Characterization, development of a field inoculation method, and fungicide sensitivity screening of the Pythium blight pathogen of snap bean (*Phaseolus vulgaris* L.)

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ABSTRACT

New Jersey, Georgia, and the Eastern Shore of Virginia (ESV) are important snap bean (*Phaseolus vulgaris* L.) growing regions, but profitability is threatened by Pythium blight. Causal agents of Pythium blight on snap bean were identified using morphological characterization and sequence analysis of the rDNA-internal transcribed spacer (ITS) regions of 100 isolates. Most isolates were *Pythium aphanidermatum* (Edson) Fitzp. (53%), and also included P*ythium deliense* Meurs (31%; all from Georgia), *Pythium ultimum* Trow (12%), *Pythium myriotylum* Drechsler (2%), *Pythium catenulatum* Matthews (1%), and unknown *Pythium* sp. (1%). To our knowledge, this is the first report of *P. deliense* in Georgia and on common bean and squash (*Cucurbita pepo* L.); as well as the first report of *P. catenulatum* on lima bean (*Phaseolus lunatus* L.) and in New Jersey. Fungicide labeling and cultivar selection for Pythium blight management is hindered by difficulties associated with conducting successful trials, because the disease occurs sporadically and clustered in the field. Three *P. aphanidermatum*-infested inoculum substrates were evaluated at three concentrations. The vermiculite/V8 juice (5:3 weight to volume) inoculum (10,000 ppg/0.3 m) consistently caused at least 50% disease in 3 field trials. Sensitivity of the Pythium blight pathogens was determined *in vitro* against five fungicides. Twenty-two *Pythium* isolates representing *P. aphanidermatum*, *P. deliense*, *P. ultimum*, and *P. myriotylum* were inoculated to media amended with each active ingredient at 0, 100 μg/ml, the concentration equivalent to the field labeled rate if applied on succulent beans at 187 L/ha, and the equivalent if applied at 374 L/ha. All isolates were completely sensitive (100% growth reduction, or GR) to all active ingredients at the labeled rates, except azoxystrobin. At 100 µg/ml azoxystrobin, one *P. deliense* isolate had 8.9% GR. All isolates had 100% GR to copper hydroxide at 100 µg/ml, and the lowest GR on mefenoxam-amended medium was 91.9%. At 100 µg/ml cyazofamid, all *P. deliense* isolates were completely sensitive and variation was observed in *P. aphanidermatum* isolates. At 100 µg/ml potassium phosphite, significant GR similarities were recorded within isolates of the same species, and less than 50% GR was observed in all *P. deliense* isolates.

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CHAPTER 1: INTRODUCTION AND OBJECTIVES

Snap bean production:

Snap bean (*Phaseolus vulgaris* L.) is a popular vegetable crop grown throughout the United States. In 2009, the National Agricultural Statistical Service (NASS) estimated 295,000 acres of the crop planted in the United States with a value of over 415 million dollars (NASS, 2010). Snap bean is an important crop on the Eastern Shore of Virginia (ESV) with approximately 5,500 acres planted in 2009. Of this acreage, 5,200 acres were harvested with a production value of 4.43 million dollars (NASS, 2010). The ESV is also home to C&E Farms in Cheriton, VA, which is one of the largest snap bean packing houses on the East Coast of the United States. C&E Farms processes approximately 1 million bushels (30 million pounds) of snap beans annually from Pennsylvania, Delaware, Maryland, Virginia and North Carolina (personal communication, Bob Colson, President C&E Farms).

The climate and soils of the temperate ESV are suitable for snap bean production. Both spring and fall crops are planted in the region, representing the majority of snap beans grown in Virginia. The spring crop is planted between the end of April to beginning of May, and depending of the cultivar’s days to maturity, harvest occurs sometime in July. The fall crop is planted in late July to August and harvested in October.

Pythium blight and other soilborne diseases of snap bean:

As the spring crop is reaching maturity, the ESV usually experiences sporadic periods of rainfall and warm temperatures. Under these environmental conditions, snap beans may be threatened by several soilborne pathogens. Of these pathogens, *Pythium* spp. can be particularly damaging by infecting aerial portions of the snap bean plant, causing a disease commonly known as Pythium blight or Pythium cottony leak. This disease on snap bean was first documented in 1931, after being observed in both Colorado and Rosslyn, Virginia in 1930 (Harter and Zaumeyer, 1931a). Damage from Pythium blight has been observed on the stems, leaves, and the bean pods, which is especially economically damaging (Fig. 1.1). In the field, disease development begins as a water-soaked lesion of stems near soil to the lower branches and pods, with eventual profuse mycelial growth over the dead cortex (Adegbola and Hagedorn, 1969; Drechsler, 1952; Harter and Zaumeyer, 1931b). Pythium blight generally occurs from the onset of flowering until harvest and is most destructive when disease onset occurs on mature pods just prior to harvest. Young, succulent tissues, like bean pods, are most susceptible to infection by *Pythium* spp., but infection may occur at any plant age under optimal growth conditions for the pathogen (Adegbola and Hagedorn, 1969). Pythium blight is a distinct disease from seed decay or damping-off diseases that occur on the seed or seedlings. Preemergence or postemergence damping-off affects the plant's crown and roots during the early stages of growth, causing the seedling to wilt and die, whereas Pythium blight affects the aerial plant parts during later growth stages (Pfender and Hagedorn, 2005). These diseases may sometimes be confused, because all can be caused by the same spp. of *Pythium*. The pathogen also causes an important post-harvest disease, known as "nesting", in which decaying beans are massed together by cottony mycelium when storage moisture and temperature are not controlled properly (Fig. 1.1; Harter and Whitney, 1927).

Pythium blight is one of three diseases commonly referred to as "white mold" by snap bean producers. The other two diseases are Sclerotinia blight, caused by *Sclerotinia* *sclerotiorum*, and Southern blight, caused by *Sclerotium rolfsii* (Fig. 1.2). Pythium blight is distinguished from other “white mold” pathogens by the presence of white fluffy mycelial growth without the production of sclerotia. Additionally, these diseases are often confused because they are favored by moist and/or humid conditions and all produce white, mycelial signs on aboveground parts of the snap bean plant. Southern blight occurs during periods of high relative humidity, coupled with an optimum temperature of 30°C (Abawi, 1989). Sclerotinia blight also thrives during humid conditions, but differs from southern blight by occurring within a cooler temperature range of 20-25°C (Steadman, 1983). Correct diagnosis of the “white mold” pathogens is important because effective chemical controls differ for each disease. *Pythium* spp. are typically controlled by pesticides specific for oomycetes. Examples of such oomyceticides are ones with the active ingredients cyazofamid, propamicarb, metalaxyl and mefenoxam. *Sclerotium rolfsii* can be controlled by products containing the active ingredients azoxystrobin, pyraclostrobin, flutalonil, and tebuconazole. Finally, *Sclerotinia* spp. are controlled by fungicides containing fluazinam, thiophanate-methyl, cyprodonil, and boscalid.

Geographical distribution and host range of Pythium blight:

Pythium blight occurs in most areas where snap beans are grown in the United States, including the Southeast and as far north as Maryland and Delaware (Drechsler, 1952). The disease is particularly problematic on the Delmarva Peninsula and in Georgia (D. B. Langston, personal communication). In 2009, Georgia ranked third in the United States in fresh market snap bean production with 16,000 harvested acres accounting for 37.73 million dollars (NASS, 2010). In addition to snap beans, intensive production of other vegetable crops occurs in these regions. The Pythium blight pathogen(s) possess(es) a wide host range, such as, scarlet runner bean (*Phaseolus coccineus* L.), cucumbers (*Cucumis sativus* L.), eggplant (*Solanum melongena* L.), potato (*Solanum tuberosum* L.), pumpkin (*Cucurbita maxima* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai var. *lanatus*), melon (*Cucumis melo* L.), and other solanaceous and cucurbit crops (Aoki et al., 2007; Blodgett, 1945; Drechsler, 1925; Dreschler, 1926; Sitterly and Keinath, 1996). This extensive host range of the pathogen on vegetable crops contributes to population buildup and the resulting severity of Pythium blight on East Coast vegetable farms. In greenhouse production, ornamental plants are often tightly spaced, and well-fertilized and watered. These conditions create high relative humidity within the structures that are conducive for Pythium blight (Braun, 1924; Cox, 1969). Additionally, Pythium blight, or cottony blight, is problematic in turfgrass foliage during periods of excessive soil moisture (Hendrix et al., 1970; Moore et al., 1963).

