately, the rate of organic matter decomposition is more important than the amount of organic matter (Gregorich et al., 2006). Similarly metals can hinder or support the basic critical functions of *Pythium* growth. The metal nickel has been observed to increase a plants ability to directly inhibit *Pythium* prior to contact with the plant root system (Ghaderian et al. 2000). Iron is another metal that indirectly suppresses *Pythium* by stimulating siderophore formation within *Pseudomonas fluorescens,* a commonly abundant soil organism (Matthijs et al. 2007). In contrast, zinc has shown to be critical in the formation of oogonia and the vegetative growth of *Pythium* (Naar, 2006).

Only in the past four years has there been a similar study exploring associations between *Pythium* communities and the soil environment (Broders et al 2009). Investigating these associations could help in understanding disease development by *Pythium*. The objective of the study was to examine the *Pythium* diversity from 138 soybean fields and the associations between the *Pythium* species collected from soybean and the characteristics of soils connected with the soybeans in North Dakota. Developing a model to calculate the probability and the presence of *Pythium* species associated with soil properties could be useful in managing the pathogen.

Materials and Methods

Collection of plants and soils

In June of 2011 and 2012 soybean seedlings and soil samples were collected from 88 and 50 soybean fields, respectively, in 20 counties in the eastern half of North Dakota, the primary soybean production area of the state (Fig. 11). Soybean fields were chosen at approximately 6 to 8 km intervals between fields or until a field was observed. Ten seedlings with roots at the first trifoliolate leaf stage were collected at random from each field with approximately 2.5 m between plants. Approximately 500 g of soil were collected at a depth of 25 cm from each field. GPS coordinates were recorded for each field. Seedlings were transported to the laboratory in coolers and were gently rinsed with lukewarm tap water to remove soil particles and lightly patted dry with paper towels. Seedlings were processed within 24 h of collection or stored at 4 °C until used the following morning. Isolations were identified as described in chapter two. Due to the fact that in 2012 *Pythium* was only isolated from seven fields, analysis was not performed using the data from the 2012 survey. Therefore all results are based on the data collected during the summer of 2011. Cation exchange capacity (CEC) and particle size analysis (sand, silt, and clay) were conducted using the Bower (1952) and Hydrometer (Tan et al. 1996) methods, respectively. The P, K, pH, electrical conductivity (EC), organic matter (OM), Zn, Fe, Cu, and calcium carbonate exchange (CCE) were analyzed by the NDSU Soil Testing Laboratory.

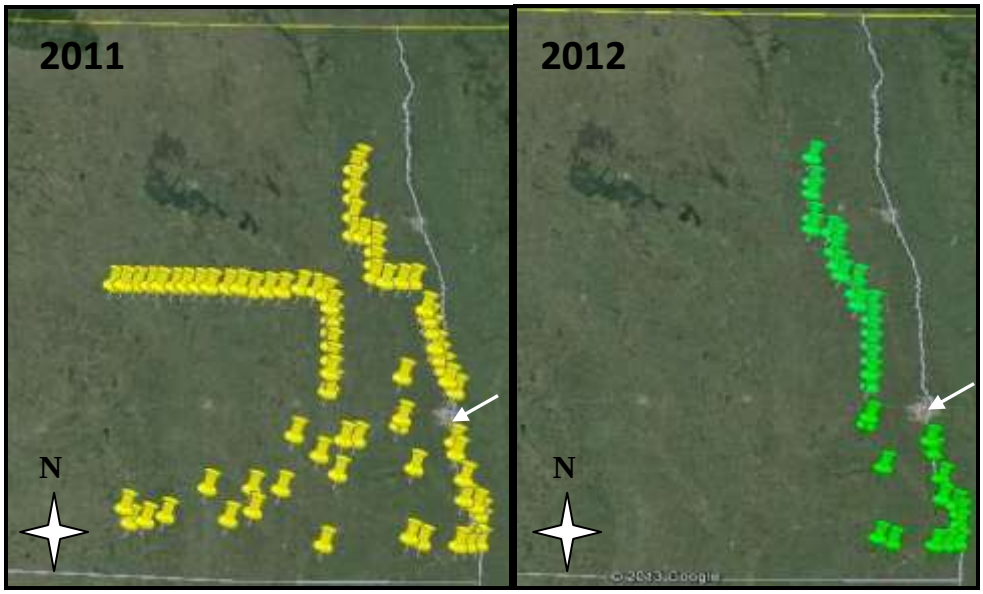

Figure 11. Map of North Dakota showing the location of the soybean fields sampled for *Pythium* in 2011 and 2012. Fargo is indicated by arrows (Google Earth).

Species diversity, evenness, and Spearman correlation

The species diversity and evenness were calculated for the entire survey using the Shannon index and evenness index E_5. The Shannon index is described as $H' = \Sigma\ pi \ln pi$, where H' is the species diversity score and *pi* is the proportion of individuals in the *i*th species (Krebs, 1999). The evenness equation is described as $E_5 = (((1/\lambda)-1)/ e^{H'}-1)$, where λ is Simpson's index (Grünwald et al. 2003). Species diversity and evenness were not calculated for each field due to only isolating one to two species on average per field. Therefore, abundance data from all fields were aggregated to determine the overall diversity and evenness indices. The data used for abundance was the number of isolates for each species per field (Appendix C). Relationships between species diversity and the 13 soil properties were evaluated using Spearman's correlation analysis (SAS version 9.1; SAS Institute, Cary, NC). A Pearson correlation analysis was performed for all 13 properties to observe any significant correlation between properties.

Cluster analysis

Due to the fact the raw data were sparse, non-linear or normally distributed, hierarchical clustering, indicator species, multi-response permutation procedures (MRPP), and logistic regression analysis were appropriate to assess the absence/presence of *Pythium* based on the 13 soil properties. PC-ORD version 6 was used to perform hierarchical clustering analysis (McCune and Mefford, 2011). Raw data was transformed using square root transformation because special treatment of zeros would not be needed, unlike log transformations (McCune and Mefford, 2011). Hierarchical clustering analysis was performed using the relative Euclidean distance measurement and Ward's method to group the fields together based on species abundance and frequency (McCune and Mefford, 2011). The dendrogram was used to define groups implementing the Dufrêne and Legendre (1997) method of pruning based on indicator species analysis (McCune and Mefford, 2011). The definition of an indicator species, according to Lindenmayer et al. (2000), is a species whose presence or absence indicates the presence or absence of a set of other species.

The indicator species analysis can be used as an objective criterion to determine the most ecologically meaningful point to prune a dendrogram from cluster analysis (McCune and Mefford, 2011). Group membership at each step of cluster formation was entered into the program where indicator values were calculated for each species at each level of grouping (McCune and Mefford, 2011). The *p*-values (generated using the Monte Carlo test) were averaged across all species; this step was repeated for all steps of clustering (McCune and Mefford, 2011). The cluster step with the smallest averaged *p*-value was determined to be the most informative level in the dendrogram (McCune and Mefford, 2011). In addition to the averaged *p*-values, the number of species shown to be significant indicators ($\alpha = 0.05$) were

tallied for each cluster step (McCune and Mefford, 2011). The more species shown to be significant indicators with the lowest averaged *p*-value was the criterion used for determining the clusters (groups) of *Pythium* species (McCune and Mefford, 2011). The cluster analysis using the criterion described, indicated that three groups of *Pythium* were present. To ascertain that the three groups were in fact dissimilar, a multi-response permutation procedure (MRPP) was performed (Mielke and Berry, 2001). The test statistic (T) was used to test the separation between groups (Mielke and Berry, 2001). The more negative the T-value the stronger the separation between groups (Mielke and Berry, 2001). The *p*-value was used in conjunction with the chance-corrected within-group agreement value (*A*); (Mielke and Berry, 2001). The *p*-value is useful in evaluating the likelihood that an observed difference between data sets is due to chance (Mielke and Berry, 2001). The smaller the *p*-value the less likely the observed differences in groups are due to chance (Mielke and Berry, 2001). The *A* value describes within-in group homogeneity, compared to the random expectation (Mielke and Berry, 2001). When the *A* value is close to one the items are identical within groups (Mielke and Berry, 2001). When the *A* value is close to zero heterogeneity within groups equals expectation by chance (Mielke and Berry, 2001).

Logistic regression

Logistic regression analysis was performed using the three cluster groups and all 11 soil properties using stepwise selection to generate multiple candidate models (SAS version 9.1; SAS Institute, Cary, NC). Logistic regression analysis requires at least 30 data points for results to be accurate (SAS version 9.1; SAS Institute, Cary, NC). Due to the fact that not all *Pythium* species were present in at least 30 different fields, performing logistic regression analysis with individual species was not appropriate. The analysis was performed in order to create an accurate model

that could be used to calculate probabilities of presence and absence of each group based on statistically significant differences in soil properties. The Akaike information criterion (AIC), and *c* (a variant of Somer's D) values were used to select the most appropriate model and evaluate the fitness and relative quality of each model for the data, in addition to the Hosmer and Lemeshow test (SAS version 9.1; SAS Iinstitute, Cary, NC). After computing the *y*-values from the logistic models the data were linearized and probabilities were calculated using the same formula ($P = e^y / (1+e^y)$); where P is probability, and e^y linearizes the logistic *y*-values (SAS version 9.1; SAS Institute, Cary, NC).

Results

Species diversity, evenness, and Spearman correlation

There were 26 *Pythium* species identified from soybean roots and three *Pythium sp.* could not be identified and appear to be potentially new species based on morphology and DNA sequence analysis when compared to sequences in GenBank. These species were recovered from 138 fields between 2011 and 2012. All *Pythium* species detected are listed in Appendix C. The species diversity index was 2.45 (Appendix C). When multiple species are present and equally abundant within a dataset the diversity index approaches the value of five, the maximum value for the Shannon diversity index. When abundance data is dominated by one species, despite the presence of other species, the Shannon diversity index will be closer to zero. The abundance used in this study was the number of isolates for each species. The evenness index was 0.69 (Appendix C). The evenness index is the ratio of the number of abundant species to the number of rarer species (Grünwald et al. 2003). The closer the index is to zero, the less evenness between species is observed; a value close to zero would be indicative of a data set that had little diversity (Grünwald et al., 2003). The evenness index in this study indicates that the *Pythium* is evenly

distributed throughout the fields sampled. The Spearman correlation analysis between the *Pythium* diversity and the 13 soil properties presented three statistically significant (at α=0.05) positive correlations between diversity and CEC, CCE, and Zn. In addition a Pearson correlation analysis was performed with the 13 soil properties. The OM and clay were omitted from the study due to the properties positive correlation with CEC. Reasons as to why OM and clay correlate with CEC are described in the discussion.

Cluster analysis

Three major communities were grouped using cluster analysis, indicator species analysis, and MRPP. The indicator species analysis showed that group or cluster step three had the smallest averaged *p*-value across all species (at α=.30) and the highest number of species shown to be significant indicators (five; *P. ultimum, P spp. unknown, P. kashmirense, P. heterothallicum,* and *P. irregulare*). The results of the MRPP had a test statistic (T) value of -32.01, α=0.00, and an *A* value of 0.16. The MRPP results indicate that the three groups were strongly separate from one another, were less likely to observe differences in the groups due to chance, and groups were heterogeneous and equaled the expectations by chance. Group one was characterized by the indicator species *P. ultimum*. Group two was characterized by *Pythium spp* (unknown; GenBank HQ643777.1) and *P. kashmirense*. Group three was characterized by *P. heterothallicum* and *P. irregulare*. Other *Pythium* species were found within each group; however these species were not significant indicators as shown by the Indicator Species Analysis (Monte Carlo test); (Appendix B).

Logistic regression

The AIC values measure the amount of information loss for each model developed for each group. The model that was selected for each group had the smallest AIC value of all the

possible candidate models developed for each group. The rank correlation of ordinal variables (*c*-value), and the accuracy of the model for fit to the data (Hosmer and Lemeshow) for group one characterized by *P. ultimum* were 0.86, and 0.8 respectively. The values were close to one, indicating that the logistic regression model extrapolated from the analysis was a good fit to the data. The logistic regression model for group one characterized by *P. ultimum* was y= -2.05 + 0.55 (Zn) (Fig. 12). Probabilities were then calculated and graphed (Fig. 12) showing that, as zinc levels increased, the probability of group one characterized by *P. ultimum*, being present within a soil increased. The actual Zn values in the data ranged from 0.23 to 4.8 ppm.

Group two characterized by *P. kashmirense* and *Pythium sp.* (unknown; GenBank HQ643777.1) was significantly correlated with the soil property CEC ($p < 0.05$). The *c*-value, and Hosmer and Lemeshow tests were 0.89 and 0.84, respectively. The logistic regression model for group two characterized by *P. kashmirense* and *Pythium sp.* (unknown; GenBank HQ643777.1) was $y = -0.95 + 0.06$ (CEC); (Fig. 13). The model indicated that as CEC increased, the probability of group two characterized by *P. kashmirense, and Pythium sp*. (unknown; GenBank HQ643777.1), being present within a soil increases. The actual CEC values in the data ranged from 11.12 to 51.24 meq/100g.

Group three characterized by *P. heterothallicum* and *P. irregulare*, significantly correlated with the soil properties CEC, and CCE ($p < 0.05$). The *c*-value, and Hosmer and Lemeshow tests were 0.95 and 0.86, respectively. The logistic regression model for group three was $y = 0.91 – 0.1173$(CEC) + 0.11(CCE); (Fig. 13). The model indicated that as the CCE increased and CEC decreases, the probability of group three characterized by *P. heterothallicum* and *P. irregulare*, being present within a soil increases. The actual CCE values in the data ranged from 0 to 39.4 %.

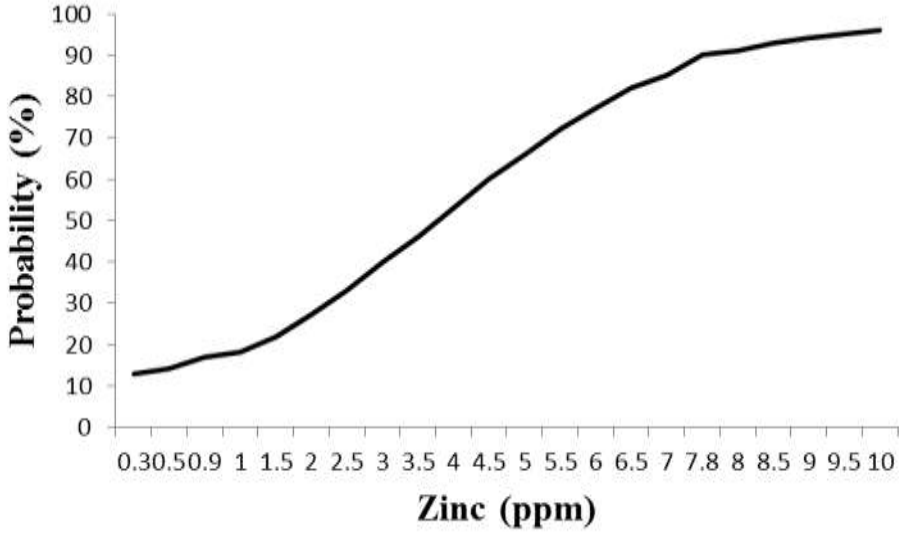

Figure 12. Probability of *Pythium* group one, characterized by *P. ultimum*, being present within a soil as zinc increases. Determined by the logistic regression model $y = -2.05 + 0.55$ (Zn).

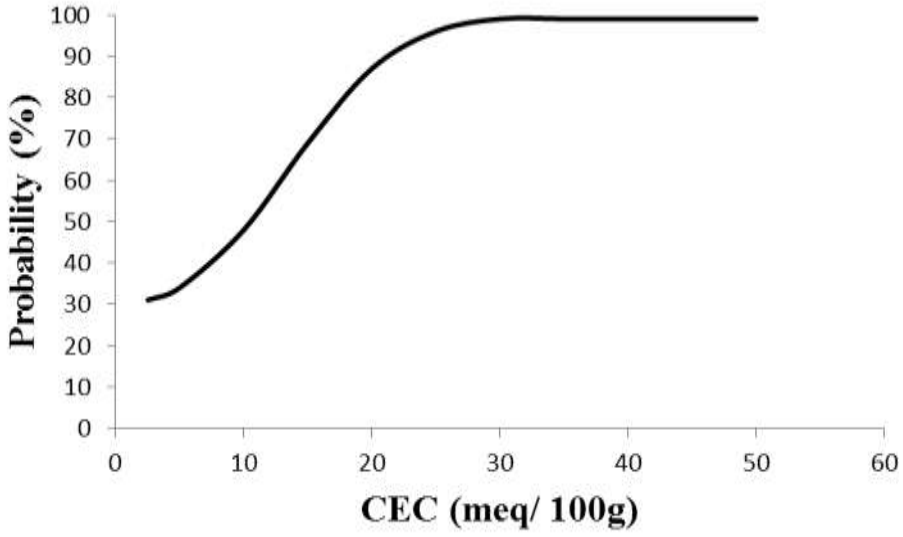

Figure 13. Probability of *Pythium* group two, characterized by *Pythium sp.* (unknown; GenBank HQ643777.1) and *P. kashmirense*, being present within a soil with increasing CEC values. Determined by logistic regression model $y = -0.95 + 0.06$ (CEC).