Epidemiology:

Pythium spp. survive as saprophytes in the soil, but because they are not strong competitors, their saprophytic growth is commonly restricted (Barton, 1961). This growth generally occurs under circumstances where other organisms have greatly reduced activity; such as, environments with high moisture and poor gas exchange (Griffin, 1963). Along with high levels of moisture, Pythium blight caused by *P. aphanidermatum* is generally observed during periods of warmer temperatures (optimal 28-35°C) (Drechsler, 1952; Harter and Zaumeyer, 1931b). Under controlled greenhouse conditions, optimal disease development occurred when inoculated plants were incubated at 95-100% relative humidity (RH) and at temperatures of 24-28°C (Adegdola and Hagedorn, 1970). Harter and Zaumeyer (1931b) compared the occurrence of Pythium blight in both Colorado and Virginia in 1930 and the role of high RH and temperature. They stated that hygrothermograph readings in Virginia were near saturation for a prolonged period of time before symptoms appeared, and similar readings were recorded near the soil line in the flood-irrigated field in Colorado. They also noted that disease symptoms were most severe when the daytime temperatures ranged between 32 to 41°C. In the presence of a susceptible host and adequate pathogen populations in the soil, optimal environmental conditions are necessary for the development of Pythium blight.

Etiology:

Oospores are the pathogen's survival structure, whereas zoospores usually serve as the primary inoculum source. Either oospores or zoosporangia can liberate zoospores when environmental conditions are optimal (Kim, 1972). Infection by the Pythium blight pathogen usually occurs during a rain or irrigation event when water splashes zoospores as well as other propagules (mycelial fragments, oospores, or sporangia) from the soil surface onto aerial parts of the plant (Kim, 1972). Kim (1972) found that among the propagule types, zoospores are the most effective primary inoculum source (Kim, 1972). Infection of aboveground bean tissue by *P. aphanidermatum* zoospores was shown to occur within 30 minutes after contact with host tissue at 25°C, and symptoms appeared after 24 to 72 hours of exposure, depending on environmental conditions (Adegbola and Hagedorn, 1969; Kim et al., 1974). Little is known about Pythium blight development on snap bean under field conditions, and gaining this knowledge is necessary to achieve better disease control.

Several species have been associated with Pythium blight symptomology in snap beans, including *P. aphanidermatum, P. ultimum, P. debaryanum, P. dissoctocum,* and *P. myriotylum* (Adegbola and Hagedorn, 1969; Dominiak and Damicone, 2006; Drechsler, 1952; Harter and

Zaumyer, 1931a). Dominiak and Damicone (2006) also collected multiple isolates of *Phytophthora drechsleri* from snap bean pods showing water soaking and/or white, cottony growth. Various *Pythium* spp. reported as the causal agent may be the result of slight differences in environmental conditions within geographical regions, since species vary in their optimal growth temperature range (van der Plaats-Niterink, 1981). Drechsler (1952) isolated *P. ultimum* from diseased plants from an irrigated snap bean field in Delaware. Adegbola and Hagedorn (1969) isolated *P. ultimum* and *P. debaryanum* from symptomatic snap beans in Wisconsin. *P. ultimum* thrives in moderately warm regions, such as Wisconsin and Delaware, growing optimally between 15-25°C (Adegbola and Hagedorn, 1969). However, the species predominately reported as the Pythium blight casual agent is *P. aphanidermatum* (Edson) Fitzp., which thrives during periods of warm temperatures (28 to 32°C) and high moisture and possesses a wide host range (Adegbola and Hagedorn, 1969; van der Plaats-Niterink, 1981). In the warmer states of Hawaii and California, *P. aphanidermatum* (syn. *P. butleri*) was isolated from symptomatic beans (Adegbola and Hagedorn, 1969). *P. aphanidermatum* may also cause disease in northeastern states, such as Delaware, during the warmer summer months (Drechsler, 1952). Confusion about the causal agent may also be the result of saphrophytic or endophytic species present on symptomatic plants, or the occurrence of a synergist pathogen complex. Therefore, isolation of *Pythium* spp. from symptomatic tissue may recover a highly virulent pathogen, a weak pathogen, or a saprophytic isolate associated with a plant with comprised vigor due to other factors (Csinos, 1979; Csinos and Hendrix, 1978). Additional research is necessary to establish the exact causal agent(s) of Pythium blight on snap beans and to explore the possible variation in strains collected from different hosts and locations.

Characterization of *Pythium* species

Morphology

Several techniques can be employed to identify *Pythium* isolates to the species level. Traditionally, researchers utilized a number of morphological criteria, including the number and attachment of antheridia (monoclinous or diclinous), the surface ornamentation of the oogonial wall (ornamented or smooth), the size of the oospore, the degree that the oospore fills the oogonium (plerotic or aplerotic), the sporangial shape (lobulate, filamentous, or spherical), and the presence or absence of hyphal swellings. Commonly used identification keys provided by van der Plaats-Niterink (1981) and Waterhouse (1968) outline the utilization of these criteria. However, many diagnosticians do not put forth the effort to identify *Pythium* isolates to species due to the time and expertise needed to produce and examine the structures required (van der Plaats-Niterink, 1981; Dick, 1990). Many oomycetes, including *Pythium* spp., exhibit overlapping similarities and tremendous intraspecific variation and plasticity in morphology, both in culture and in the field making species identification using morphological characteristics difficult (Francis and St. Clair, 1997; Martin, 2000).

Molecular techniques

Since the 1980s, molecular techniques have increasingly improved the ability to identify fungal isolates to species. Several tools have been used in recent years to identify *Pythium* spp., including restriction fragment length polymorphisms (RFLP) of genomic and mitochondrial DNA (Martin and Kistler, 1990; Harvey et al., 2001), RFLPs of the ITS regions and random amplified polymorphic DNA (RAPDs) (Herrero and Klemsdal, 1998; Kageyama et al., 1998), amplified fragment length polymorphism (AFLP) fingerprinting (Garzon et al., 2005), isozyme analysis (Mugnier, 1995), single-stand conformational polymorphism analysis of the internal transcribed spacer 1 (Kong et al., 2004), and the development of species-specific primers for the detection of multiple species (Arif et al., 2010; Wang et al., 2003). The ITS regions 1 and 2 of ribosomal DNA are most commonly used to separate *Pythium* spp. (Al-Sa'di et al., 2007; Bailey et al., 2002; Klassen et al., 1996; Moorman et al., 2002). The sequence analysis of this region is so reliable because the rate of accumulation of mutations often correlates with the rate of speciation (Bruns et al., 1991; Chen et al., 1992; Kageyama et al., 1997). In recent years, ITS sequence analysis is widely performed by researchers, and this sequence information is freely accessible through realms such as NCBI's GenBank database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/genbank/). This abundant information provides a powerful tool for performing comparative sequence alignment between unknown *Pythium* isolates and previously identified ones (Cooke et al., 2000). However, some *Pythium* spp. are so closely related that they cannot be distinguished using the ITS region. For example, *P. aphanidermatum* and *P. deliense* only differ in ITS sequence by 2-3%, and this similarity may be explained by a recent speciation event (Levesque and de Cock, 2004). Between the two species, certain morphological characteristics differ slightly: the average oospore diameter, the shape of the oogonial stalk, and sporangial shape and tendency to form in bunches (van der Plaats-Niterink, 1981). Although *P. aphanidermatum* and *P. deliense* are similar in host range, temperature optimum, and ITS sequence, they were considered distinct species based on morphological differences (Chen et al., 1991; van der Plaats-Niterink, 1981). Thus, to reliably distinguish *P. aphanidermatum* and *P. deliense,* morphological characterization is necessary.