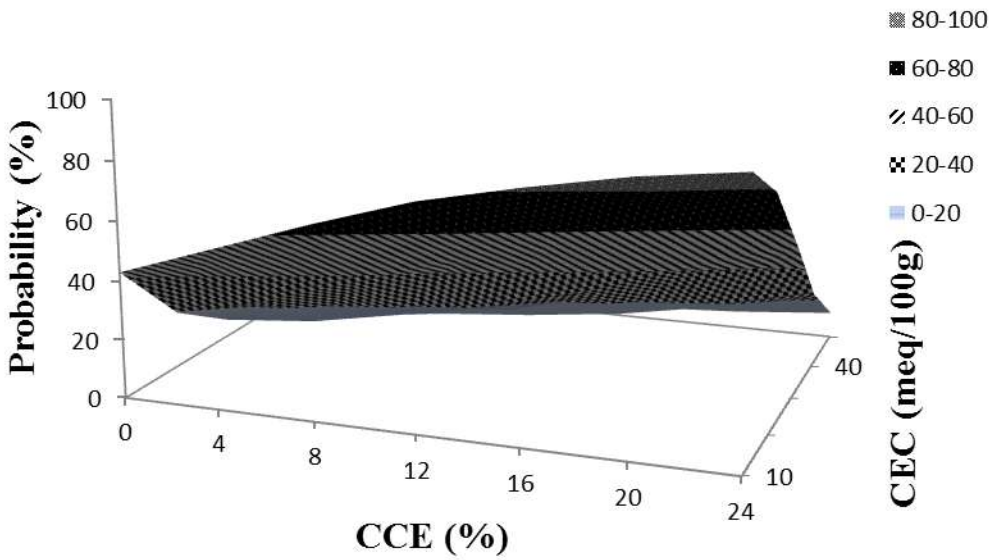

Figure 14. Probability of *Pythium* group three, characterized by *P. heterothallicum* and *P. irregulare*, being present within a soil as CCE and CEC values increase. Determined by logistic regression model $y = 0.91 – 0.12$ (CEC) + 0.11 (CCE).

Discussion

Associations between soil properties and the structure of fungal communities have been well documented since the 1950's (Coleman et al. 2004). Cation exchange capacity (CEC), percent sand, silt, clay and other edaphic properties have been shown to influence species diversity directly and indirectly (Six et al. 2004). The CEC is a measurement that assesses a soil's capacity to exchange ions, which is based on the sum of exchange sites contributed by both OM and clay (Coleman et al. 2004), which comprise the colloidal fraction of soil. The size and shape (< 1 µm in diameter) of the colloidal fraction give the soil a large amount of reactive surface area (Brady and Weil, 2010). The negative charges found predominantly on the clay and OM can adsorb and release cations (positively charged ions); (Brady and Weil, 2010). The static electrical charge keeps the positively charged nutrients from being washed away by water (Brady and Weil, 2010). The static charge also makes nutrients (K^+, Ca^+, H^+ and etc.) available for

uptake by plants and microorganisms (Brady and Weil, 2010). However the static electrical charge is a weak bond to the surface of the colloids (Brady and Weil, 2010). The adsorbed cations are continuously vibrating and oscillating due to surrounding cations within the soil solution (Brady and Weil, 2010). The adsorbed cations will break away from the colloidal surface and into soil solution (Brady and Weil, 2010). The cation is then replaced by another equally charged cation from solution (Brady and Weil, 2010). This process is referred to as the cation exchange. A soil with high CEC will have a higher availability of nutrients for soil microorganisms (Brady and Weil, 2010). Research has shown that soils with higher amounts of OM and clay have greater diversity of organisms due to higher amounts of essential nutrients (Coyne, 1999; Donaldson and Deacon, 1993; Fierer et al., 2003; and Gardner and Hendrix, 1973). Organic matter would invite an array of different organisms including *Pythium* and the antagonistic organisms *Pseudomonas fluoresces* and *Trichoderma spp*. In the presence of the two antagonistic species, *Pythium* diversity and abundance does decline (Ghaderian et al. 2000; Matthijs et al., 2007). In an environment with lower levels of OM, *Pythium* diversity and abundance have been shown to increase (Stone et al., 2001). Previous studies have indicated that increased amounts of clay may lower levels of oxygen and decrease porosity (Gardner and Hendrix, 1972; Donaldson and Deacon, 1992, 1993; Fiere et al. 2003; Hillel, 1998). These are factors which have been shown to decrease species diversity and dispersal of primary inoculum of *Pythium* (Donaldson and Deacon, 1992). The primary inoculum of *Pythium* is the zoospore which requires water to travel to plant root systems (Donaldson and Deacon, 1993).

The logistic regression model indicated that the presence of group two characterized by *Pythium sp.* (unknown; GenBank HQ643777.1) and *P. kashmirense*, increases with increasing values of CEC. There results corroborate the results of Broders et al. 2009, who also described

CEC as a significant soil property that influenced *Pythium* community structure. In addition, previous research has indicated a correlation between CEC and OM with the hyphae density of other facultative saprophytes such as *Fusarium sp.* and *Aspergillus sp.* (Beare et al. 1993). Soils from the fields where *Pythium* group two characterized by *Pythium sp.* (unknown; GenBank HQ643777.1) and *P. kashmirense* was most predominant had higher OM content (six to eight %) and clay percentages ranging between 12-20 % compared to the other fields (Appendix C). Higher amounts of organic matter and clay could explain the presence of group two in the field. Although increasing OM in soil has generally been considered a method of suppressing *Pythium*, recent studies have found that highly decomposed OM can be colonized by *Pythium* when the organism becomes a facultative saprophyte (Boehm et al., 1993; Gregorich et al., 2006; and Stone et al., 2001). In contrast to group two characterized by *Pythium sp.* (unknown; GenBank HQ643777.1) and *P. kashmirense* as CEC values decreased the presence of group three characterized by *P. heterothallicum* and *P. irregulare* increased. Soils from the fields where group three characterized by *P. heterothallicum* and *P. irregulare* were most predominant had the highest clay percentages and lowest levels of OM among the fields sampled. The reason that lower CEC values are associated with greater presence of *Pythium*, group three may be due to lower interspecific competition between *Pythium* and other organisms. Lower amounts of OM and higher clay content will decrease oxygen, porosity, and species diversity within a soil. Organic matter and percent clay are both important because the two properties provide much of the nutrients needed for survival of various organisms (Coleman et al. 2004). Although OM and clay were not significant soil properties, CEC was, and therefore indirectly alluded to the importance of OM and percent clay in the study.

The results of this study clearly show that soil zinc and calcium carbonate influence the probability of two *Pythium* groups, characterized by *P. ultimum* (group one) and *Pythium sp.* (unknown; GenBank HQ643777.1) and *P. kashmirense* (group two) being present in a soil. Zinc was a significant factor in the presence of *Pythium* group one characterized by *P. ultimum*. Previous research indicated zinc was critical in the formation of the oogonium and the vegetative growth of *Pythium* (Lenney and Klemmer, 1966). In addition, zinc has also been observed to inhibit the antagonistic activity of *Trichoderma sp*. on *Pythium* (Naar, 2006). For group three characterized by *Pythium sp.* (unknown; GenBank HQ643777.1) and *P. kashmirense*, calcium carbonate increased the probability of the detection of the group in the soil (Fig. 14). Calcium carbonate may affect zoospore activity. Zoospores locate specific regions of roots by chemotaxis (Donalson and Deacon, 1993). One of the cations that regulate the motility of zoospores is Ca^{2+} (Donalson and Deacon, 1993). Previous research indicated a central role of Ca^{2+} in the adhesion and the germination of encysted zoospores (Donalson and Deacon, 1992). Calcium carbonate is a salt. The source of naturally occurring salts in soil come from the primary or parent material (Pepper et al., 2006). The fields where group three characterized by *Pythium sp.* (unknown; GenBank HQ643777.1) and *P. kashmirense*, were predominantly found located in the Red River Valley. The parent material for much of the Red River Valley is composed of shale and limestone. Limestone is the primary source of calcium carbonate in the fields located in the Red River Valley (NRCS, 2013). In addition to parent material, the management of soil also affects levels of salinity with a soil (Pepper et al., 2006). In areas with a shallow saline groundwater table, such as the Red River Valley, heavy rainfall will raise the water table to the soil surface, resulting in little to no downward movement of excess salts (Pepper et al., 2006). The water

evaporates and the salts remain within the soil increasing concentrations of salts such as calcium carbonate (Pepper et al., 2006).

Management strategies for pathogenic *Pythium* have included fungicides, and cultural practices. Much of the biology of the genus *Pythium* is well understood; however very little is understood of how *Pythium* is affected by the soil environment. Our research is one of few studies to address associations between *Pythium* presence and absence and soil edaphic properties (Broders et al. 2009). Addressing such associations has led to the development of probability models that could predict the presence of certain *Pythium* species within a field depending on the soil properties. Previous research has shown that not all *Pythium* species are controlled by the same fungicidal treatments and cultural practices (Broders et al 2009). The ability to predict the presence of certain species can make selecting a proper management strategy more efficient. Future research should be conducted on the practicality of each probability model in field situations.

Conclusion

Previous research has indicated associations between soil properties and soil microorganisms (Beare et al. 1993). Assuming there are no such associations between soil properties and *Pythium* communities would be contradictory to prior studies suggesting otherwise. This research has shown associations between soil properties and three *Pythium* communities characterized by five different indicator species. The associations were used to develop models that predict probabilities of presence and absence of the three *Pythium* communities based on soil properties. For presence of group one characterized by *P. ultimum*, the probability increased when soil zinc levels increased. For group two, characterized by *Pythium sp*. (unknown; GenBank HQ643777.1) and *P. kashmirense*, probability increased with

higher levels of CEC. For group three, characterized by *P. irregulare* and *P. heterothallicum*, probability increased with higher levels of calcium carbonate and lower levels CEC. Future research should test the accuracy of these probability models in the field.

REFERENCES

Abad, Z.G., H.D. Shaw, and L.T. Lucas. 1994. Characterization and pathogenicity Pythium species isolated from turfgrass with symptoms of root and crown rot in North Carolina. APS 84: 913-921.

Adams, R.B. 1971. *Pythium apanidermatum* oospore germination as affected by time, temperature, and pH. Phytopathology. 61:1149-1150.

Agrios, G. N. 2005. Plant Pathology. Elsevier Academic Press. New York, NY.

Allain-Boulé, N., R. Tweddell, M. Mazzola, R. Bélanger, and C.A. Lévesque. 2004. *Pythium attrantheridium* sp. nov.:taxonomy and comparison with related species. Mycol. Res. 108:795-805.

Al-Sadi, A,M., N.A. Al-Habsi, K.S.Al-Kiyumi, F.A.Al-Said, S.A.Al-Rawahy, M. Ahmed, and M.L.Deadman. 2010. Effect of salinity on growth, reproduction and pectolytic enzyme production by *Pythium aphanidermatum*: a serious soil borne pathogen of vegetable crops in Oman. A Monograph on Management of Salt-Affected Soils and Water for Sustainable Agriculture. 95-98.

Al-Sadi, A.M., R.S. Al-Masoudi, N. Al-Habsi, F.A.Al-Said, S.A.Al-Rawahy, M.Ahmed, and M.L.Deadman. 2010. Effect of salinity on *Pythium* damping-off of cucumber and on the tolerance of *Pythium aphanidermatum*. Plant Path.59:112-120.

Ash, M. 2012. Soybeans and oil crops. [online]. Available at http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/related-data-statistics.aspx. Washington, D.C.

Bainbridge, A. 1970. Sporulation by *Pythium ultimum* at various soil moisture tensions. Trans. Brit. Mycol. Soc. 55:485-488.

Bala, K., G.P. Robideau, N. Desauliners, A.W.A.M de Cock, C.A. Levesque. 2010. Taxonomy, DNA barcoding and phylogeny of three new species of *Pythium* from Canada. Persoonia 25:22-31.

Barton, R. 1958. Occurrence and establishment of *Pythium* in soils. Trans. Brit. Mycol. Soc. 41:207-222.

Beare, M.H., B.R. Pohlad, D.H. Wright, and D.C. Coleman. 1993. Residue placement and fungicide effects on fungal communities in conventional and no-tillage soils. Soil Sci. Soc. Am. J. 57: 392-399.

Biesbrock, J.A. and F.F. Hendrix. 1970. Influences of soil water and temperature on root necrosis of peach caused by Pythium spp. Phytopathology 60:880-882.

Bienapfl, J. C., Malvick, D. K., and Percich, J. A. 2010. Identification and characterization of fungal communities associated with soybean roots in Minnesota. Phytopathology 100:S14.

Brady, N. and R. Weil. 2010. Elements of the nature and properties of soils. Orentice Hall, Upper Saddles River, NJ.

Broders, K.D., P. E. Lipps, P. A. Paul, and A. E. Dorrance. 2007. Characterization of *Pythium spp.* Associated with Corn and Soybean Seed and Seedling Disease in Ohio. Amer. Phyto. Soc. 91: 727-735.

Broders, K.D., M.W. Wallhead, G.D. Austin, P.E. Lipps, P.A. Paul, R.W. Mullen, and A.E. Dorrance. 2009. Association of soil chemical and physical properties with Pythium species diversity, community composition, and disease incidence. Phytopathology 99: 957-967.

Bower, C.A., R.F. Reitemeier, and M. Fireman. 1952. Exchangeable cation analysis of saline and alkali soils. Soil Sci. 73: 251-261.

Brown, G.E. and B.W. Kennedy. 1965. *Pythium* pre-emergence damping-off of soybeans in Minnesota. Pl. Dis. Reptr. 49:646-647.

Campbell, W.A., and F.F. Hendrix. 1967. A new heterothallic *Pythium* from southern United State. Mycologia. 59:274-278.

Chase, T.E. and P.B. Bartlett. 2013. Oomycetes isolated from soybeans with damping-off in South Dakota. Phytopathology. 103 (Suppl. 2):S2.25

Chi, C.C. and E.W. Hansn. 1962. Interralated effects of environment and age of alfalfa and red clover seedlings on susceptibility to *Pythium debaryanum*. Phytopathology. 52:985-989.

Coleman, D.C., D.A. Crossley Jr., and P.F. Hendrix. 2004. Fundamentals of soil ecology. Elsevier Inc. New York, NY.
Coyne, M. 1999. Soil microbiology: an exploratory approach. Delmar Publishers. Albany, NY.

Davies J.L. and Wobeser, G.A. 2010. Systemic infection with *Mortierella wolfii* following abortion in a cow. *Can Vet J.* 51: 1391–3.

de Cock, A.W.A.M. and C.A. Lévesque. 2004. New species of *Pythium* and *Phytophthora*. Studies in Mycology. 50:481-487.
Dick, M.W. 1990. Key to *Pythium*. University of Reading Press, Reading, UK.

Donaldson, S.P. and J.W. Deacon. 1992. Role of calcium in adhesion and germination of zoospore cysts of *Pythium*: a model to explain infection of host plants. J. of Gen. Micro. 138:2051-2059.

Donaldson, S.P. and J.W. Deacon. 1993. Changes in motility of *Pythium* zoospores induced by calcium and calcium-modulating drugs. Mycol.Res. 97:877-883.

Dufrêne, M. and P. Legendre. 1997. Species assemblages and indicator species: the need for a flexible asymmetrical approach. Ecological Monographs. 61:53-73.

Fiere, N., J. P. Schimel, and P.A. Holden. 2003. Variations in mircrobial community composition through two soil depth profiles. Soil Bio. and Biochem. 35: 167-176.

Gardner, D.E., and F.F. Hendrix Jr. 1972. Carbon dioxide and oxygen concentrations in relation to survival and saprophytic growth of *Pythium irregular* and *Pythium vexans* in soil. Can. J. Bot. 51:1593-1598.

Ghaderian, Y.S.M.m A.J.E. Lyon, and A.J.M. Baker. 2000. Seedling mortality of metal hyperaccumulator plants resulting from damping off by *Pythium spp*. New Phytol. 146:219-224.

Source: "Fargo, ND" coordinates. 46°52'43.65" N 96°47'30.66" W. Google Earth. April 9 2013. *Accessed* November 2 2013.

Gregorich, E.G., M.H. Beare, U.F. McKim, and J. O. Skjemstad. 2006. Chemical and biological characterisitics of physically uncomplexed organic matter. Soil Sci. Soc. Am. J. 70:975-985.

Hendrix, F.F. and W.A. Campbell. 1973. Pythiums as plant pathogens. Anni. Rev. Phytopathol. 11:77-98.

Hillel, D. 1998. Environmental soil physics. Academic press. San Diego, CA.

Ivarson, K. C. and Mack, A. R. 1972. Root surface mycoflora of soybean in relation to soil temperature and moisture in a field environment. Can. J. Soil Sci. 52: 199-208 1972

Jiang, Y.N., J.S. Haudenshield, and G.L. Hartman. 2012. Characterization of *Pythium spp*. from soil samples in Illinois. Can. J. Plant Pathol. 34:448-454.

Johnson, L.F., and J.H. Doyle. 1986. Relationships of seedling disease of cotton to characteristics of loessial soils in Tennessee. Phytopathology 76:286-290.

Kang, S., Mansfield, M. A., Park, B., Geiser, D. M., Ivors, K. L., Coffey, M. D., Grünwald, N. J., Martin, F. N., Lévesque, C. A., and Blair, J. E. 2010. The promise and pitfalls of sequence-based identification of plant pathogenic fungi and oomycetes. Phytopathology 100:732-737.

Killebrew, J. F., Roy, K. W., and Abney, T. S. 1993. *Fusaria* and other fungi on soybean seedlings and roots of older plants and interrelationships among fungi, symptoms, and soil characteristics. Can J Plant Pathology 15:139-146.

Kirkpatrick, M. T., C. S., Rothrock, J.C. Rupe, and E. E. Gbur. 2006. The effect of *Pythium ultimum* and soil flooding on two soybean cultivars. Plant Dis. 90:597-602.

Kobayashi, S. S. Uzuhashi, M. Tojo, M. Kakishima.2010. Characterization of *Pythium nunn* newly recorded in Japan and its antagonistic activity against *P. ultimum* var. *ultimum*. J. Gen. Plant Pathol. 76:278-283.

Larkin, R.P., J.T. English, and J.D. Mihail. 1995. Identification, distribution, and comparatice pathogenicity of *Pythium sp*. associated with alfalfa seedlings. Soil Biol. Biochem. 27:357-364.

Leach, L.D. 1947. Growth rates of host and pathogen as factors determining the severity of preemergence damping-off. J. Ag. Res. 75:161-179.

Lenney, J.F. and H.W. Klemmer. 1966. Factors controlling sexual reproduction and growth in *Pythium graminicola*. Nature. 209:1365-1366.

Levesque, C.A., and A.W.A.M de Cock. 2004. Molecular phylogeny and taxonomy of the genus *Pythium*. Mycological Res. 108:1363-1383.

Lewis, J.A., and R.D. Lumsden. 1984. Reduction of preemergence damping-off of peas caused by *Pythium ultimim* with calcium oxide. Can. J. Plant Pathol. 6:227-232.

Lifshitz, R., and J.G. Hancock. 1983. Saprophytic development of *Pythium ultimum* in soil as a function of water matric potential and temperature. Phytopathology, 73:257-261.

Lifshitz, R., M. Dupler, Y. Elad, R. Baker. 1984a. Hyphal interactions between a mycoparasite, *Pythium nunn* and several soil fungi. Can. J. Microbiol. 30:1482-1487.

Lifshitz, R., B.Sneh, R. Baker. 1984b. Soil suppressiveness to plant pathogenic *Pythium* species. Phytopathology 74:1054-1061.

Lindenmayer, David B.; C.R. Margules, D.B. Botkin. 2000. Indicators of biodiversity for ecologically sustainable forest management. Conservation Biology 14: 941–950.

Lindsay, W.l. 1979. Chemical equilibria in soils. John Wiley and Sons, New York. NY.

Lipps, P.E. 1980. The influence of temperature and water potential on asexual reproduction by *Pythium sp.* Associated with snow rot on wheat. Phytopathology. 70:794-797.

Martin, F.N. and J.G. Hancock. 1986. Association of chemical and biological factors in soils suppressive to *Pythium ultimum*. Phytopathology. 76:1221-1231.

Martin, F.N. and J.G. Hancock. 1987. The use of *Pythium oligandrum* for biological control of preemergence damping-off caused by *P. ultimum*. Phytopathology 77:1013-1020.

Martin, F.N. and P.W. Tooley. 2003. Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encodes cytochrome oxidase I and II genes. Mycologia. 95:269-284.

Matthiesen, R.L., and A.E. Robertson. 2013. Pathogenicity of *Pythium* species affecting corn and soybean in Iowa at three temperatures using two assay methods. Phytopathology. 103(Suppl. 2):S2.92.

Matthijs, S., K. A. Tehrani, G.Laus, R.W. Jackson, R.M. Cooper, and P. Cornelis. 2007. Thiquinolobactin, a *Pseudomonas* siderophore with antifungal and anti-*Pythium* activity. Environ. Micro. 9:425-434.

McCune, B. and M.J. Mefford. 2011. PC-ORD. Multivariate Analysis of Ecological Data. Version 6. MjM Software, Gleneden Beach, OR.

McLeod, A., W.J. Botha, J.C. Meitz, C.F.J. Spies, Y.T. Tewoldemedhin, L. Mostert. 2009. Morphological and phylogenetic analysis of *Pythium* species in South Africa. Mycol. Res. 113:933-951.

Middleton, J.T. 1943. The taxonomy , host range, and geographic distribution of the genus *Pythium*. Lancaster Press Inc. Lancaster, PA.

Mielke, P.W., Jr. 1984. Meterological applications of permutation techniques based on distance functions. Pp.813-830. In P.R. Krishnaiah & P.K. Sen, eds., Handbook of Statistics, Vol. 4 Elsevier Science Publications.

Naar, Z. 2006. Effect of cadmium, nickel, and zinc on the antagonistic activity of *Trichoderma* Spp. against *Pythium irregular* Buisman. Acta Phyto, et Entom, Hung.41:193-202.

Paul, B. 2002. *Pythium terrestris*, a new species isolated from France, its ITS region, taxonomy and its comparison with related species. FEMS Microbiol. Lett. 212:255-260.

Paul, B. and K. Bala. 2008. A new species of *Pythium* with inflated sporangia and coiled antheridia, isolated from India. FEMS Microbiol. Lett. 282:251-257.

Paul, B., R. Mathew, B. Kanak, A. Paul, M. Henry, F.Lefort, and L. Belbahri. 2008. Morphology, taxonomy, and phylogenetic analysis of a new species of *Pythium* isolated from France. Fungal Diversity. 28:55-63.

Pepper, I., C.Gerba, and M. Brusseau. 2006. Environmental and pollution science. Elsevier Inc. Burlington, MA.

Plaats-Niterick, A.J. van der. 1981. Monograph of the genus *Pythium*. Studies in Mycology. 21:1-242.

Rasmussen, S.L. and M.E, Stanghellini. 1998. Effect of salinity stress on develioment of Pythium blight in Agrostis palustris. Phytopathology.78:1495-1497.

Robideau, G.P., A.W.A.M. de Cock, M.D. Coffey, H. Voglmayr, H. Brouwer, K. Bala, D.W. Chitty, N. Désaulniers, Q.A. Eggertson, C.M.M. Gachon, C.Hu, F.C. Küpper, T.L. Rintoul, E.Sarhan, E.C.O.Verstappen, Y. Zhang, P.J.M. Bonants, J.B.Ristaino, and C. A. Lévesque. 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Mol. Eco. Res. 11:1002-1011.

Rojas, A., J.L. Jacobs, S. Napieralski, C.A. Bradley, T. Chase, P.D. Esker, L. Giesler, D.Jardine, B.D. Nelson, D. Malvick, S. Markell, A.E. Robertson, J.C. Rupe, L.Sweets, K. Wise, and M.I. Chilvers. 2013. Diversity of oomycetes associated with soybean seedling diseases in the U.S. Phytopathology. 103(Suppl.2):S2.123

SAS Institute Inc. 2011. Base SAS®9.3 Procedures Guide. Cary, NC: SAS Institute Inc.

Schlub, R.L. and J.L. Lockwood. 1981. Etiology and epidemiology of seeding rot of soybean by *Pythium ultimum*. Phytopathology. 71:134-138.

Schroeder, K. L., Martin, F. N., de Cock, W. A. M., Lévesque, C. A., Spies, C. F. J., Okubara, P. A., and Paulitz, T. C. 2013. Molecular Detection and Quantification of *Pythium* Species: Evolving Taxonomy, New Tools, and Challenges. Plant Dis. 97: 4-20.

Six, J., H. Bossuyt, S. Degryze, and K. Denef. 2004. A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. Soil and Tillage Research 79:7-31.

Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Web Soil Survey. 2013. [online] Available at http://websoilsurvey.nrcs.usda.gov/. Washington, D.C.

Sparrow, F.K. 1931. The classification of *Pythium*. Science. 73:41-42.

Stanghellini, M.E. and J.G. Hancock. 1971. Radial extent of the bean spermosphere and its relation to the behavior of *Pythium ultimum*. Phytopathology. 61:165-168.

Stone, A.G., S.J. Raina, and H.A.J.Hoitink. 2001. Particulate organic matter composition and *Pythium* damping-off of cucumber. Soil Sci. Soc. Am. J. 65:761-770.

Stone, A.G. S.J. Scheuerell, and H.M. Darby. 2004. Suppression of soilborne diseases in field agricultural systems: organic matter management, cover cropping, and other cultural practices.Dept. pf Horticulture, Oregon State University, Corvallis OR.

Tambong, J.T., A.W.A.M de Cock, N.A. Tinker, C.A. Levesque. 2006. Oligonucleotide array for identification and detection of *Pythium* species. Applied and Environ. Micro. 72:2691-2706.

Tamm, L., B. Thurig, C. Bruns, J.G. Fuchs, U. Kopke, M. Laustela, C. Leifert, M. Mahlberg, B. Nietlispach, F. Weber, A. Fliebach. 2010. Soil type, management history, and soil amendments

influence the development of soil-borne (*Rhizoctonia solani*, *Pythium ultimum*) and air-borne (*Phytophthora infestans*, *Hyaloperonospora parasitica*) diseases. Eur. J. Plant Pathol. 127:465-481.

Tan, K. H. 1996. Soil Sampling, preparation, and analysis. MarcelDekker, Inc., New York.

Tsao, P.H. and G. Ocana. 1969. Selective isolation of species of *Phytophthora* from natural soils on an improved antibiotic medium. Nature. 223:636-638.

Vilgalys, R. LAB. 2013. rDNA primer sequences for fungal sequencing. [online] Available at http://biology.duke.edu/fungi/mycolab/. Dept. of Biology Duke University, Durham, NC.

Wei, L., A.G. Xue, E.R. Cober, C. Babock, J. Zhang, S. Zhang, W. Li, J. Wu, and L.Liu. 2011 Pathogenicity of *Pythium* species causing seed rot and damping-off in soybean under controlled conditions. Phytoprotection. 91:3-10.

Weiland, J.E. 2011. Influence of isolation method on recovery of *Pythium* species from forest nursery soils in Oregon and Washington. Plant Dis. 95:547-553.

Zhang, B.Q., W.D. Chen, and X.B. Yang. 1998. Occurrence of *Pythium* species in long-term maize and soybean monoculture and maize/soybean rotation. Mycol. Res. 102:1450-1452.

Zhang, B.Q., and X.B. Yang. 2000. Pathogenicity of *Pythium* populations from corn-soybean rotation fields. Plant Dis. 84:94-99.

Zhang, H., Wu, X., Li., G. and Qin, P. 2011. Interactions between arbuscular mycorrhizal fungiand phosphate-solubilizing fungus (*Mortierella* sp.) and their effects on *Kostelelzkya virginica* growth and enzyme activities of rhizosphere and bulk soils at different salinities. Biol Fertil Soils 47:543–554.

Zitnick-Anderson, K. and B. Nelson. 2012a. Characterization and identification of *Pythium* from soybean roots in North Dakota. Phytopathology. 102:S4.145.

Zitnick-Anderson, K, and B. Nelson. 2012b. Stimulation of sexual structure production by *Pythium*. Phytopathology. 102:S4.145.

APPENDIX A

Figure A1. *P. attrantheridium* A. terminal wavy oogonial wall B. aplerotic wall thickness between 2-4µm C. terminal globose sporangia D. radial growth pattern on PDA.

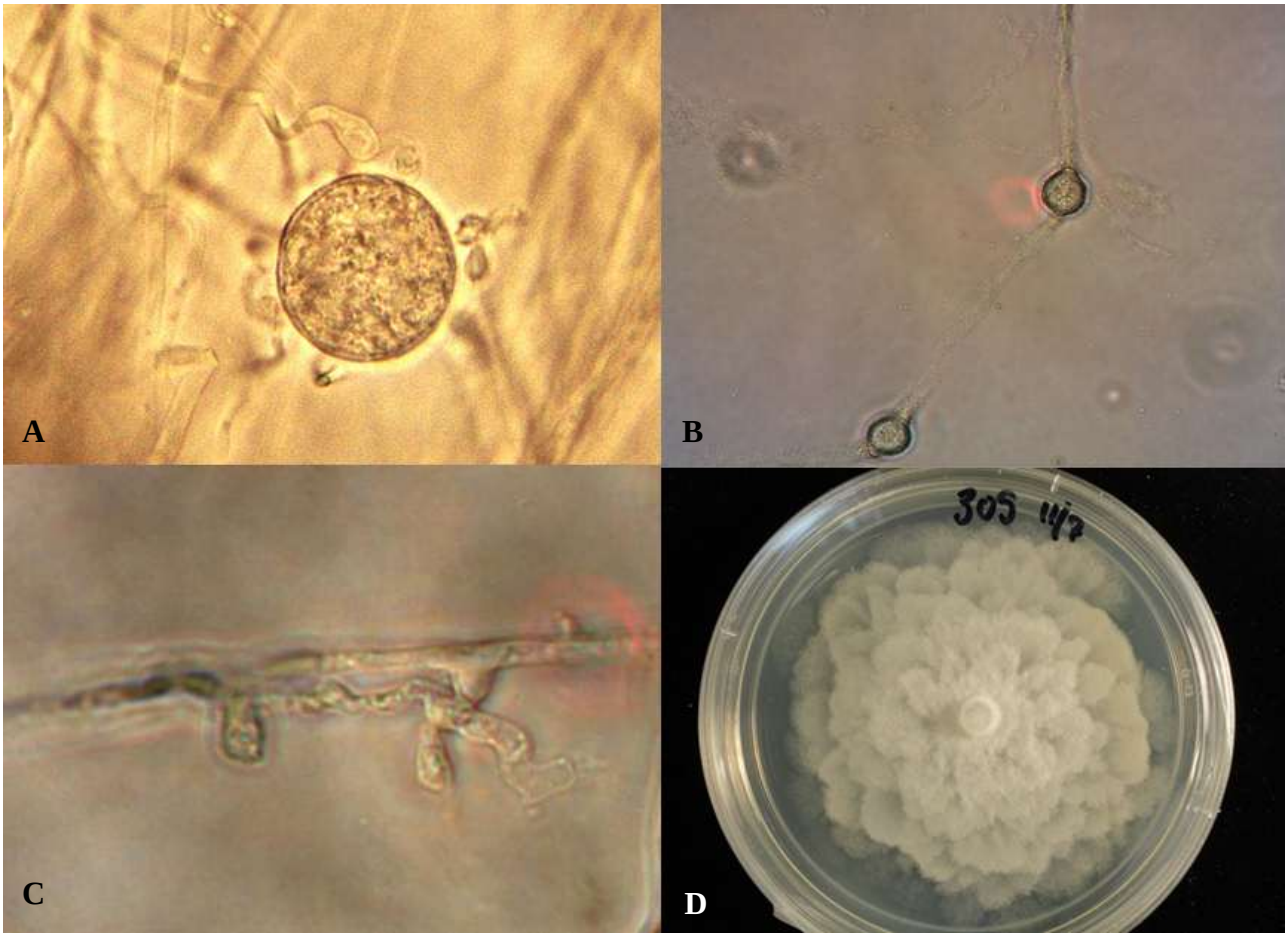

Figure A2. *P. kashmirense* A. oogonium with multiple antherida attachments B. chained sporangia C. inflated sporangia D. chrysanthemum growth pattern on PDA.