Accurate identification of the Pythium blight pathogen to species would greatly assist in selecting successful control strategies, and molecular techniques are an essential tool to achieve this goal. The pathogen populations causing this disease in a particular region must be determined, because different *Pythium* spp. vary in their sensitivity to different fungicides, particularly single-site inhibitors (Broders et al., 2007; Kato et al., 1990).

Management strategies:

Cultural practices

Snap bean producers rely primarily upon cultural practices to avoid significant yield losses due to Pythium blight. Damage caused by *Pythium* spp. can be avoided by planting into well-drained soils (Pfender and Hagedorn, 2005). In general, propagules (especially oospores) of *Pythium* spp. tend in increase in fields in continuous monoculture, which can pose a threat of serious crop losses (Hendrix and Campbell, 1973). Therefore, crop rotation is perhaps the most important cultural practice for reducing Pythium blight pressure; however, it is not an effective option when employed alone due to the pathogen’s wide host range. Cultivar selection can decrease disease incidence, especially in combination with fungicide applications (Damicone et al., 2008). These cultivars usually exhibit high bean set that keep susceptible pod tissue from touching the soil, where the inoculum source resides (personal communication, J. P. Damicone). Cropping histories of *Pythium*-susceptible vegetables in snap bean producing areas have led to high endemic levels of *Pythium* spp. in many regions. Alternative management strategies can be implemented after a better understanding of the etiology and epidemiology of Pythium blight on snap beans has been elucidated. Improved disease management tactics are necessary to achieve better disease control, higher yields and sustainable production levels on the ESV, Georgia, New Jersey and other vegetable producing regions.

Chemical control

In recent years, significant crop losses and even complete crop failures in important snap bean growing areas have occurred due to Pythium blight. Once *Pythium* spp. become established in the soil, oospores are produced, which can survive in soil for 8 months to 12 years (Hoppe, 1959, 1966; Lumsden and Ayers, 1975). These survival structures are difficult to eliminate except with soil fumigants, such as methyl bromide, chloropicirin, and metam sodium (Collins et al., 2004; Hendrix et al., 1970; Martin, 1999). However, management by fumigation is cost-prohibitive for most snap bean producers. In-season foliar fungicides are an economical control option; however, the only product currently possessing a Section 3 Federal label for Pythium blight control is Prophyt 6.7F (potassium phosphite; Helena Chemical Company, Collierville, TN). In one field trial, potassium phosphite was shown to effectively manage Pythium blight on snap bean caused by *P. aphanidermatum* (Rideout et al., 2010). In 2008, Ridomil Gold Copper 65WP (mefenoxam and copper hydroxide; Syngenta Crop Protection, Greensboro, NC), which is effective against many oomycetes, received a section 24(c) label for use on snap beans in Delaware, Georgia, Maryland, and Virginia (Rideout et al., 2010). Ranman 400SC (cyazofamid; FMC Corporation, Philadelphia, PA) is also an effective oomycete fungicide and has shown efficacy in previous field trials against snap bean Pythium blight (Rideout et al., 2010). Cyazofamid is currently being evaluated by USDA's IR-4 program and is expected to receive a label for use on PCL of snap bean in the near future (www.csrees.usda.gov/funding/rfas/minor_crop.html). Azoxystrobin (Quadris 2.08SC; Syngenta

Crop Protection, Greensboro, NC) is labeled for use on various snap bean diseases, but, is not specifically labeled for control of Pythium blight. In a field trial conducted by Rideout et al. (2010), Quadris applied at rates of 0.27 kg a.i./ha and at 0.18 kg a.i./ha in combination with ProPhyt 6.7F (2.35 kg a.i./ha) were effective at reducing disease levels. All of the mentioned fungicides have provided suppression of Pythium blight caused by *P. aphanidermatum* on snap bean in limited trials, but control of other Pythium blight causal agents has not yet been determined (Damicone and Trent, 2004; Rideout et al., 2010).

Pythium species sensitivity to fungicides

Understanding a pathogen's *in vitro* sensitivity to fungicides can be critical for constructing long-term disease and fungicide resistance management strategies. Since a fungicide's mode of action may involve preventative, curative, or vapor activity, knowledge of their direct affect on pathogen growth is necessary (Wong and Wilcox, 2001). Most fungicides are formulated to be stable under a wide range of environmental conditions, so when a product is properly applied according to the label and is still not efficacious, it may be due to pathogen insensitivity (Sukul and Spiteller, 2000). In some cases, this pathogen insensitivity may not be the result of resistance development, but due to inherent characteristics within related species (Broders et al., 2007; Kato et al., 1990). In previous studies, sensitivity levels were consistent within isolates of the same species and different from isolates of other species. For example, Broders et al. (2007) showed that species that produce globose sporangia or hyphal swellings are generally less sensitive to azoxystrobin than those producing filamentous sporangia**.** Cook et al. (2009) showed that exposure to phosphorous acid had a slight but significantly different effect *in vitro* on two *Pythium* spp. causing Pythium blight on turfgrass. *P. aphanidermatum* isolates demostrated EC_{50} values between 36 to 172 μg/mL and *P. myriotylum* isolates demonstated EC_{50} values ranging from 124 to 221 μg/mL.

Variations in fungicide sensitivity may also be the result of resistance development within species populations. In many cases, resistance to a fungicide originates from naturally occurring, rare mutations existing in a population before even being exposed to the fungicide (Dagget et al., 1993; Kousik and Keinath, 2008). Loss in sensitivity to fixed copper bactericides has been reported for bacterial spot and speck of tomato and pepper, but this observation has not been made for *Pythium* spp. (Cuppels and Elmhirst 1999; Marco and Stall, 1983). Similarly, development of insensitivity to azoxystrobin has been reported for several fungal pathogens, including *Pythium* spp. (Broders et al., 2007; Mavroeidi and Shaw, 2005; Vinelli and Dixon, 2002; Wong and Wilcox, 2002). Pathogens within the Peronosporales have been effectively controlled by phenylamide fungicides since their introduction in the late 1970s (Morton and Urech, 1988). Fungicides in this group target the organism's ribosomal RNA polymerases, and due to this specific mode of action there is a high risk for the development of insensitive pathogen populations (Brent and Hollomond, 1998). Isolates of *Pseudoperonospora cubensis* insensitive to the phenylamide metalaxyl were collected from cucumber just 2 years after its introduction, and insensitivity by a *Pythium* spp. was first reported on turfgrass in 1984 (Reuveni et al., 1980; Sanders, 1984). An isomer of metalaxyl, mefenoxam (Ridomil Gold; Syngenta Crop Protection, Greensboro, NC), has been used more extensively in recent years to manage foliar oomycete pathogens. Resistance to mefenoxam has been observed in *P. aphanidermatum* on poinsettia and geranium in greenhouse production (Moorman et al., 2002; Moorman and Kim, 2004). Insensitivity to mefenoxam by *Phytophthora capsici* in cucurbit and pepper isolates, and *Phytophthora erythroseptica* and *Pythium ultimum* in potato isolates has been observed (Lamour and Hausbeck, 2000; Parra and Ristiano, 2001; Lamour and Hausbeck, 2003; Taylor et al., 2002; Taylor et al., 2006). Isolates of *Phytophthora capsici* that were insensitive to cyazofamid were collected from watermelon in 2008 (Kousik and Keinath, 2008). Because of the presence of mefenoxam and cyazofamid insensitivity in closely related pathogen species, the screening of Pythium blight isolates for sensitivity to mefenoxam, cyazofamid, and additional efficacious agrochemicals is warranted.