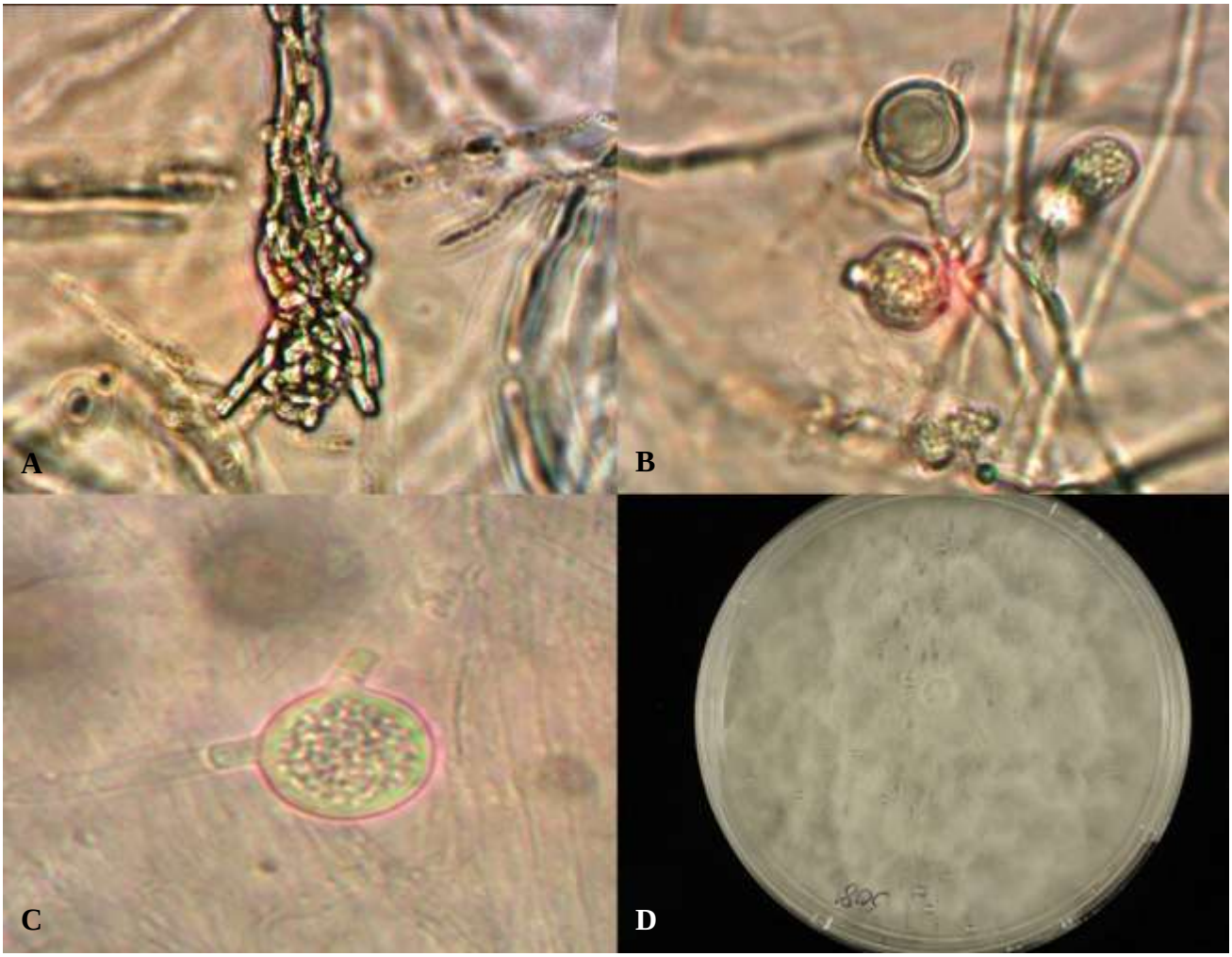

Figure A3. *P. oopapillum* A. sporangia filamentous and inflated B. thick walled oospore with a papillae C. intercalary, smooth globose sporangia D. chrysanthemum growth pattern on PDA.

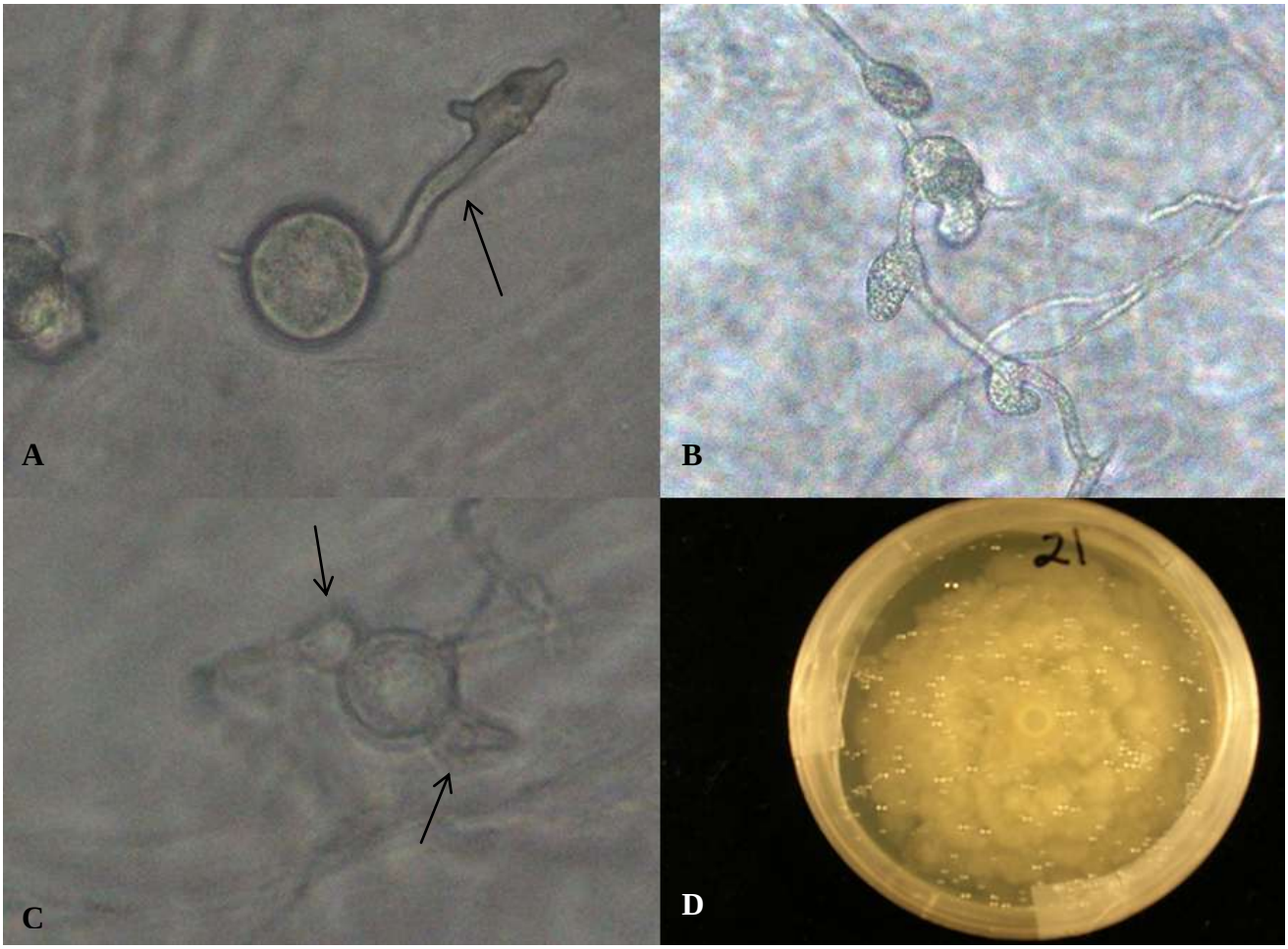

Figure A4. *P. rostratifingens* A. sporangia with zoospore discharge tube (indicated by arrow) B. chained sporangia C. mainly two antherida per oogonia (indicated by arrows); paragynous/hypogynous D. chrysanthemum growth pattern on PDA.

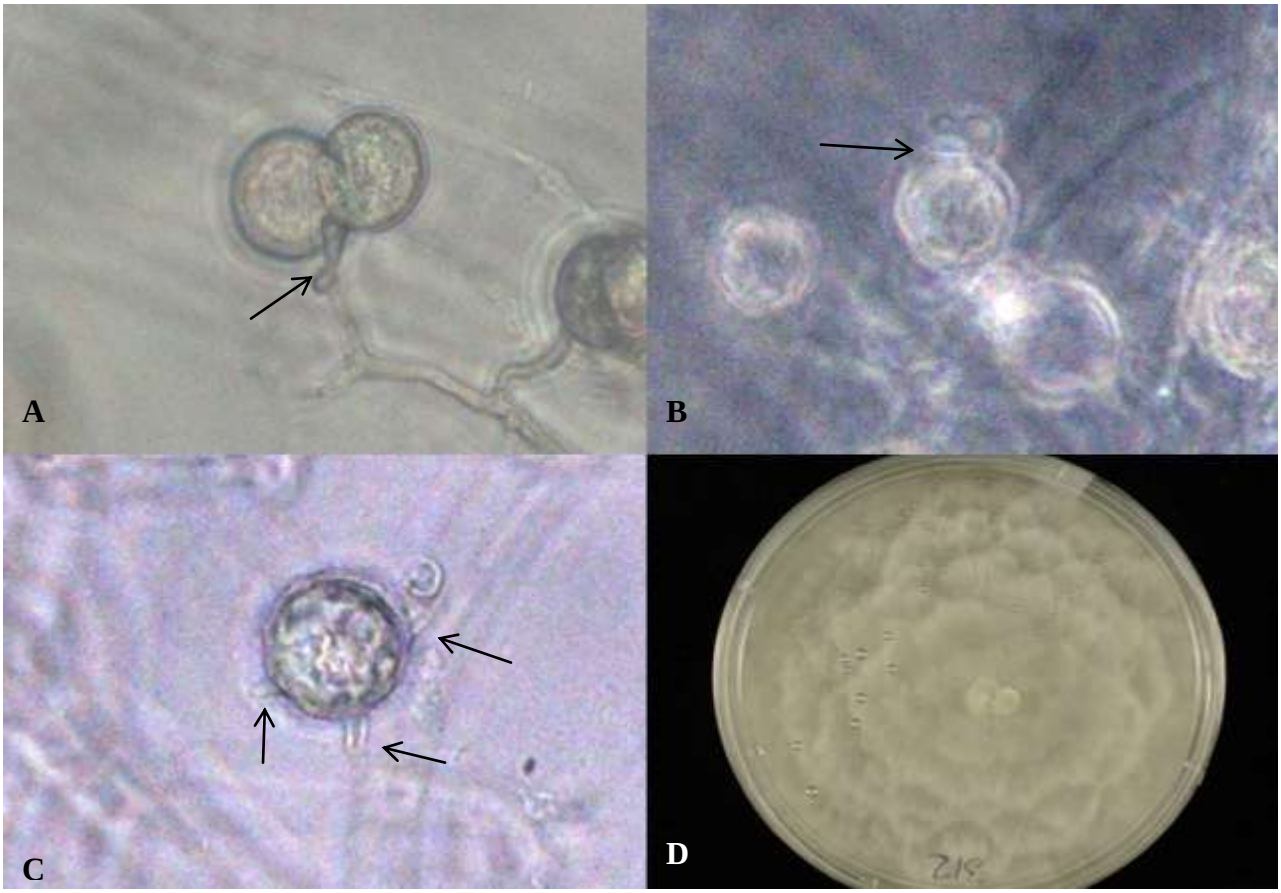

Figure A5. *P. terrestris* A. hypogynous antheridia (indicated by arrow) B. sporangia with truncated zoospore discharge tube (indicated by arrow) C. ornamented oogonia with papillae (indicated by arrow) D. chrysanthemum growth pattern on PDA.

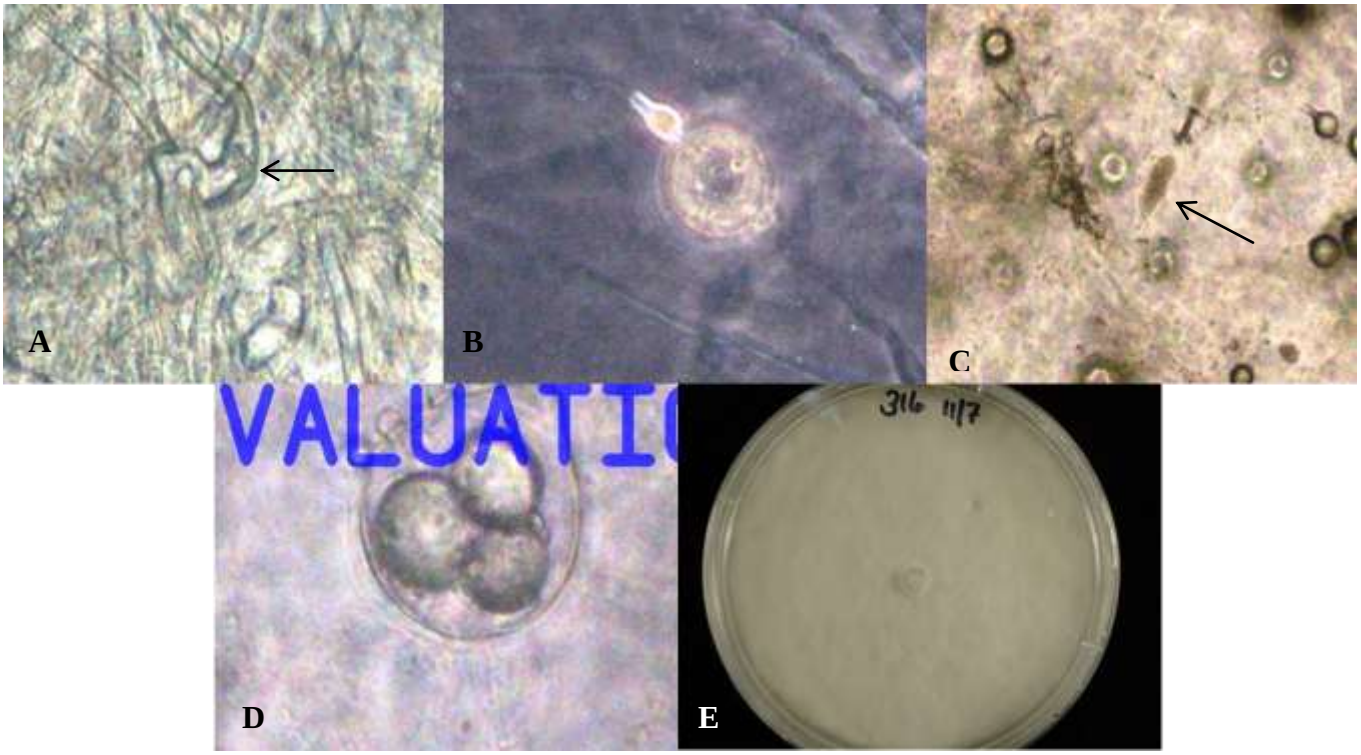

Figure A6. *P. viniferum* A. sickle shaped sporangia attached to oogonium B. hypogynous antheridia C. elongated oogonium D. oogonium with three oospores E. arachnid growth pattern on PDA.

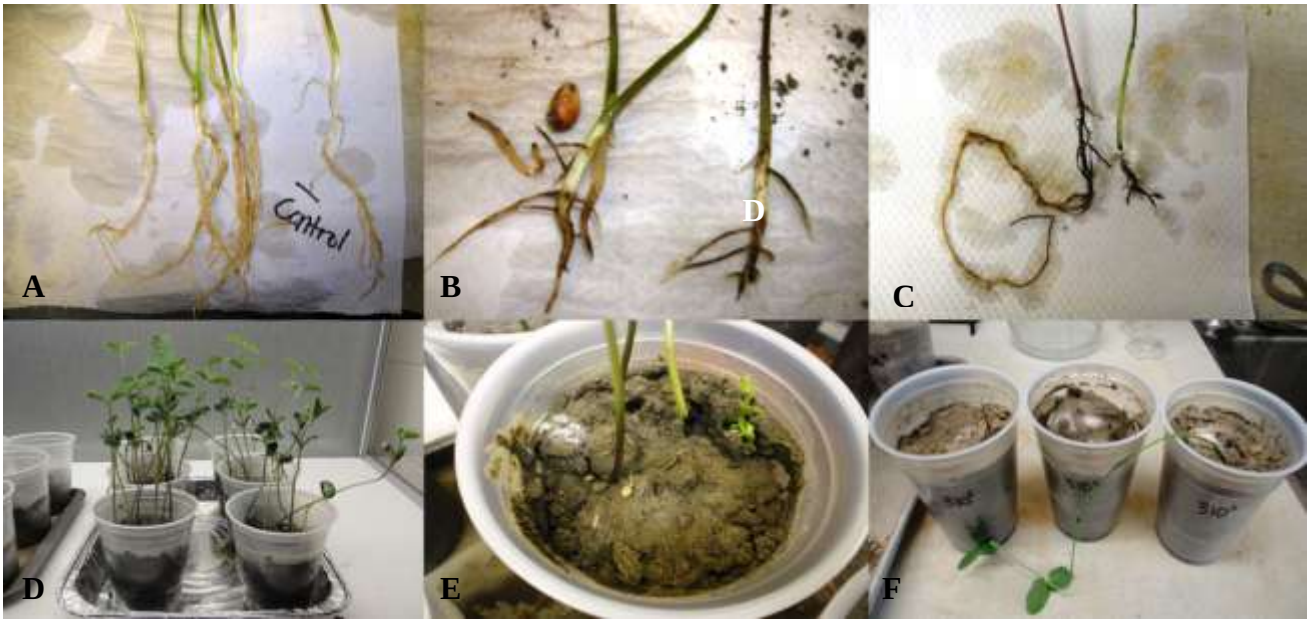

Figure A7. Pathogenicity of *P.terrestris* on soybean cultivar Barnes. A. (-) control B. *P. terrestris* C. (+) control D. (-) control E. *P. terrestris* F. (+) control.