Experimentation for determining effective management practices

Previous attempts have been made to evaluate the efficacy of fungicides, resistant cultivars and cultural practices against Pythium blight on snap bean, but determining the most effective materials and practices has been difficult due to inconsistent disease pressure in field trials (Damicone, 2004; Damicone and Trent, 2004; Rideout et al., 2008). The sporadic and clustered nature of this disease in the field has lead to inconsistent efficacy results and, in some cases, failed trials. Previous research has documented the variable nature of this pathosystem. Rideout et al. (2008) determined that fungicide applications did not reduce disease incidence or postharvest pod rot associated with Pythium spp. when compared to the nontreated control. In a study by Damicone and Trent (2004), applications of Reason 500SC (fenamidone; Bayer CropScience, RTP, NC), Ranman 400F, Ridomil Copper 70W, Kocide 2000DF (53.8% copper hydroxide; DuPont Crop Protection, Wilmington, DE), and Phostrol 6.7S (mono- and dibasic sodium, potassium and ammonium phosphites; NuFarm Americas, Inc., Burr Ridge, IL) reduced levels of Pythium blight. However, yields were not significantly impacted by disease. A repeat of this study conducted by Damicone (2004) determined no significant differences in disease ratings or yield between the nontreated control and fungicide treatments. The development of a reliable and repeatable field inoculation technique will aid in producing more consistent results. Subsequently, researchers and industry personnel will better understand which fungicides are most likely to suppress levels of Pythium blight in field settings.

Techniques have been developed for field inoculation of *Pythium* spp. causing disease in other cropping systems, which involve spreading a pathogen-infested substrate onto the soil surface or incorporating into the soil (Cummings, 2007; Feng et al., 1999; Yin and Hoy, 1998). Prior research has shown that a successful inoculation technique involves the encouragement of the pathogen's oospore resting structures within the substrate. Oospores are resilient structures that serve as *Pythium* spp. chief mechanism for survival over longer periods, since the mycelium of the pathogen quickly lyses as its food source is depleted (Stanghellini and Hancock, 1971). However, techniques that encourage primary mycelial production of oomycetes, on substrates such as rice grains, were successful in a greenhouse setting and only require 5-7 days of preparation (Holmes and Benson, 1994). High soil moisture levels and warm temperatures are also important factors to encourage Pythium blight pathogen infection. For techniques that produce oospores as its inoculum propagule, germination of oospores is highly dependent on a high water potential (at or near soil capacity) and a suitable temperature (Lumsden and Ayers, 1975; Stanghellini and Burr, 1973). Stanghellini and Burr (1973) also showed that high soil moisture increased nutrient availability necessary for *P. aphanidermatum* oospore germination. Techniques from past studies were only successful when experimental field trials were irrigated the day before and immediately following inoculation (Feng et al., 1999). Regardless of the inoculum substrate, adequate soil moisture is necessary for initial establishment of *Pythium* spp. in the soil, as well as its propagule production and ability to cause disease.

Objectives:

The primary goal of this research was to assemble an isolate collection representative of Pythium blight pathogen populations and determine the species of the causal agent(s) and their frequency. This information will aid in identifying the most efficacious fungicides for control use, as well as aid in predicting potential disease epidemics according to weather conditions. *Pythium* spp. differ in both sensitivity to different fungicides and in their optimal temperature for growth and ability to cause disease. This research also focused on identifying loss of sensitivity, if any, of the Pythium blight pathogen(s) to fungicides commonly used in production of snap beans and other vegetables. A reliable inoculation technique in field experimental trials will help researchers identify efficacious fungicides, as well as tolerant or resistant snap bean cultivars that will alleviate damage caused by Pythium blight.

The research objectives were:

1. Morphological and molecular identification of *Pythium* spp. associated with Pythium blight symptoms on different hosts from Virginia, Georgia, and New Jersey.
2. To develop a repeatable field inoculation technique of the Pythium blight pathogen on snap bean.
3. To screen *Pythium* isolates from different locations and hosts *in vitro* for sensitivity to oomycete fungicides, with the active ingredients azoxystrobin, cyazofamid, mefenoxam, copper hydroxide, and potassium phosphite.

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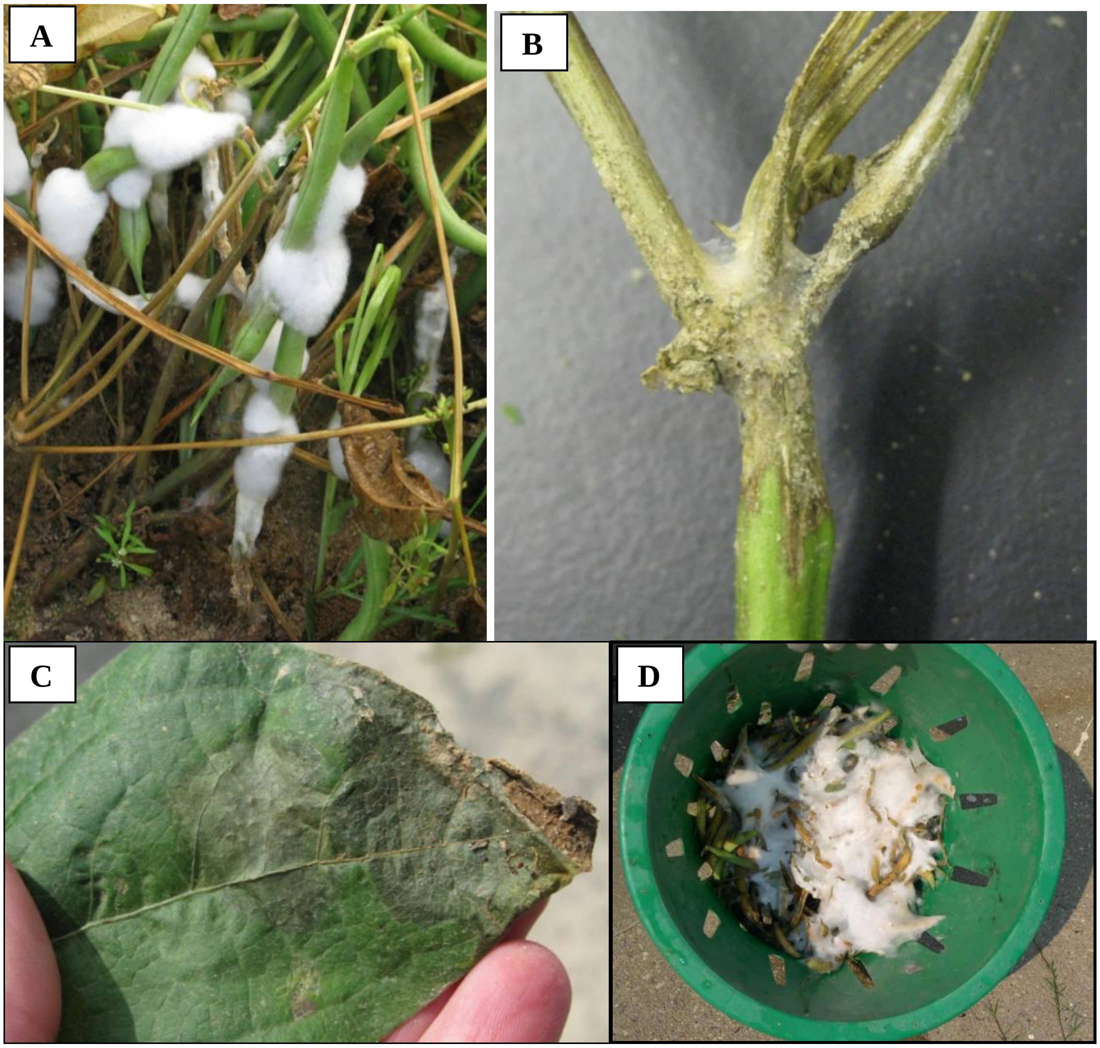

Fig. 1.1. Pythium blight symptoms and signs commonly develop in the field as **A)** a water-soaked lesion and cottony mycelial growth on the snap bean pods, **B)** at the bottom branch nodes and stem, and **C)** on the leaf. **D)** It can manifest as a post-harvest pathogen and cause "nesting" in snap beans.