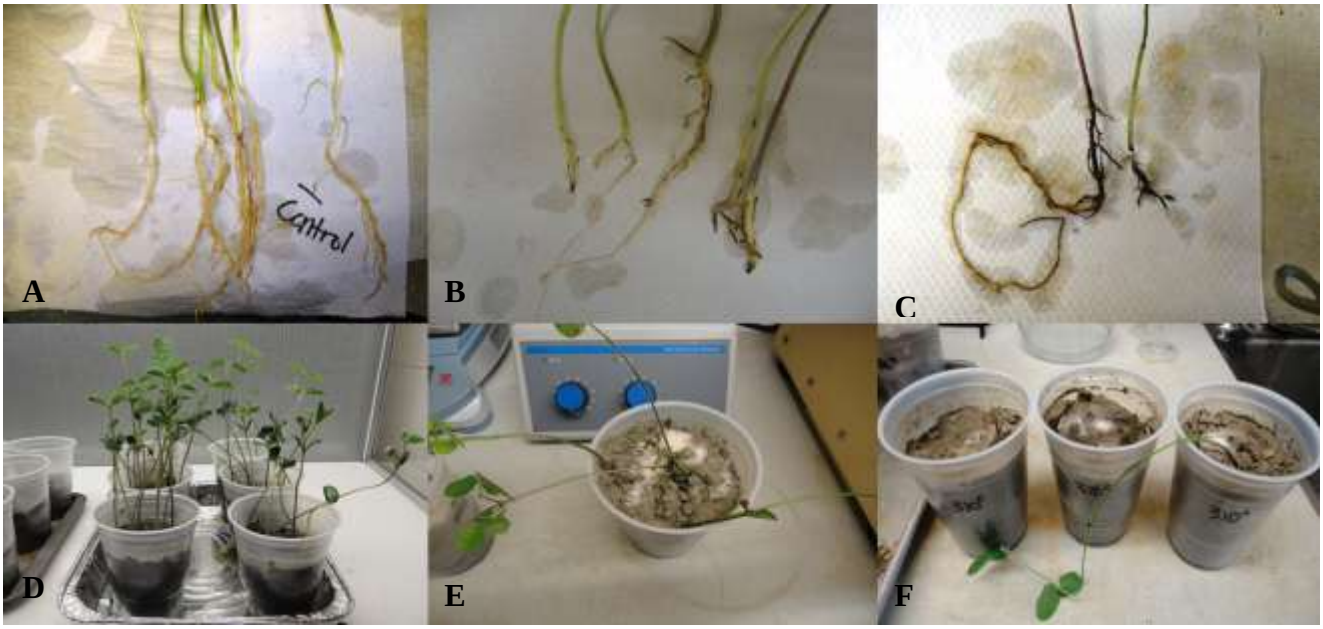

Figure A8. Pathogenicity of *Pythium* sp. (unknown; GenBank HQ643777.1) on soybean cultivar Barnes A. (-) control B. *Pythium* sp. (unknown; GenBank HQ643777.1) C. (+) control D. (-) control E *Pythium* sp. (unknown; GenBank HQ643777.1) F. (+) control.

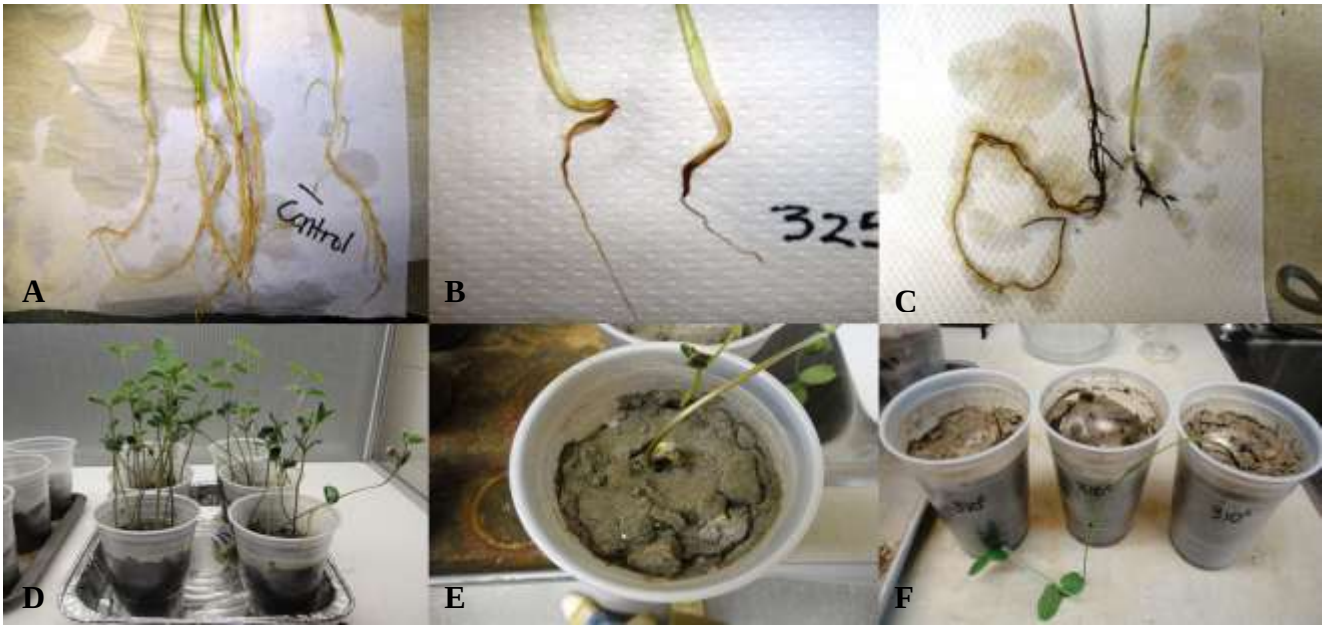

Figure A9. Pathogenicity of *P. kashmirense* on soybean cultivar Barnes A. (-) control B. *P. kashmirense* C. (+) control D. (-) control E. *P. kashmirense* F. (+) control.

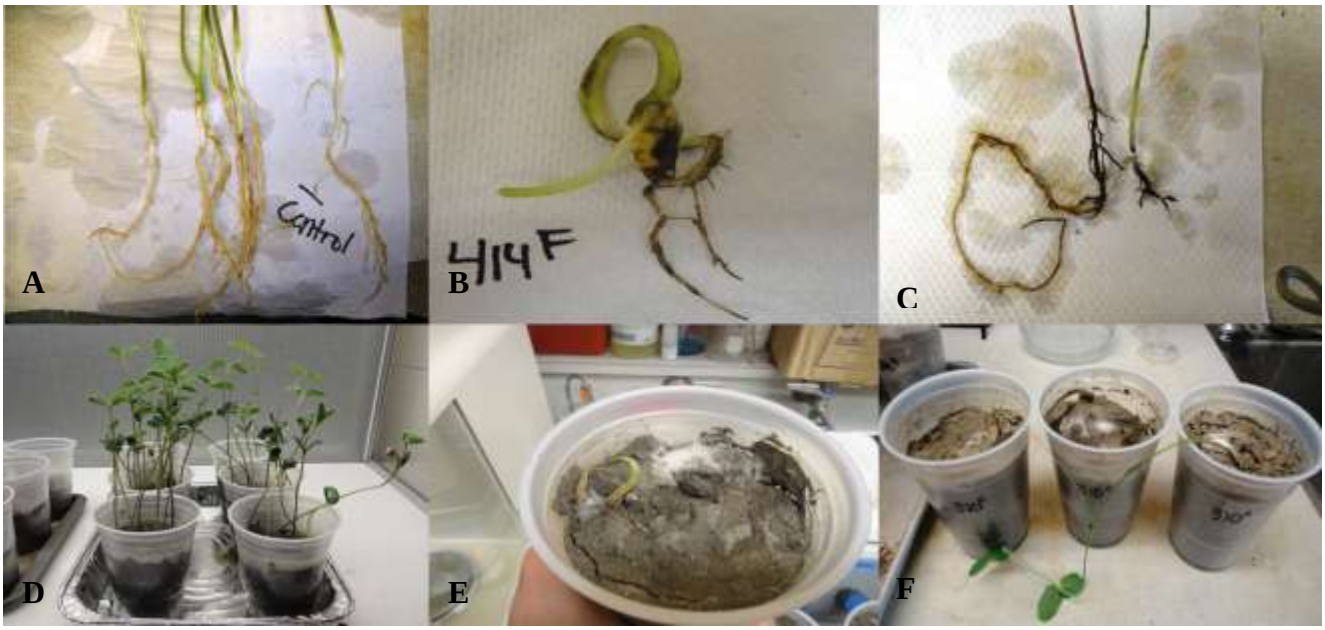

Figure A10. Pathogenicity of *P. minus* on soybean cultivar Barnes A. (-) control B. *P. minus* C. (+) control D. (-) control E. *P. minus* F. (+) control.

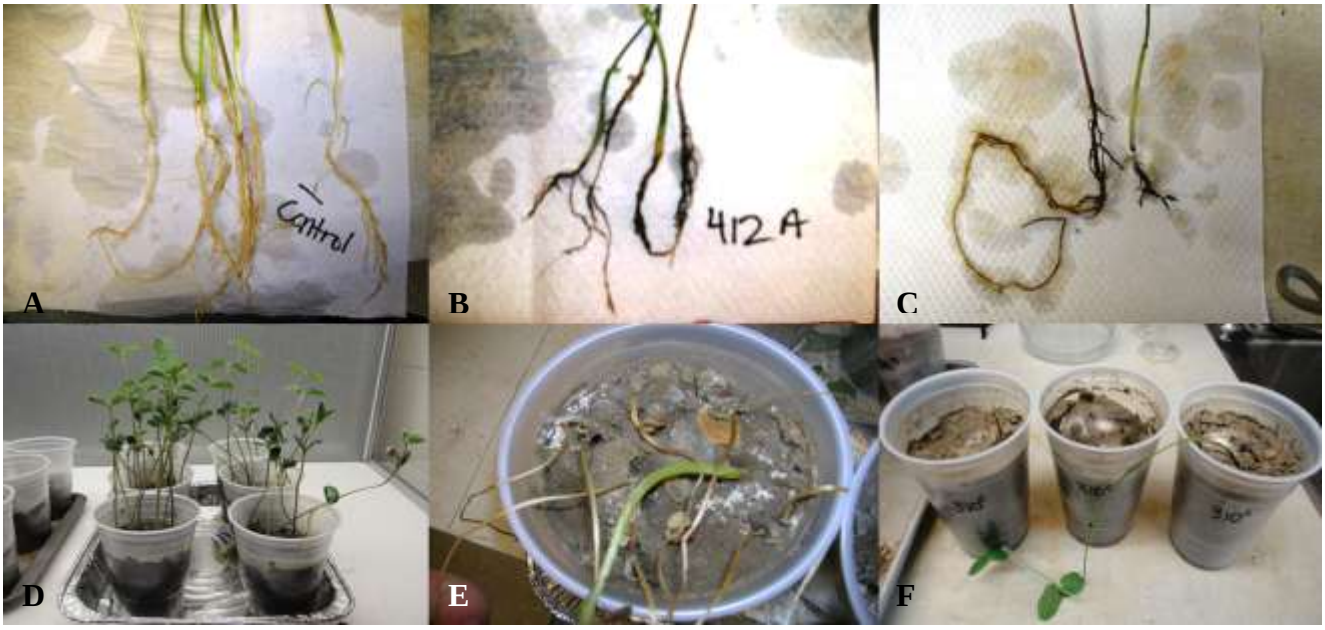

Figure A11. Pathogenicity of *P. violae* on soybean cultivar Barnes A. (-) control B. *P. violae* C. (+) control D. (-) control E. *P. violae* F. (+) control**.**

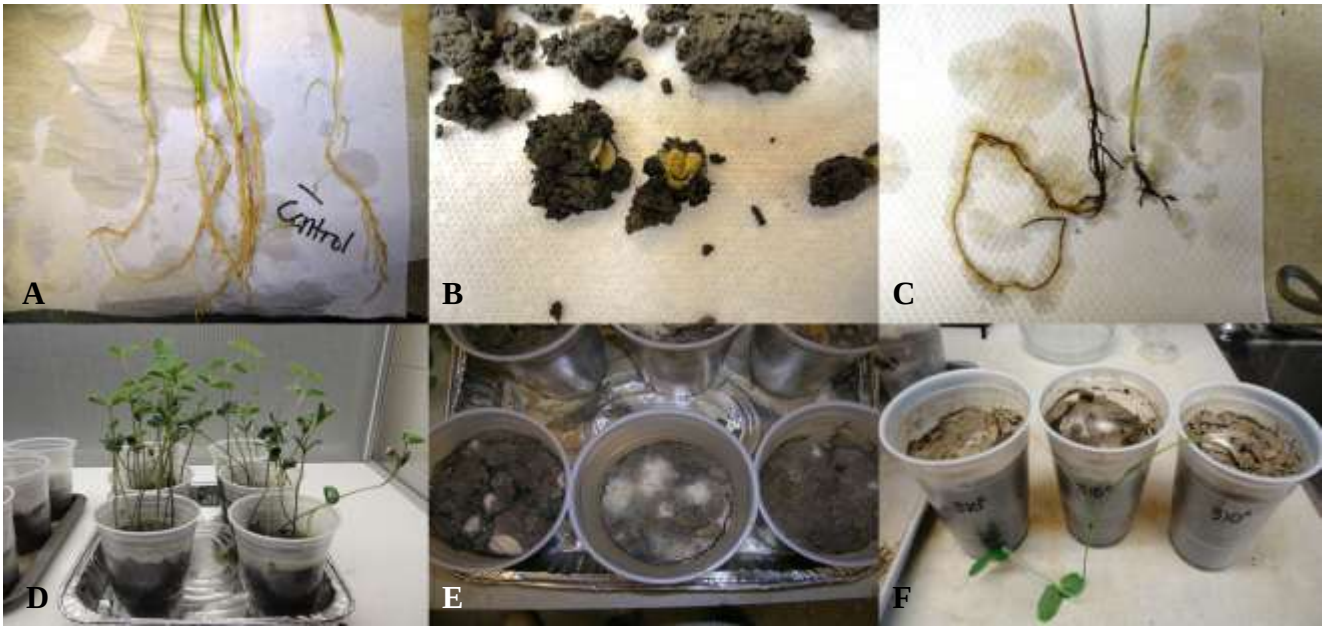

Figure A12. Pathogenicity of *P. viniferum* on soybean cultivar Barnes A. (-) control B. *P. viniferum* C. (+) control D. (-) E. *P. viniferum* F. (+) control.