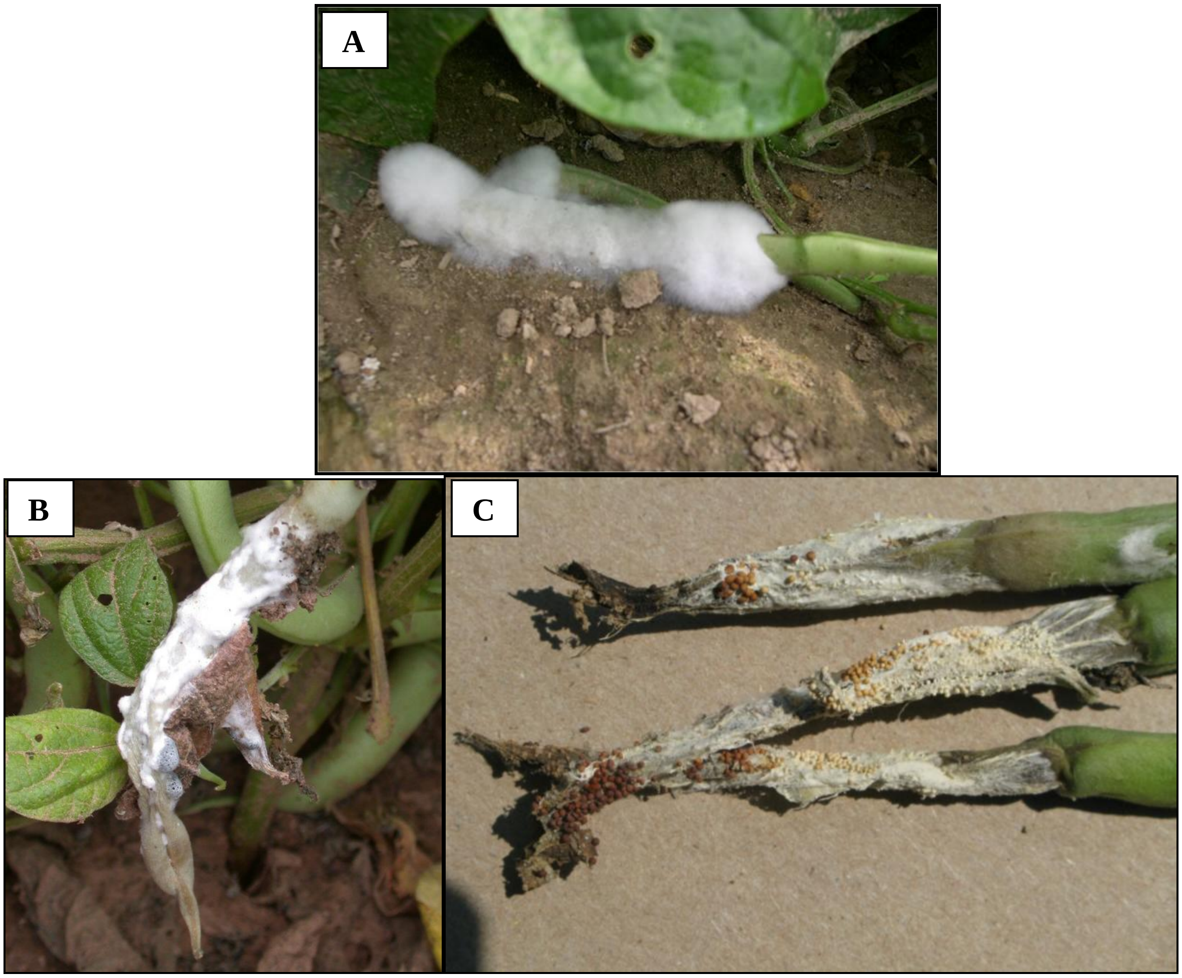

Fig. 1.2. Pythium blight signs **A)** may develop in the field, and is commonly confused with other 'white molds' such as **B)** Sclerotinia blight caused by *Sclerotinia sclerotiorum* and **C)** Southern blight caused by *Sclerotium rolfsii*.

CHAPTER 2: CHARACTERIZATION OF THE PYTHIUM BLIGHT PATHOGEN OF SNAP BEAN IN EASTERN U.S. STATES

Abstract:

New Jersey, Georgia, and the Eastern Shore of Virginia (ESV) are important snap bean (*Phaseolus vulgaris* L.) growing regions, but profitable yields are threatened by Pythium blight, one of the most severe snap bean diseases in the U.S. Although this disease is well documented, the species of *Pythium* causing this disease have not been well characterized. This information is important for determining management strategies, since *Pythium* spp. differ in their sensitivity to fungicides and differ in their response to environmental conditions. Isolates were collected to identify the causal agent(s) of Pythium blight on snap beans. Because of the pathogen's wide host range and distribution, isolates were recovered from different hosts, including other legumes, cucurbits and solanaceous crops, in the snap bean-growing areas listed above. Isolates were collected from soil by baiting and by isolating from symptomatic plants. For each isolate, pathogenicity on snap beans was verified and the isolate was characterized by morphology and sequence analysis of the rDNA-internal transcribed spacer (ITS) regions. Because of the ITS sequence similarity of *P. deliense* to *P. aphanidermatum*, identification of *P. deliense* isolates was confirmed by polymerase chain reaction (PCR) using *P. deliense* specific primers for the rDNA-ITS region. All ESV isolates were identified as *Pythium aphanidermatum*, except for two *P. myriotylum* and four *P. ultimum* isolates. *P. aphanidermatum*, *P. ultimum,* and *P. catenulatum* were recovered from New Jersey crops. *P. aphanidermatum* and *P. ultimum* were also isolated from symptomatic plants in Georgia, as well as multiple isolates of *P. deliense*. To our knowledge this is the first time *P. deliense* has been reported in Georgia and the first report of

this species on common bean and squash. Also, to our knowledge, this is the first report of *P. catenulatum* on lima bean and in New Jersey. This research confirms that multiple *Pythium* species are responsible for Pythium blight symptoms on snap beans.

Introduction:

Pythium blight has become an increasingly important disease in snap bean (*Phaseolus vulgaris* L.) production in the eastern U.S., and is particularly problematic on the Delmarva Peninsula and in Georgia (Langston, 2010; Rideout et al., 2010). In the field, disease development begins as a water-soaked lesion, followed by profuse mycelial growth over the dead cortex. These symptoms may occur on the stems, leaves, and/or the bean pods, which results in severe economic loss (Adegbola and Hagedorn, 1969). The Pythium blight pathogen(s) possess(es) a wide host range, including other legumes, cucurbit and solanaceous crops. The extensive host range of the pathogen contributes to population buildup in soils and results in increases in severity of Pythium blight on East Coast vegetable farms. Although this disease is well documented, limited information is available that clarifies which spp. of *Pythium* are most responsible for disease losses. Several species have been associated with Pythium blight symptomology in snap beans, including *P. aphanidermatum, P. ultimum, P. debaryanum, P. dissoctocum,* and *P. myriotylum* (Adegbola and Hagedorn, 1969; Dominiak and Damicone, 2006; Drechsler, 1952; Harter and Zaumyer, 1931). Dominiak and Damicone (2006) also collected multiple isolates of *Phytophthora drechsleri* from snap bean pods showing water soaking and/or white, cottony growth. Various *Pythium* spp. reported as the causal agent may be the result of slight differences in environmental conditions within geographical regions, since species vary in their optimal growth temperature range (van der Plaats-Niterink, 1981).