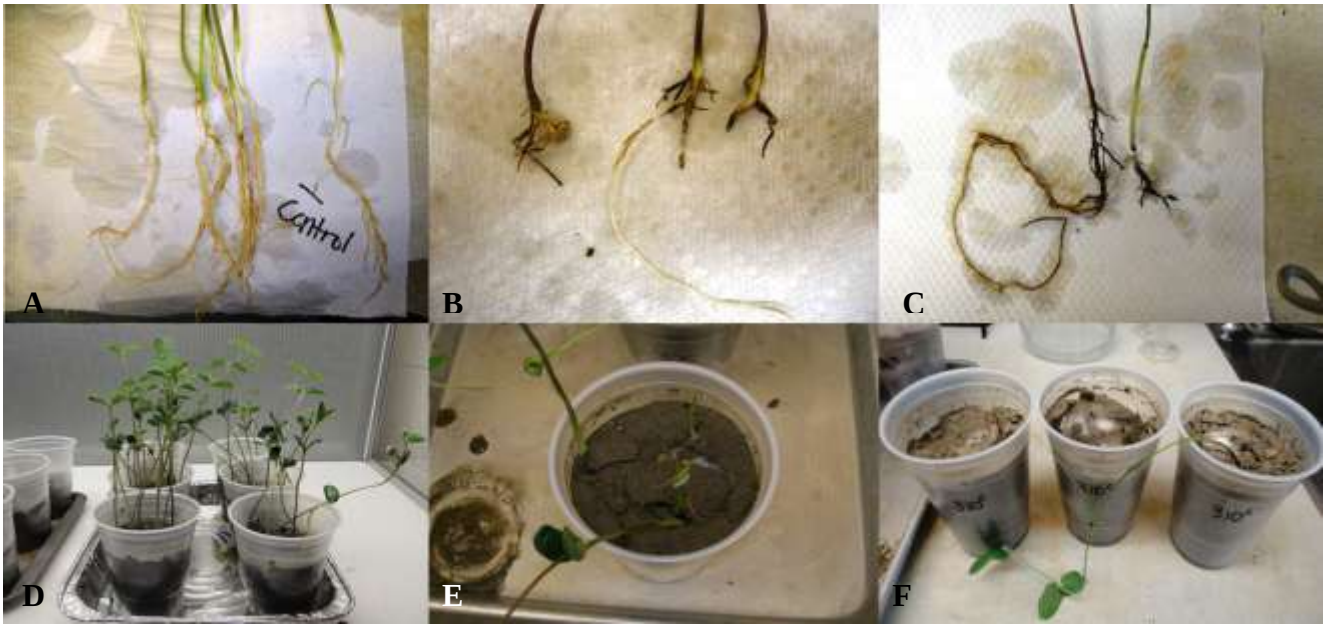

Figure A13. Pathogenicity of *P. periilum* on soybean cultivar Barnes A. (-) control B. *P. periilum* C. (+) control D. (-) control E. *P. periilum* F. (+) control.

APPENDIX B

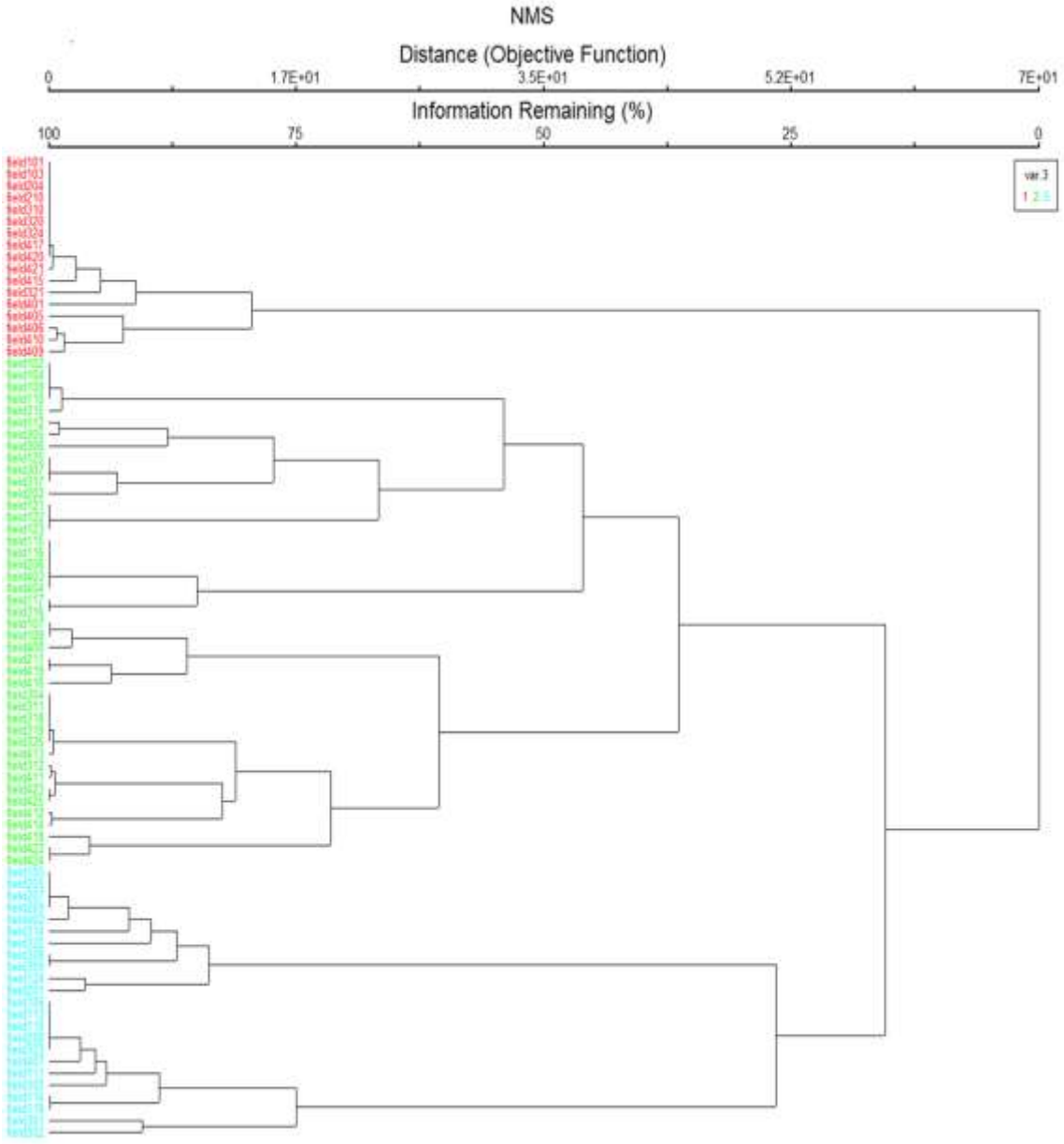

Figure B1. Cluster analysis dendrogram. Three *Pythium* groups were determined using inidcator species analysis. Group one is highlighted in green. Group two is highlighted in blue. Group three is highlighted in red.

```
****************************** Indicator Values
******************************
PC-ORD, 6.08
23 Oct 2013, 14:54:03

Indicator values calculated with method of Dufrêne, M. &
P. Legendre.  1997.  Species assemblages and indicator
species: the need for a flexible asymmetrical approach.
Ecological Monographs 67:345-366.


NMS

        Groups were defined by values of: var.3
        Input data has:    83 field    by    23 species


INDICATOR VALUES (% of perfect indication,
based on combining the above values for relative abundance
and relative frequency)
                                  Group
                    Sequence:     1    2    3
                  Identifier:     1    2    5
              Number of items:   17   43   23
    Column      Avg Max MaxGrp
    1 rost        3   8      1    8    2    0
    2 perii       5  13      1   13    0    1
    3 inf         3   7      1    7    0    1
    4 ult        30  87      1   87    2    0
    5 unk        12  35      2    0   35    2
    6 hete       18  53      5    0    0   53
    7 irr        16  48      5    0    0   48
    8 att         5  13      2    1   13    0
    9 syl         5   8      5    0    7    8
   10 perp        5  12      2    0   12    2
   11 arr         2   7      2    0    7    0
   12 kash        5  14      2    0   14    0
   13 min         3   9      5    0    0    9
   14 oop         2   7      2    0    7    0
   15 inter       1   4      5    0    0    4
   16 lut         2   5      2    0    5    0
   17 dic         2   5      2    0    5    0
   18 col         2   7      2    0    7    0
   19 orth        1   3      5    0    1    3
   20 diss        2   5      2    0    5    0
   21 nunn        1   3      5    0    1    3
   22 vin         2   5      1    5    1    0
   23 vio         2   4      1    4    1    0
       Averages   6  16           5    5    6
```

Figure B 2. Results from indicator species analysis showing the abundance and frequency of each indicator species for each group (cluster).

MONTE CARLO test of significance of observed maximum indicator value for species
4999 permutations.
Random number seed: 4828

Column	Maxgrp	Observed Indicator Value (IV)	IV from randomized groups Mean	S.Dev	p *
1 rost	1	7.5	5.7	3.12	0.2699
2 perii	1	13.2	6.3	3.15	0.0566
3 inf	1	6.5	5.7	3.06	0.3129
4 ult	1	86.8	16.9	4.35	0.0002
5 unk	2	34.9	15.6	4.28	0.0016
6 hete	5	52.6	11.8	4.01	0.0002
7 irr	5	47.8	9.7	3.71	0.0002
8 att	2	12.8	8.1	3.57	0.0988
9 syl	5	8.3	9.1	3.65	0.5087
10 perp	2	12.0	8.7	3.75	0.1516
11 arr	2	7.0	4.8	2.81	0.3295
12 kash	2	14.0	6.9	3.36	0.0506
13 min	5	8.7	4.5	2.09	0.1134
14 oop	2	7.0	4.9	2.83	0.3045
15 inter	5	4.3	3.6	1.43	0.4835
16 lut	2	4.7	4.4	2.07	0.3783
17 dic	2	4.7	4.5	2.07	0.3857
18 col	2	7.0	4.8	2.77	0.2875
19 orth	5	3.0	4.5	2.09	0.8620
20 diss	2	4.7	4.4	2.14	0.4965
21 nunn	5	3.4	4.4	2.16	0.8092
22 vin	1	4.5	4.4	2.06	0.4843
23 vio	1	3.6	4.4	2.09	0.6539
Averages		15.6080	6.88	2.90	0.3061

Figure B3. Results of the Monte Carlo test used in the indicator species analysis. Group (cluster) step three had the lowest averaged *p*-value and the most species (five) to be significant indicators.

```
****************** Multi-Response Permutation Procedures (MRPP)
******************
PC-ORD, 6.08
 7 Oct 2013, 18:57:18


NMS

        Groups were defined by values of: var.3
        Input data has:      83 field    by     23 species
        Weighting option: C(I) = n(I)/sum(n(I))
        Distance measure: Euclidean (Pythagorean)

     GROUP:     1
Identifier:     1
      Size:    17       3.6764290    = Average distance
Members:
 field101 field103 field204 field210 field310 field320 field321 field324
 field401 field405 field406 field409 field410 field415 field417 field420
 field421

     GROUP:     2
Identifier:     2
      Size:    43       6.7982626    = Average distance
Members:
 field102 field104 field107 field108 field109 field110 field112 field115
 field116 field117 field121 field122 field123 field125 field202 field206
 field211 field304 field305 field306 field307 field311 field312 field315
 field316 field317 field318 field319 field325 field403 field404 field408
 field411 field412 field413 field414 field416 field418 field419 field422
 field423 field424 field425

     GROUP:     3
Identifier:     5
      Size:    23       6.1450625    = Average distance
Members:
 field105 field106 field111 field113 field114 field118 field119 field124
 field201 field205 field207 field208 field209 field301 field302 field303
 field308 field309 field314 field322 field323 field402 field407

        Test statistic: T =      -32.007222
           Observed delta =       5.9778436
           Expected delta =       7.1639123
        Variance of delta =      0.13731681E-02
        Skewness of delta =     -0.77285326

        Chance-corrected within-group agreement, A =    0.16556159
          A = 1 - (observed delta/expected delta)
          Amax = 1 when all items are identical within groups (delta=0)
          A = 0 when heterogeneity within groups equals expectation by chance
          A < 0 with more heterogeneity within groups than expected by chance

        Probability of a smaller or equal delta, p =    0.00000000
```

Figure B4. Results of the MRPP. Statistical test T-value was -32. *A* was 0.16. *p*-value was 0.0. All three criteria indicate that the three groups developed from the indicator species and cluster analysis were separate and significantly different from one another.

APPENDIX C

Table C1. GPS coordinates for fields 101-125 from 2011.

GPS 2011	Latitude			Longitude		
Field	Degrees(°)	minutes	suffix (N/S)	Degrees(°)	minutes	suffix (W/E)
101	46	38.1	N	96	40.731	W
102	46	58.621	N	97	13.211	W
103	46	58.6	N	97	13.346	W
104	46	44.812	N	97	14.218	W
105	46	42.523	N	97	8.112	W
106	46	14.112	N	97	9.798	W
107	46	5.422	N	97	3.921	W
108	46	3.922	N	97	49.001	W
109	46	3.011	N	97	46.214	W
110	46	3.1	N	96	42.442	W
111	46	3.422	N	96	37.042	W
112	46	9.412	N	96	36.152	W
113	46	13.211	N	96	36.521	W
114	46	15.722	N	96	38.978	W
115	46	18.04	N	96	43.241	W
116	46	24.702	N	96	44.41	W
117	46	33.094	N	96	47.711	W
118	46	37.504	N	96	49.124	W
119	46	40'5.72	N	96	49'6.56	W
121	46	40'18.89	N	96	48'38.31	W
122	46	40'57.47	N	96	48'1.41	W
123	46	41'35.16	N	96	47'52.02	W
124	46	42'29.54	N	96	47'58.94	W
125	46	43'33.38	N	96	47'49.81	W

Table C2. GPS coordinates for fields 201-213 from 2011.

GPS 2011	Latitude			Longitude		
Field	Degrees(^{o})	minutes	suffix (N/S)	Degrees(^{o})	minutes	suffix (W/E)
201	46	21.301	N	98	43.958	W
202	46	21.301	N	98	43.958	W
203	46	21.331	N	98	23.137	W
204	46	21.339	N	98	9.602	W
205	46	26.504	N	97	43.237	W
206	46	37.816	N	97	40.606	W
207	46	67.795	N	97	34.615	W
208	46	53.771	N	96	48.197	W
209	46	53.771	N	98	33.918	W
210	46	10.842	N	99	3.264	W
211	46	11.664	N	99	12.648	W
212	46	9.882	N	99	19.95	W
213	46	9.15	N	99	22.542	W

Table C3. GPS coordinates for fields 301-325 from 2011.

GPS 2011	Latitude			Longitude		
Field	Degrees(°)	minutes	suffix (N/S)	Degrees(°)	minutes	suffix (W/E)
301	46	57.143	N	96	51.584	W
302	47	0.832	N	96	54.257	W
303	47	4.458	N	96	56.463	W
304	47	8.221	N	96	57.8	W
305	47	13.449	N	96	59.783	W
306	47	17.373	N	97	1.243	W
307	47	20.742	N	97	2.431	W
308	47	29.855	N	97	8.323	W
309	47	29.928	N	97	14.116	W
310	47	31.396	N	97	21.593	W
311	47	35.544	N	98	27.19	W
312	47	39.706	N	98	27.191	W
314	47	43.571	N	98	27.214	W
315	47	44.652	N	98	27.154	W
316	47	45.206	N	98	32.933	W
317	47	51.124	N	98	37.404	W
318	47	57.393	N	98	37.441	W
319	47	2.138	N	98	37.433	W
320	48	6.896	N	98	37.378	W
321	48	9.072	N	98	37.375	W
322	48	27.454	N	99	34.516	W
323	47	27.435	N	99	31.423	W
324	48	27'31.97'	N	99	36'20.66"	W
325	48	27'6.68"	N	99	37'20.49"	W

Table C4. GPS coordinates for fields 401-425 from 2011.

GPS 2011	Latitude			Longitude		
Field	Degrees(°)	minutes	suffix (N/S)	Degrees(°)	minutes	suffix (W/E)
401	46	53.77	N	96	48.236	W
402	46	55.405	N	97	47.201	W
403	46	59.762	N	97	47.466	W
404	47	3.865	N	97	47.439	W
405	47	8.71	N	97	47.438	W
406	47	12.94	N	97	47.453	W
407	47	17.027	N	97	47.378	W
408	47	21.268	N	97	47.632	W
409	47	25.173	N	97	50.176	W
410	47	26.502	N	97	54.702	W
411	47	25.663	N	98	0.438	W
412	47	26.568	N	98	5.539	W
413	47	26.574	N	98	10.647	W
414	47	26.529	N	98	17.661	W
415	47	26.523	N	98	24.873	W
416	47	27.408	N	98	30.946	W
417	47	27.407	N	98	37.054	W
418	47	27.43	N	98	45.178	W
419	47	27.439	N	98	50.869	W
420	47	27.455	N	98	56.856	W
421	47	27.454	N	99	1.877	W
422	47	27.494	N	99	8.958	W
423	47	27.517	N	99	13.641	W
424	47	27.491	N	99	20.276	W
425	47	27.501	N	99	26.181	W

Table C5. GPS coordinates for fields 1-20 from 2012.

GPS 2012	Latitude			Longitude		
Field	Degrees(^{o})	minutes	suffix (N/S)	Degrees(^{o})	minutes	suffix (W/E)
1	46	54.739	N	97	13.215	W
2	46	58.633	N	97	13.195	W
3	47	2.988	N	97	13.141	W
4	47	7.804	N	97	13044	W
5	47	12.143	N	97	13.026	W
6	47	16.335	N	97	13.108	W
7	47	20.744	N	97	13083	W
8	47	21.05	N	97	18.252	W
9	47	24.679	N	97	19.598	W
10	47	29.012	N	97	19.193	W
11	47	30.533	N	97	25054	W
12	47	33.828	N	97	27.21	W
13	47	38.799	N	97	28.373	W
14	47	42.286	N	97	27.164	W
15	47	44.642	N	97	29.949	W
16	47	45.514	N	97	37.32	W
17	47	48.999	N	97	37.449	W
18	47	57.104	N	97	37.43	W
19	48	0.754	N	97	37.432	W
20	48	8.643	N	97	37.365	W

Table C6. GPS coordinates for fields 21-38 from 2012.