Drechsler (1952) isolated *P. ultimum* collected from an irrigated bean field in Delaware. Adegbola and Hagedorn (1969) isolated *P. ultimum* and *P. debaryanum* from symptomatic snap beans in Wisconsin. *Pythium ultimum* thrives in moderately warm regions, such as Wisconsin and Delaware, growing optimally between 15-25°C (Adegbola and Hagedorn, 1969). However, the sp. predominately reported as the Pythium blight casual agent is *P. aphanidermatum* (Edson) Fitzp., which thrives during periods of warm temperatures (28 to 32°C) and high moisture and possesses a wide host range (Adegbola and Hagedorn, 1969; van der Plaats-Niterink, 1981). *P. aphanidermatum* may also cause disease in northern states, such as Delaware, during the warmer summer months (Drechsler, 1952).

Snap bean producers rely primarily upon cultural practices, such as reduced planting densities, avoidance of poorly drained soils, and crop rotation, to avoid significant yield losses due to Pythium blight (Baldwin, 1990). Cultivar selection can decrease disease incidence, especially in combination with fungicide applications (Damicone et al., 2008). However, the only product currently possessing a Section 3 Federal label for Pythium blight control is ProPhyt 6.7F (potassium phosphite; Helena Chemical Company, Collierville, TN), which has been shown to effectively manage Pythium blight on snap bean caused by *P. aphanidermatum* in at least one study (Rideout et al., 2010). In 2008, Ridomil Gold Copper 65WP (mefenoxam and copper hydroxide; Syngenta Crop Protection, Greensboro, NC), which is effective against many oomycetes, received a section 24(c) label for use on snap beans in Delaware, Georgia, Maryland, and Virginia (Damicone and Trent, 2003; Rideout et al., 2010). Cyazofamid (Ranman 400SC; FMC Corporation, Philadelphia, PA) is also an effective fungicide for oomycetes and has shown efficacy in previous field trials against Pythium blight on snap bean (Rideout et al., 2010). Cyazofamid is currently being evaluated by USDA's IR-4 program and is expected to receive a label for use on Pythium blight of snap bean in the near future. In order to identify the most effective chemical control regimen, the pathogen populations in a particular region must be characterized, because *Pythium* spp. vary in their sensitivity to different fungicides, particularly single-site inhibitors (Broders et al., 2007; Kato et al., 1990). Even within species, variations in sensitivity to fungicides are observed, which is usually the result of resistance development within pathogen populations (Moorman et al., 2002; Moorman and Kim, 2004; Taylor et al., 2002).

Several techniques can be employed to identify *Pythium* isolates to the species level. Traditionally, researchers emphasized a number of morphological criteria outlined in identification keys (Dick, 1990; van der Plaats-Niterink, 1981; Waterhouse, 1968). These criteria include the number and attachment of antheridia (monoclinous or diclinous), the surface ornamentation of the oogonial wall (ornamented or smooth), the size of the oospore, the degree that the oospore fills the oogonium (plerotic or aplerotic), the sporangial shape (lobulate, filamentous, or spherical), and the presence or absence of hyphal swellings. Many oomycetes, including *Pythium* spp., exhibit overlapping similarities and tremendous intraspecific variation and plasticity in morphology, both in culture and in the field making species identification using morphological characteristics difficult (Francis and St. Clair, 1997; Martin, 2000). The morphology of some cosmopolitan *Pythium* spp. can be variable, depending on the region of the world from which it is isolated (van der Plaats-Niterink, 1981).

Several molecular tools have been used in recent years to identify *Pythium* spp., including restriction fragment length polymorphisms (RFLP) of genomic and mitochondrial DNA (Martin and Kistler, 1990; Harvey et al., 2001), RFLPs of the internal transcribed spacer (ITS) regions and random amplified polymorphic DNA (RAPDs) (Herrero and Klemsdal, 1998;

Kageyama et al., 1998), amplified fragment length polymorphism (AFLP) fingerprinting (Garzon et al., 2005), isozyme analysis (Mugnier, 1995), single-strand conformational polymorphism analysis of the internal transcribed spacer 1 (Kong et al., 2004), and the development of species-specific primers for the detection of multiple species (Arif et al., 2010; Wang et al., 2003). The ITS regions 1 and 2 of ribosomal DNA are most commonly used to separate *Pythium* spp. (Al-Sa'di et al., 2007; Bailey et al., 2002; Klassen et al., 1996; Moorman et al., 2002). The sequence analysis of this region is very reliable because the rate of accumulation of mutations often correlates with the rate of speciation (Bruns et al., 1991; Chen et al., 1992; Kageyama et al., 1997). In recent years, ITS sequence analysis has been widely used by researchers, and this sequence information is freely accessible through databases such as NCBI's GenBank (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/genbank/). Genetic sequence databases provide a powerful tool for performing comparative sequence alignment between unknown *Pythium* isolates and previously identified ones (Cooke et al., 2000). However, the ITS region of some *Pythium* spp. is so similar that it is not useful for separating some *Pythium* spp. For example, *P. aphanidermatum* and *P. deliense* only differ 2-3% in their ITS sequences; this similarity may be explained by a recent speciation event (Levesque and de Cock, 2004). Between the two species, certain morphological characteristics differ slightly: the average oospore diameter, the shape of the oogonial stalk, and sporangial shape and tendency to form in bunches (van der Plaats-Niterink, 1981). Although *P. aphanidermatum* and *P. deliense* are similar in host range, temperature optimum, and ITS sequence, they were considered distinct species based on morphological differences (Chen et al., 1991; van der Plaats-Niterink, 1981). Thus, to reliably distinguish *P. aphanidermatum* and *P. deliense*, morphological characterization is necessary.

Confusion about the causal agents of Pythium blight may also be the result of saphrophytic or endophytic species present on symptomatic plants or the occurrence of a synergist pathogen complex. The approach taken in this research was to determine the causal agents by accumulating multiple isolates from symptomatic tissue and field soil, characterize each isolate to species, and verify pathogenicity. The objectives of this research were to (i) establish the causal agent(s) of Pythium blight on snap bean and (ii) examine variation in the frequency of each species collected from different hosts and locations on the Eastern Shore of Virginia and other snap bean producing regions, such as Georgia and New Jersey, where Pythium blight is a serious problem.

Materials and Methods:

Isolate collection from soil

During the summers of 2008 and 2009, soil samples were collected from several agricultural fields in Virginia and Georgia where snap bean Pythium blight had been previously reported (Table 2.1). According to results from previous studies, the highest densities of *P. aphanidermatum* oospores occur in the top 15 cm of soil (Stanghellini et al., 1982). Samples were collected with a soil borer to a 30 cm depth and air-dried on butcher paper for 2 days at approximately 25°C. The soil was sieved (no. 2; Humboldt Manufacturing Co., Chicago, IL) to remove most plant material. Two techniques were used to recover *Pythium* spp. from the soil. The first baiting method used snap bean as bait and also verified pathogenicity. Three 3-week old snap bean seedlings of the cultivar ‘Bronco’ (Seminis Vegetable Seeds, St. Louis, MO) were transplanted into a 3:1 soil:vermiculite mixture (Bojac sandy loam soil; Horticultural grade vermiculite; Industries Inc., North Bloomfield, OH) in 20 cm diameter plastic pots. The soil was moistened to field capacity, and plants were covered with a perforated transparent plastic bag to maintain relative humidity above 95%. Plants were placed in a growth chamber and the temperature under the plastic bag was maintained at 32°C until symptoms appeared. Symptomatic tissue was surface disinfested with a 10% bleach solution for 1 min. and transferred to PARF (pimaricin, ampicillin, rifampicin, and fludioxonil). This medium is similar to PARP selective medium (pimaricin, ampicillin, rifampicin, and PCNB) (Eckert and Tsao, 1962), except PCNB was replaced with fludioxonil (Maxim 4FS, Syngenta Crop Protection, Greensboro, NC), which provides selection against *Rhizoctonia* and *Fusarium* spp. (fludioxonil was used in place of PCNB because of health and environmental concerns linked to PCNB). PARF was made by preparing corn meal agar (CMA) (BD Diagnostics, Sparks, MD; 17 g/L dH_2O) and adding antibiotic suspensions after the agar cooled to 45-50°C. Pimaricin (>95% a. i., Sigma Chemical Co., St. Louis, MO; 20 µg/ml) was added to prevent fungal growth. Rifampicin (Rifampicin SV, sodium salt, Sigma Chemical Co.; 10 µg/ml) and ampicillin (>95% a. i., Sigma Chemical Co.; 250 µg/ml) were added to prevent bacterial growth. Fludioxonil (Maxim 4FS; Syngenta Crop Protection, Greensboro, NC) was added to a final concentration of 60 µg/ml.