GPS 2012	Latitude			Longitude		
Field	Degrees(°)	minutes	suffix (N/S)	Degrees(°)	minutes	suffix (W/E)
21	46	37.132	N	96	48.821	W
22	46	58.646	N	97	13.156	W
23	46	44.836	N	97	13.366	W
24	46	42.556	N	97	14.512	W
25	46	14.324	N	97	8.143	W
26	46	5.711	N	97	9.364	W
27	46	3.977	N	97	3.802	W
28	46	3.112	N	97	49.5	W
29	46	3.08	N	96	46.663	W
30	46	3.1	N	96	42.148	W
31	46	3.455	N	96	37.052	W
32	46	9.602	N	96	36.96	W
33	46	13.073	N	96	36.985	W
34	46	15.855	N	96	38.409	W
35	46	18.14	N	96	43.853	W
36	46	24.782	N	96	44.14	W
37	46	33.194	N	96	47.713	W
38	46	37.554	N	96	49.238	W

Table C7. Number of isolates for each species per field from 2011.

field	species	# of isolates
field101	*P. ult.*	30
field102	*P.kash.*	30
field103	*P. ult.*	30
field104	*P.kash.*	30
field105	*P. irr.*	30
field106	*P. het.*	30
field107	*P. sp. unk.*	15
field107	*P. sly.*	15
field108	*P. sp. unk.*	9
field108	*P. sly.*	21
field109	*P.kash.*	10
field110	*P.kash.*	20
field111	*P. orth.*	10
field111	*P. nunn*	7
field112	*P. perp.*	5
field112	*P. lut.*	24
field112	*P. dic.*	10
field112	*P. col.*	7
field112	*P. diss.*	4
field113	*P. het.*	30
field114	*P. het.*	17
field114	*P. sly.*	13
field115	*P.att.*	30
field116	*P.att.*	30
field117	*P. sp. unk.*	15
field117	*P.att.*	15
field118	*P. het.*	22
field118	*P. sly.*	8
field119	*P. het.*	30
field121	*P. oop.*	30
field122	*P. oop.*	10
field123	*P. oop.*	17
field124	*P. irr.*	17
field124	*P. sly.*	13
field125	*P. perp.*	30

Table C7. Number of isolates for each species per field from 2011. (cont.)

field	species	# of isolates
field201	*P. irr.*	17
field201	*P. sly.*	9
field201	*P.inter.*	30
field202	*P. ult.*	14
field202	*P. perp.*	16
field204	*P. ult.*	30
field205	*P. irr.*	30
field206	*P.att.*	30
field207	*P. irr.*	30
field208	*P. sp. unk.*	14
field208	*P. het.*	16
field209	*P. sp. unk.*	6
field209	*P. irr.*	17
field210	*P. ult.*	18
field210	*P. sp. unk.*	7
field211	*P. sp. unk.*	8
field211	*P. sly.*	16

Table C7. Number of isolates for each species per field from 2011. (cont.)

field	species	# of isolates
field301	*P. sp. unk.*	11
field301	*P. minus*	30
field302	*P. het.*	22
field302	*P. perp.*	8
field302	*P. minus*	30
field303	*P. sp. unk.*	12
field303	*P. het.*	18
field304	*P. sp. unk.*	16
field305	*P. perp.*	6
field305	*P. lut.*	20
field305	*P. col.*	4
field306	*P. perp.*	10
field306	*P. dic.*	10
field306	*P. col.*	8
field306	*P. orth.*	7
field306	*P. diss.*	11
field306	*P. nunn*	2
field307	*P. perp.*	16
field307	*P. hyp.*	14
field308	*P. het.*	13
field308	*P. irr.*	17
field309	*P. ult.*	30
field309	*P. het.*	9
field309	*P. irr.*	21
field310	*P. ult.*	30
field311	*P. ult.*	12
field311	*P. sp. unk.*	30
field312	*P. ult.*	14
field312	*P. sp. unk.*	18
field312	*P. het.*	1
field314	*P. irr.*	16

Table C7. Number of isolates for each species per field from 2011. (cont.)

field	species	# of isolates
field315	*P. vini.*	3
field315	*P. vio.*	5
field315	*P. deb.*	3
field316	*P. sp. unk.*	12
field316	*P.att.*	18
field317	*P. perp.*	30
field318	*P. sp. unk.*	30
field319	*P. ult.*	6
field319	*P. sp. unk.*	30
field320	*P. ult.*	15
field321	*P. het.*	9
field322	*P. irr.*	25
field322	*P. perp.*	5
field323	*P. ult.*	19
field323	*P. het.*	30
field324	*P. ult.*	19
field325	*P. ult.*	17
field325	*P. sp. unk.*	30

Table C7. Number of isolates for each species per field from 2011. (cont.)

field	species	# of isolates
field401	*P.att.*	13
field402	*P. sp. unk.*	8
field402	*P. irr.*	20
field403	*P.att.*	25
field404	*P. ult.*	30
field404	*P.att.*	19
field405	*P. ult.*	30
field406	*P. ult.*	30
field407	*P. het.*	30
field408	*P. ult.*	30
field408	*P. sp. unk.*	11
field408	*P. sly.*	16
field408	*P.terr.*	3
field409	*P. ult.*	30
field410	*P. ult.*	18
field411	*P. ult.*	7
field411	*P. sp. unk.*	12
field412	*P. sp. unk.*	23
field413	*P. sp. unk.*	21
field414	*P. ult.*	16
field414	*P. sp. unk.*	30
field415	*P. vini.*	5
field415	*P. vio.*	2
field415	*P. deb.*	2
field416	*P. ult.*	30
field416	*P. sly.*	30

Table C7. Number of isolates for each species per field from 2011. (cont.)

field	species	# of isolates
field417	*P. ult.*	30
field418	*P. arr.*	30
field419	*P. ult.*	30
field419	*P. sly.*	23
field419	*P.terr.*	3
field420	*P. ult.*	27
field421	*P. ult.*	5
field421	*P. sp. unk.*	2
field422	*P. ult.*	6
field422	*P. sp. unk.*	9
field422	*P. arr.*	20
field423	*P. ult.*	11
field423	*P. sp. unk.*	23
field424	*P. ult.*	8
field424	*P. sp. unk.*	8
field424	*P. arr.*	20
field425	*P. ult.*	8
field425	*P. sp. unk.*	22

Table C8. Number of isolates for each species per field from 2012.

field	species	# of isolates
32	*P. rost.*	30
31	*P. rost.*	30
19	*P.inf.*	27
17	*P.inf.*	30
17	*P. het.*	21
19	*P. het.*	22
8	*P.att.*	30
9	*P. peri.*	30

Table C9. BLAST parameters for each species.

species	Query Length	Query Cov.	e-value	max. ident
P. ultimum	850	100	0	100
Pythium sp.	879	100	0	99
P. heterothallicum	775	100	0	99
P. irregulare	912	100	0	99
P. attrantheridium	836	100	0	99
P. sylvaticum	928	100	0	99
P. perplexum	891	100	0	99
P. arrhenomanes	846	100	0	100
P. kashmirense	867	95	0	99
P. minus	826	100	0	99
P. oopapillum	815	100	0	99
P. intermedium	793	100	0	99
P. lutartium	808	100	0	99
P.diclinum	845	99	0	100
P. coloratum	875	99	0	100
P. orthogonon	755	100	0	99
P. dissotocum	891	100	0	99
P. hypogynum	874	100	0	99
P. nunn	796	100	0	99
P. viniferum	879	96	0	99
P. violae	856	98	0	99
P. terrestris	914	100	0	99
P. aristosporum	865	100	0	99
P. debaryanum	913	100	0	99
P. rostratifingens	875	100	0	99
P. periilum	800	100	0	99

Table C10. Survival and Emergence data for each *Pythium* species and controls. RE represents the average number of seedlings to survive or emerge. The high and low confidence intervals are represented by CI.

Survival					**Emergence**			
Species	**RE**	**lower CI**	**higher CI**	**RE**	**RE**	**lower CI**	**Higher CI**	**RE**
*P.ultimum**	0	0	0	0	0	0	0	0
P.debaryanum	0.11	-0.14	0.36	0.11	0.11	-0.14	0.36	0.11
P.minus	0.22	-0.02	0.07	0.22	0.22	-0.02	0.07	0.22
P.irregulare	0.44	-0.58	1.46	0.44	0.44	-0.33	1.22	0.44
P.viniferum	0.44	-0.33	1.22	0.44	0.44	-0.58	1.46	0.44
P.sylvaticum	0.44	-0.33	1.22	0.44	0.55	-0.12	1.23	0.55
P.violae	0.55	-0.12	1.23	0.55	1	-0.38	2.38	1
P.lutarium	0.55	-0.12	1.23	0.55	1.33	-0.15	2.82	1.33
P.kashmirense	0.77	-0.042	1.97	0.77	1.55	0.77	2.33	1.55
P.heterothallicum	1	-0.38	2.38	1	1.77	0.11	3.44	1.77
P.intermedium	1.33	-0.15	2.82	1.33	1.88	-0.19	3.97	1.88
P.periilum	1.44	0.28	2.6	1.44	3.22	2.33	4.12	3.22
P.perplexan	1.55	0.77	2.33	1.55	3.44	1.76	5.12	3.44
P.diclinum	1.77	0.11	3.44	1.77	3.44	2.89	4.44	3.44
P.hypogenum	1.88	-0.19	3.97	1.88	4.22	2.6	5.84	4.22
P.terrestris	3.22	2.02	4.42	3.22	4.22	2.67	6.21	4.22
P.attrantheridium	3.22	2.33	4.12	3.22	4.44	2.67	6.21	4.44
P.dissotocum	3.44	2.89	4.44	3.44	4.77	3.4	6.15	4.77
P.sp. HQ643777.1	3.44	1.76	5.12	3.44	5.12	4.45	5.8	5.12
P.inflatum	4.22	2.67	6.21	4.22	7.11	5.37	8.84	7.11
P.oopapillum	4.77	3.4	6.15	4.77	7.44	6.28	8.6	7.44
P.orthogonon	7.77	6.7	8.85	7.77	7.77	6.7	8.85	7.77
P.nunn	8.11	7.13	9.1	8.11	8.11	7.13	9.1	8.11
P.rostratifingens	8.44	7.66	9.22	8.44	8.44	7.66	9.22	8.44
Negative Control	9.5	9.5	9.5	9.5	9.5	9.5	9.5	9.5

Table C11. Shannon and Evenness Index calculations.

Shannon Index and E_5 Index

Species	Abundance (# of isolates)	Pi	Pi*(ln(Pi))	Pi^2
P. ultimum	543	0.221181	-0.33371	0.111364
P. sp. HQ643777.1	426	0.173523	-0.30392	0.092365
P.heterothallicum	307	0.125051	-0.25999	0.067592
P. irregulare	240	0.09776	-0.22732	0.051672
P. attrantheridium	180	0.07332	-0.19158	0.036703
P. sylvaticum	164	0.066802	-0.18077	0.032677
P. perplexum	126	0.051324	-0.15241	0.023229
P. arrhenomanes	70	0.028513	-0.10143	0.010288
P. kashmirense	92	0.037475	-0.12307	0.015146
P. minus	50	0.020367	-0.07931	0.006289
P. oopapillum	57	0.023218	-0.08737	0.007633
P. intermedium	30	0.01222	-0.05383	0.002897
P. lutarium	44	0.017923	-0.07208	0.005196
P. diclinum	20	0.008147	-0.03919	0.001536
P. coloratum	19	0.007739	-0.03762	0.001415
P. orthogonon	17	0.006925	-0.03444	0.001186
P. dissotocum	15	0.00611	-0.03115	0.00097
P. nunn	9	0.003666	-0.02056	0.000423
P. viniferum	8	0.003259	-0.01866	0.000348
P. violae	7	0.002851	-0.01671	0.000279
P. terrestris	6	0.002444	-0.0147	0.000216
P. aristodporum	6	0.002444	-0.0147	0.000216
P. debaryanum	5	0.002037	-0.01262	0.000159
P. hypogynum	14	0.005703	-0.02947	0.000868

Total	2455
Natural log of sample size	7.80588204
	2455
SW index	2.43656843
$e^{H'}=N_1$	11.4337376
Sum of Pi^2	0.012063282
G=1/Sum of Pi^2	8.28961828
$E_5=G-1/N_1-1$	0.69865838

Table C12. Spearman correlation for species diversity and soil properties.

	species	CEC	P	K	pH	EC	OM	Zn	Fe	Cu	CCE	Sand	Silt	Clay
species	1	-0.2961	-0.069	-0.0647	0.10529	-0.0938	-0.2095	-0.1806	-0.0059	-0.1332	0.1892	0.05923	-0.0636	-0.1198
		0.0057	0.0007	<.0001	<.0001	<.0001	<.0001	<.0001	0.7697	<.0001	<.0001	<.0001	0.0017	<.0001
CEC	-0.29609	1	0.30095	0.33979	0.16464	0.44762	0.4043	-0.0597	-0.1709	0.55573	0.23712	-0.4247	-0.0682	0.46345
	0.0057		<.0001	<.0001	<.0001	<.0001	<.0001	0.0032	<.0001	<.0001	<.0001	<.0001	0.0008	<.0001
P	-0.06899	0.30095	1	0.09132	-0.1653	0.23926	0.14443	0.22777	0.07186	0.12857	-0.067	-0.144	-0.1144	0.21638
	0.0007	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	0.0004	<.0001	0.0009	<.0001	<.0001	<.0001
K	-0.06474	0.33979	0.09132	1	0.01204	0.23437	0.41599	0.02404	0.15906	0.6067	0.09543	-0.3469	0.05523	0.33879
	<.0001	<.0001	<.0001		0.5528	<.0001	<.0001	0.236	<.0001	<.0001	<.0001	<.0001	0.0065	<.0001
pH	0.10529	0.16464	-0.1653	0.01204	1	0.56417	0.08099	-0.5969	-0.7425	0.20681	0.8277	-0.0265	-0.0731	0.13919
	<.0001	<.0001	<.0001	0.5528		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.1922	0.0003	<.0001
EC	-0.09376	0.44762	0.23926	0.23437	0.56417	1	0.33779	-0.4276	-0.4886	0.55786	0.57723	-0.3798	-0.0156	0.46736
	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.4434	<.0001
OM	-0.20951	0.4043	0.14443	0.41599	0.08099	0.33779	1	-0.056	-0.1289	0.53979	0.12664	-0.3683	0.02026	0.44032
	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.0058	<.0001	<.0001	<.0001	<.0001	0.318	<.0001
Zn	-0.18058	-0.0597	0.22777	0.02404	-0.5969	-0.4276	-0.056	1	0.61373	0.03093	-0.4318	-0.035	0.07737	-0.0905
	<.0001	0.0032	<.0001	0.236	<.0001	<.0001	0.0058		<.0001	0.1274	<.0001	0.0848	0.0001	<.0001
Fe	-0.00594	-0.1709	0.07186	0.15906	-0.7425	-0.4886	-0.1289	0.61373	1	0.04888	-0.6147	-0.0507	0.17287	-0.1044
	0.7697	<.0001	0.0004	<.0001	<.0001	<.0001	<.0001	<.0001		0.0159	<.0001	0.0124	<.0001	<.0001
Cu	-0.13324	0.55573	0.12857	0.6067	0.20681	0.55786	0.53979	0.03093	0.04888	1	0.30531	-0.5596	0.03725	0.59189
	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.1274	0.0159		<.0001	<.0001	0.0663	<.0001
CCE	0.1892	0.23712	-0.067	0.09543	0.8277	0.57723	0.12664	-0.4318	-0.6147	0.30531	1	-0.133	-0.0195	0.18418
	<.0001	<.0001	0.0009	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	0.3379	<.0001

Table C12. Spearman correlation for species diversity and soil properties. (cont.)