The second method for baiting Pythium spp. from soil used a potato tuber bait method described by Stanghellini and Kronland (1985) with some modifications. Soil samples were air-dried and sieved as described previously. In a 100 x 15 mm Petri dish, 24 g of soil was moistened to field capacity, and 5 pieces of potato tuber tissue (1 cm^2 and 3 mm thick) were placed on the soil. A 0.5 cm water agar (Fisher Scientific, Waltham, MA) plug was placed on top of each potato piece. Samples were incubated for 12-15 hours at 30°C. Water agar slices were placed on PARF selective media for recovery of the pathogen.

Isolate collection from symptomatic plants

All remaining isolates were recovered from symptomatic plant tissue from various hosts and locations during 2007 through 2010. Isolates were predominantly recovered from snap beans, but isolates were also recovered from other legumes, cucurbits, and solanaceous crops. Hyphal tip transfers of isolates were made in duplicate to CMA slants. Hyphal tip cultures were grown at 22°C for 3 days then covered by sterile water and placed in long-term storage at room temperature (Table 2.4).

Pathogenicity tests

After species identification, pathogenicity on snap bean was verified for each isolate. For each isolate, 4 replications were conducted. For inoculum preparation, each isolate was grown on CMA for 2 days at 22°C. Snap bean pods (cultivar 'Bronco') were surface disinfested in 5% sodium hypochlorite for 1 min and rinsed twice in sterile water. Agar plugs (3-mm diameter) were cut from the leading edge of the growing culture and immediately placed on a non-wounded snap bean pod. Each agar plug was covered with a 0.5 ml Eppendorf™ microfuge tube cap and the edge of the cap was sealed with petroleum jelly. Pods were then placed in a plastic Ziploc™ bag lined with moistened sterile paper towels and sealed to retain high humidity. Pods were incubated at 25°C for 4 days. The presence of water-soaked lesions surrounding the inoculation sites verified pathogenicity. Ten symptomatic bean pods were arbitrarily selected, and the pathogen was re-isolated to complete Koch's postulates.

Morphological characterization

All isolates were morphologically identified according to characteristics described by van der Platts-Niterink (1981) and Waterhouse (1968). Stimulation of sexual and asexual structures necessary for morphological characterization was carried out with grass blade culture as described by Waterhouse (1968). Isolates were grown on CMA for 3 days at 22°C and 4 agar plugs were transferred from the leading edge of the colony into a 100 x 15 mm Petri dish. Sterile water was added to the level of the agar. Four to six 0.5-cm pieces of tall fescue (*Festuca arundinacea* Schreb. cultivar Jaguar) blades, previously boiled in sterile water for 10 min, were placed in each Petri dish. Dishes were incubated at 22°C under continuous fluorescent light (44 watt) for 2 to 4 days. For each isolate the sporangial morphology, presence of hyphal swellings, oogonial surface ornamentation, oogonial fill, type of antheridial attachment, and presence of zoospores were recorded (Table 2.3 and Appendix A). For each isolate, 20 oogonia representative of the isolate were selected, and the diameter of the oospores and number of antheridia per oogonium were measured. These characteristics were viewed using an Olympus BX41 microscope (Olympus Imaging America Inc., Center Valley, PA), with images captured by an Olympus DP11 camera attachment (Fig. 2.1). Isolates were compared to previously characterized reference isolates of *P. aphanidermatum*, *P. myriotylum, P. ultimum*, and *P. deliense.*

DNA purification

DNA was extracted from isolates P001-P016 using the Biosprint 15™ (Qiagen, Germantown, MD) following the Biosprint™ plant extraction protocol. Isolates were grown on V8 agar (150 ml V8 juice, 3 g $CaCO_3$, 15 g agar, 850 ml dH_2O) for 4 days at 22°C. A sterile scalpel blade was used to scrape approximately 50 mg of mycelium from the culture, which was placed in a 2.0 ml screw-cap tube containing 2 glass beads (2-mm diameter). Ethanol (100%) was added to cover the mycelium in the tube and the tube was incubated overnight at 4°C. Tubes were centrifuged for 2 min at 16,000 g, the ethanol discarded, and the mycelium frozen by placing tubes in liquid nitrogen (approximately -150°C). The samples were macerated using a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK) for 30 sec at 2,500 rpm. Maceration was repeated twice with samples re-frozen in liquid nitrogen between each maceration. DNA was extracted using the Biosprint 15™ following the Biosprint™ plant extraction protocol.

DNA of remaining isolates (P021-P107) was extracted using the FastPrep®-24 (MP Biomedicals, Solon, OH) and FastDNA® kit (MP Biomedicals, Solon, OH), as described by Paulitz and Adams (2003). Isolates were grown on potato dextrose agar (PDA) agar at room temperature for 2 days. Three agar plugs were taken from the outer edge of the colony and placed in 15 ml of potato dextrose broth (PDB) in a Petri dish (100 x 15 mm). Cultures were allowed to grow until mycelium covered most of the dish, typically 7 to 10 days. Mycelial mats were washed with sterile water, blotted dry with sterile filter paper, and placed in a 2.0 ml screw-cap tube containing 1 ceramic bead (6.3mm diameter) and Lysing Matrix A (garnet particles), which is included in the FastDNA® kit. DNA was extracted following the FastDNA® kit protocol for yeast, algae, and fungi, with modification. One milliliter of buffer CLS-Y was added to each tube and the samples were macerated in the FastPrep on speed 4 for 40 sec. Samples were incubated at 22°C for 5 min and processed a second time on speed 4 for 40 sec. Samples were again incubated at 22°C for 5 min and then spun at 14,000 g for 5 min. Supernatant (600 µl) and 600 µl of Binding Matrix were transferred to a 2.0 ml microcentrifuge tube and inverted to mix. Samples were placed on an Innova 2000™ platform shaker (New

Brunswick Scientific, Edison, NJ) at room temperature for 5 min at 60 rpm. Samples were then spun at 14,000 g for 1 min and the supernatant discarded. The pellet was gently resuspended by adding 500 µl of buffer SEWS-M, using the force of the liquid from the pipet tip. Samples were spun at 14,000 g for 1 min and the supernatant discarded. Samples were spun for a second time at 14,000 g for 1 min, with the residual liquid being removed with a pipet tip. DNA was eluted by gently resuspending Binding Matrix in 100 µl of buffer DES. Samples were then incubated for 5 min at 55°C in a water bath. Samples were centrifuged at 14,000 g for 1 min, and eluted DNA was transferred to clean microcentrifuge tubes. Samples were stored at -20°C. DNA in each sample was quantified using the Qubit® fluorometer (Invitrogen, Carlsbad, CA) and the Quant-iT™ dsDNA BR assay kit (Invitrogen). DNA quantification was performed by following instructions included in the kit.