	species	CEC	P	K	pH	EC	OM	Zn	Fe	Cu	CCE	Sand	Silt	Clay
Sand	0.05923 <.0001	-0.4247 <.0001	-0.144 <.0001	-0.3469 <.0001	-0.0265 0.1922	-0.3798 <.0001	-0.3683 <.0001	-0.035 0.0848	-0.0507 0.0124	-0.5596 <.0001	-0.133 <.0001	1	-0.545 <.0001	-0.6373 <.0001
Silt	-0.06355 0.0017	-0.0682 0.0008	-0.1144 <.0001	0.05523 0.0065	-0.0731 0.0003	-0.0156 0.4434	0.02026 0.318	0.07737 0.0001	0.17287 <.0001	0.03725 0.0663	-0.0195 0.3379	-0.545 <.0001	1	-0.1604 <.0001
Clay	-0.1198 <.0001	0.46345 <.0001	0.21638 <.0001	0.33879 <.0001	0.13919 <.0001	0.46736 <.0001	0.44032 <.0001	-0.0905 <.0001	-0.1044 <.0001	0.59189 <.0001	0.18418 <.0001	-0.6373 <.0001	-0.1604 <.0001	1

Table C13. Pearson correlation for the soil properties

	CEC	P	K	pH	EC	OM	Zn	Fe	Cu
CEC	1	0.16063 0.1444	0.28209 0.0093	0.35019 0.0011	0.32658 0.0024	0.1914 0.0812	-0.08883 0.4216	-0.3351 0.0018	0.51654 <.0001
P	0.16063 0.1444	1	0.14177 0.1983	-0.03476 0.7536	0.29892 0.0057	0.00143 0.9897	0.14745 0.1807	-0.00891 0.9359	0.15256 0.1659
K	0.28209 0.0093	0.14177 0.1983	1	0.06862 0.5351	0.1441 0.191	0.36161 0.0007	0.04642 0.675	0.02124 0.8479	0.55923 <.0001
pH	0.35019 0.0011	-0.03476 0.7536	0.06862 0.5351	1	0.48771 <.0001	0.16897 0.1244	-0.54282 <.0001	-0.88959 <.0001	0.2079 0.0577
EC	0.32658 0.0024	0.29892 0.0057	0.1441 0.191	0.48771 <.0001	1	0.20934 0.056	-0.39574 0.0002	-0.47747 <.0001	-0.41707 <.0001
OM	0.1914 0.0812	0.00143 0.9897	0.36161 0.0007	0.16897 0.1244	0.20934 0.056	1	-0.04461 0.687	-0.11511 0.2971	-0.41599 <.0001
Zn	-0.08883 0.4216	-0.14745 0.1807	-0.04642 0.675	-0.54282 <.0001	-0.39574 0.0002	-0.04461 0.687	1	-0.50711 <.0001	-0.05898 0.5941
Fe	-0.3351 0.0018	-0.00891 0.9359	-0.02124 0.8479	-0.88959 <.0001	-0.47747 <.0001	-0.11511 0.2971	-0.50711 <.0001	1	-0.10458 0.3438
Cu	0.51654 <.0001	0.15256 0.1659	0.55923 <.0001	0.2079 0.0577	0.41707 <.0001	0.41599 <.0001	-0.05898 0.5941	-0.10458 0.3438	1
CCE	0.25784 0.0179	-0.08859 0.4229	-0.07649 0.4892	-0.45208 <.0001	-0.32017 0.003	-0.05088 0.6458	-0.16251 0.1397	-0.31077 0.004	-0.24847 0.0227
Sand	-0.37437 0.0005	-0.08364 0.4494	-0.3147 0.0036	-0.03984 0.719	-0.28182 0.0094	-0.34922 0.0011	-0.02563 0.817	-0.00445 0.9679	-0.45029 <.0001
Silt	-0.11313 0.3055	-0.0712 0.5198	0.0332 0.7643	-0.07 0.5269	0.00202 0.9854	0.03261 0.7684	-0.10709 0.3323	-0.15476 0.1598	-0.08148 0.4612
Clay	-0.55693 <.0001	-0.10292 0.3515	-0.30697 0.0045	-0.14089 0.2011	-0.2777 0.0105	-0.31519 0.0035	-0.08199 0.4584	-0.15744 0.1526	-0.55186 <.0001

Table C13. Pearson correlation for the soil properties. (cont.)

	CCE	Sand	Silt	Clay
CEC	0.25784 0.0179	-0.37437 0.0005	-0.11313 0.3055	0.55693 <.0001
P	-0.08859 0.4229	-0.08364 0.4494	-0.0712 0.5198	0.10292 0.3515
K	0.07649 0.4892	-0.3147 0.0036	0.0332 0.7643	0.30697 0.0045
pH	0.45208 <.0001	-0.03984 0.719	-0.07 0.5269	0.14089 0.2011
EC	0.32017 0.003	-0.28182 0.0094	-0.00202 0.9854	0.2777 0.0105
OM	0.05088 0.6458	-0.34922 0.0011	-0.03261 0.7684	0.31519 0.0035
Zn	-0.16251 0.1397	-0.02563 0.817	-0.10709 0.3323	-0.08199 0.4584
Fe	-0.31077 0.004	-0.00445 0.9679	-0.15476 0.1598	-0.15744 0.1526
Cu	0.24847 0.0227	-0.45029 <.0001	-0.08148 0.4612	0.55186 <.0001
CCE	1	-0.17067 0.1206	-0.14978 0.1739	0.12773 0.2469
Sand	-0.17067 0.1206	1	-0.57158 <.0001	-0.5271 <.0001
Silt	-0.14978 0.1739	-0.57158 <.0001	1	-0.3257 0.0025
Clay	0.12773 0.2469	-0.5271 <.0001	-0.3257 0.0025	1

Table C14. Soil properties and presence/absence data for *Pythium* groups one, two, and three.

Field	CEC	P	K	pH	EC	OM	Zn	Fe	Cu	CCE	Sand	Silt	Clay	Grp 1	Grp 2	Grp 3
101	31.67	33	360	7.7	1.3	3.3	1.13	12.2	0.88	8.1	6.6	74.4	19	1	0	0
102	46.97	84	750	7.5	2.8	5.9	1.35	18.8	1.65	8.9	7.1	51	41.9	0	1	0
103	34.95	46	450	7.6	0.97	3.9	4.31	19.9	1.21	13.8	5.9	65.8	28.3	1	0	0
104	30.22	24	315	7.9	1	4.1	0.84	15.6	1.69	0	19.5	61.5	19	0	1	0
105	44.05	17	475	7.7	2.1	5.8	1.3	8.6	1.54	38.2	7.9	87	5.1	0	0	1
106	14.20	42	450	7.6	2.15	5.9	1.21	11.9	1.9	1.4	26.8	24.2	49.1	0	0	1
107	28.40	14	480	7.7	0.89	5.8	1.22	11.9	1.05	10.1	19.7	64.9	15.4	0	1	0
108	38.59	20	155	8	0.26	1.2	2.02	7.8	0.28	9.7	51	47.7	1.3	0	1	0
109	41.14	54	195	8.1	1.3	2.6	0.89	6.9	0.67	17.1	20.2	69.6	10.2	0	1	0
110	44.78	45	230	7.9	1.7	4	0.25	11.3	1.27	7.7	18.3	62.7	19	0	1	0
111	35.68	93	315	7.9	1.6	3.6	1.75	11.9	1.36	21.5	5.8	73.7	20.6	0	0	1
112	26.94	10	270	7.6	0.54	3.4	1.59	9.8	1.4	10.1	18.6	70	11.4	0	1	0
113	27.67	33	415	6.9	1.3	5.2	1.04	29.9	1.98	1.6	2	48.5	49.5	0	0	1
114	40.41	21	350	7.8	1	5.7	1.76	16	1.82	19.5	19.6	43.6	36.8	0	0	1
115	49.15	9	375	7.8	0.9	5.8	2.25	11.9	1.26	0.4	16.1	52.2	31.7	0	1	0
116	41.30	57	460	7.9	0.82	4.5	1.31	13.8	1.47	11	5.2	58.7	36.1	0	1	0
117	51.08	15	445	7.9	2.2	3.8	0.95	17	1.53	25.6	22.9	38.4	38.7	0	1	0
118	54.34	12	413	7.9	0.83	4.4	1.35	11.2	1.33	21.5	15.6	45	93.4	0	0	1
119	39.67	19	395	8	0.8	4.6	1.65	11.2	1.4	39.4	10.3	51.1	38.7	0	0	1
121	42.39	36	270	6	0.34	2.7	1.39	36.5	0.76	0	11.9	34.1	54.1	0	1	0
122	51.08	61	230	7.1	0.99	4.8	3.25	12.5	0.96	0.8	10.4	45.7	43.9	0	1	0
123	54.34	40	220	6.1	0.31	2.9	2.6	33	0.57	0	8.4	37.4	54.3	0	1	0
124	39.67	44	205	7.7	2.6	6.3	0.8	7.5	0.89	3.2	17	42.4	40.6	0	0	1
125	42.39	21	245	7.8	1	5.7	0.92	16	1.82	2.7	26.8	24.2	49.1	0	1	0
201	14.67	20	170	5.7	0.18	2.1	2.32	57.5	0.43	0	39.3	58.2	2.5	0	0	1
202	33.69	22	280	6.2	0.32	4.8	1.37	44	0.77	0	10.3	73.3	16.5	0	1	0
203	30.43	31	330	5.9	0.39	5.1	2.07	48	1.19	0	6	64.8	29.2	1	0	0
204	26.08	11	240	6.6	0.37	4.7	1.97	36	0.83	0	23.4	57.6	19	0	0	0
205	22.17	15	267	6.4	0.35	4.6	1.45	40	0.89	0	14.6	57.1	28.3	0	0	1
206	25.54	10	230	5.4	0.39	6	2.44	39.5	0.96	0	13.7	69.9	16.5	0	1	0
207	20.65	19	370	6	0.26	5.2	4.2	59	0.81	0	20.8	60.2	19	0	0	1
208	23.37	24	310	5.8	0.28	4.3	2.32	54	0.77	0	15.9	70.2	13.9	0	0	1
209	23.91	6	390	7.7	0.44	3.2	0.36	9.4	0.55	0.4	20.4	64.2	15.4	1	0	1
210	22.82	22	330	5.7	0.28	3.6	0.8	52.5	0.58	0	31.8	51.8	16.5	0	0	0
211	26.63	13	420	5.9	0.32	4.1	1.32	42.5	0.97	0	20	53.2	26.6	0	1	0
301	13.04	27	160	7.6	0.35	3.9	0.75	8.1	0.51	2.2	28.4	56.6	15	0	0	1
302	25.00	28	230	7.8	0.98	5	0.75	7.7	0.79	5.7	21.8	54.2	24.1	0	0	1
303	26.63	17	320	7.5	0.38	5	0.67	9.6	0.56	0.7	28.6	48.9	22.5	0	0	1

Table C14. Soil properties and presence/absence data for *Pythium* groups one, two, and three (cont.)

Field	CEC	P	K	pH	EC	OM	Zn	Fe	Cu	CCE	Sand	Silt	Clay	Grp 1	Grp 2	Grp 3
304	32.06	36	350	7.6	0.42	6.1	2.5	9.9	1.22	1	28.4	52.6	19	0	1	0
305	28.61	42	300	5.4	0.2	6.1	3.55	80	1.23	0	23.4	57.6	19	0	1	0
306	34.51	24	680	7.3	0.32	4.8	0.91	40	0.64	0.6	32	52.6	15.4	0	1	0
307	33.65	42	205	6.7	0.2	4.4	3.75	26	0.51	0	24.7	61.4	13.9	0	1	0
308	25.90	13	290	7.4	0.22	4.9	2.45	52	0.88	0.8	7.9	87	5.1	0	0	1
309	26.63	104	365	7.6	2.18	4.9	1.03	9.3	0.94	0.8	25.5	47.9	26.6	1	0	1
310	13.56	46	700	5.9	0.39	6.3	2.8	63	0.91	0	14.1	69.4	16.5	0	0	0
311	45.10	58	380	7.1	0.56	6.2	1.35	12	0.75	0.6	33.2	53.9	12.8	0	1	0
312	11.41	9	490	7.8	0.53	5.2	0.61	7.6	0.82	1.9	17.3	59.6	23.1	0	1	0
314	21.19	45	485	5.5	0.19	4.6	2.7	77	0.59	0	33.9	54.7	11.4	0	0	1
315	20.65	99	335	7.7	1.71	2.9	0.76	6.1	0.51	1	43	43	13.9	0	1	0
316	22.28	19	290	7.6	1.76	3.6	0.71	4.6	0.42	0.7	34.6	51.5	13.9	0	1	0
317	15.76	34	270	7.7	0.59	4	0.7	5.8	0.4	0.89	48.6	45.1	6.3	0	1	0
318	21.74	19	265	7.6	0.34	4.3	0.81	6.4	0.32	0.4	43.1	48.1	8.9	0	1	0
319	26.08	29	205	7.9	0.7	5.4	0.96	10	0.79	1.5	38.9	48.3	12.8	1	1	0
320	20.11	53	270	6	0.32	4.4	1.69	49	0.87	0	27.8	63.3	8.9	1	0	0
321	49.99	36	190	7.6	0.33	2.3	1.81	5.8	0.53	1.4	24.8	51.2	24.1	0	0	0
322	14.67	20	170	5.7	0.18	2.1	2.32	58	0.43	0	55.1	43.6	1.3	0	0	1
323	27.71	23	445	7.9	2.2	3.8	0.95	17	1.53	25.6	27.1	59	13.9	1	0	1
324	28.45	42	300	5.4	0.2	6.1	3.55	80	1.23	0	27.3	41.8	30.9	0	0	0
325	27.11	30	345	5.9	0.17	4.2	1.75	70	0.78	0	2.2	77.1	20.6	1	1	0
401	44.56	24	680	7.4	0.72	7.1	1.39	8.1	1.86	0.5	2.4	51.2	46.5	0	0	0
402	33.15	44	205	7.7	2.6	6.3	0.8	7.5	0.89	3.2	8.1	65.3	26.6	0	0	1
403	47.82	30	460	7.3	0.68	6.1	1.23	14	1.46	0.6	2.8	50.2	47	0	1	0
404	30.97	70	110	7.9	0.7	1.9	0.48	6.8	0.47	1.2	54.7	35.4	10	1	1	0
405	46.19	38	720	7.5	0.75	7.7	1.81	7.5	1.85	0.3	17.7	35.3	47	1	0	0
406	46.73	42	450	7.6	2.15	5.9	1.21	12	1.9	1.4	12.5	33.2	54.3	0	0	0
407	41.30	27	400	7.6	0.75	6.2	0.99	9.4	0.95	0.2	4.7	61.1	34.3	0	0	1
408	35.86	15	270	7.6	2.1	4.7	0.72	7.7	0.62	1.7	11.9	63.1	25	1	1	0
409	47.82	31	415	7.6	2.68	5	0.69	8.8	1.77	0.6	2.6	59.5	37.9	1	0	0
410	39.67	43	700	7.4	0.85	6	2.64	13	1.31	2.1	14.9	56.8	28.3	0	0	0
411	28.26	28	240	6.2	1.74	4.1	1.75	31	0.77	0	5.4	73.1	21.6	0	1	0
412	26.08	45	370	6.2	0.95	4.7	4.85	35	1.11	0	6.6	74.4	19	0	1	0
413	33.15	44	205	7.7	2.6	6.3	0.8	7.5	0.89	3.2	0.8	75.2	24.1	0	1	0

Table C14. Soil properties and presence/absence data for *Pythium* groups one, two, and three (cont.)

Field	CEC	P	K	pH	EC	OM	Zn	Fe	Cu	CCE	Sand	Silt	Clay	Grp 1	Grp 2	Grp 3
414	18.48	41	410	6.4	0.52	3.3	3.85	34	0.55	0	37.2	51.4	11.4	1	1	0
415	13.59	51	250	5.3	0.39	2.4	3.05	43	0.5	0	47	43.1	10	0	0	0
416	19.56	36	270	6	0.34	2.7	1.39	37	0.76	0	48.5	38.7	12.8	1	1	0
417	33.69	61	230	7.1	0.99	4.8	3.25	13	0.96	0.8	15.8	62.7	21.6	0	0	0
418	24.45	40	220	6.1	0.31	2.9	2.6	33	0.57	0	51.5	37.1	11.4	0	1	0
419	18.48	22	100	8.1	0.91	12.3	0.71	5	0.45	20	21.5	50.2	28.3	1	1	0
420	17.93	22	250	7.4	0.19	3.9	3.2	9.5	0.41	0.7	56.3	33.7	10	1	0	0
421	41.30	10	310	7.7	1.69	5.4	0.88	10	1.29	1.3	63.5	27.6	8.9	0	0	0
422	51.08	37	505	7.6	0.87	6.4	1.39	16	1.78	1.2	61.9	29.3	8.9	0	1	0
423	54.34	90	650	7	0.95	8	2.7	20	1.7	0.6	1.4	63.6	35	0	1	0
424	39.67	24	480	7.7	0.93	5.3	0.79	14	1.64	2.2	2.2	77.1	20.6	0	1	0
425	42.39	100	465	5.8	1.45	4.9	2.1	57	2.35	0	3.4	49.6	47	0	1	0