Sequence analysis

All 100 isolates were molecularly characterized by comparative alignment of the sequenced internal transcribed spacer region (ITS) of the ribosomal DNA (Cooke et al., 2000). DNA was amplified using the primers ITS6 and ITS4 (Table 2.2; White et al. 1990). PCR reaction components consisted of 2.5 µl of 10X PCR buffer , 0.75 µl of 50 mM $MgCl_2$, 0.25 µl of 10 mM dNTPs (Invitrogen™), 2.5 µl of 10 µM of each primer, 0.25 µl of Platinum *Taq* DNA polymerase (Invitrogen™, 5 u/ µl) and nuclease-free water to bring to 25 µl volume. PCR programs consisted of 1 cycle at 95°C for 2 min; 30 cycles of 95°C for 20 sec, 53°C for 25 sec, and 72°C for 50 sec; and a final extension of 72°C for 10 min. An aliquot (8 µl) of each PCR reaction was run on a 1.5% agarose gel by electrophoresis at 80V for 45 min. Gels were stained with ethidium bromide and visualized with UV light (302 nm). The PCR amplification resulted in a band of approximately 845 base pairs.

The MinElute® PCR Purification Kit (Qiagen, Germantown, MD) was used to prepare the PCR products for the sequencing reaction; the manufacturer's protocol was followed All samples were sequenced by the Virginia Bioinformatics Institute Core Lab (Blacksburg, VA). Sequence data for both complementary strands were obtained: partial sequence of the 18S ribosomal RNA gene, complete sequence of the internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, and partial sequence of the 28S ribosomal RNA gene. Sequences were manually edited, and a consensus sequence obtained using the SDSC Workbench (workbench.sdsc.edu; San Diego Supercomputer Center, La Jolla, CA). Alignments were performed using Clustal W (Higgins et al., 1994). Each isolate sequence was compared with other ITS sequences using GenBank's Basic Local Alignment Search Tool (BLAST).

***P. deliense* identification using species-specific PCR primers**

Several isolates were putatively identified as *P. deliense* using morphological characteristics. Due to high ITS sequence similarity, this species cannot be distinguished from *P. aphanidermatum* by ITS sequence comparison (van der Plaats-Niterink, 1981; Levesque and de Cock, 2004). Therefore, DNA of putative *P. deliense* isolates was amplified using the species-specific primers PD1F and PD1R designed from the rDNA-ITS region (Table 2.2; Arif et al., 2010). A *P. aphanidermatum* isolate (P090) was used as a negative control and a previously identified *P. deliense* reference isolate (P165, Table 2.4) was used as a positive control. PCR reaction components consisted of 2.5 µl of 10X PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.25 µl of 10 mM dNTPs (Invitrogen™), 2.5 µl 10 µM of each primer, 0.25 µl of Platinum *Taq* DNA polymerase (Invitrogen™, 5 u/ µl) and a volume of nuclease-free water to bring to 25 µl. PCR programs consisted of 1 cycle at 94°C for 3 min; 35 cycles of 94°C for 20 sec, 53°C for 15 sec, and 72°C for 20 sec; and a final extension of 72°C for 3 min. An aliquot (8 µl) of each PCR reaction was run on a 1.5% agarose gel by electrophoresis at 90V for 45 min. Gels were stained with ethidium bromide and visualized with UV light (302 nm). The PCR amplification resulted in a band of approximately 143 base pairs.

Results:

Species identification

A total of 100 *Pythium* isolates were collected from 2007 through 2010 and were identified to species using morphological characterization and sequence analysis. Isolates were recovered by culturing symptomatic legume, cucurbit, and solanaceous tissue and soil baiting. Pathogenicity on snap bean was verified for all isolates.

Isolate identification by morphology revealed 5 *Pythium* spp. and one unidentified *Pythium* spp. isolate as the causal agents of Pythium blight of snap bean (Table 2.3). Slight variability was observed within each species, including sporangial morphology, number of attached antheridia, type of antheridial attachment, and oospore size. However, a number of consistent differences between species allowed for morphological identification. These differentiating characteristics included: presence of hyphal swellings in *P. ultimum* isolates (Fig. 2.1); filamentous sporangia, and the number of antheridia and their attachment type of *P. myriotylum*; the spherical, presence of hyphal swellings (Fig. 2.1), plerotic oogonial fill, and the number of antheridia and attachment type of *P. catenulatum*. *P. aphanidermatum* and *P. deliense* had similar sporangial morphology—unbranched, terminal lobate sporangia were most commonly observed in isolates of both species (Fig. 2.2 and Fig. 2.3). The oogonial stalk of *P. deliense* isolates, however, was noticeably curved toward the antheridial stalk (Fig. 2.3). *P. deliense* consistently produced a smaller average oospore diameter (16.5 μm) than *P. aphanidermatum* (21.5 μm). These characteristics distinguished this sp. from *P. aphanidermatum*.

The sequence of the ITS 1, 5.8S ribosomal RNA gene, and ITS 2 was obtained for each isolate. Multiple isolates of the same sp. all had 100% similarity in sequence, including those collected from different locations. Isolate identification to species was verified with 99-100% sequence similarity when compared with other ITS sequences using GenBank's BLAST analysis. Sequence analysis corresponded with the identifications assigned using morphological characterization in all cases. The ITS sequence of the unidentified *Pythium* sp. (isolate P056) did not align with any existing sequences in GenBank (Appendix B). This sp. was identified to the genus level by morphological characteristics and results from the BLAST search showed the highest sequence similarity with *P. orthogonon* with only 93% maximum identity.

P. aphanidermatum and *P. deliense* isolates sequences were highly similar (98%, Fig. 2.4). Therefore, identification of *P. deliense* isolates was confirmed by molecular characterization using species-specific PCR primers from the ITS region (Arif et al., 2010) (Fig. 2.5).

Frequency and geographical distribution of species collected

Isolates were collected from 3 eastern U.S. states: 49 from Virginia, 40 from Georgia, and 11 from New Jersey (Fig. 2.6). Isolates of the same species but collected from different locations still showed similarity in both morphological characteristics (Table 2.3) and ITS sequence.

P. deliense was only collected from Georgia, *P. catenulatum* only from New Jersey, and *P. myriotylum* only from Virginia. Both *P. aphanidermatum* and *P. ultimum* were collected from all three locations. The most commonly isolated species was *P. aphanidermatum* (53%), followed by *P. deliense* (31%) and *P. ultimum* (12%). *P. myriotylum* and *P. catenulatum* were less commonly found (2% and 1%, respectively).

Discussion:

Though snap bean production in the eastern U.S. heavily relies upon cultural practices to prevent the detrimental effects of Pythium blight, chemical control is an important management tactic. Determining the species causing Pythium blight will aid in choosing the most efficacious fungicides for control; this work confirms that multiple *Pythium* spp. cause Pythium blight. Because optimal growth temperatures and host range differ amongst *Pythium* spp., understanding the species associated with disease will also help determine the risk for disease outbreaks according to the weather and the crop rotational options.

P. aphanidermatum, P. ultimum, and *P. myriotylum* were previously identified as pathogens causing Pythium blight on snap bean (Adegbola and Hagedorn, 1969; Dominiak and Damicone, 2006; Drechsler, 1952; Harter and Zaumyer, 1931). The high rate of recovery of *P. aphanidermatum* in our sampling was expected, because Pythium blight is usually observed during the warm summer months during which time optimal temperatures for this species occur. Infection by the Pythium blight pathogen usually occurs during a rain or irrigation event when water splashes propagules (mycelial fragments, oospores, sporangia, or zoospores) from the soil onto aerial parts of the plant (Kim, 1972). Kim (1972) found that among the propagule types, zoospores are the most effective primary inoculum source and found *P. aphanidermatum* to be the only causal agent of Pythium blight in Maryland snap bean fields (Kim, 1972). Of the five species identified as causal agents in this research, all but *P. ultimum* var. *ultimum* produce zoosporangia and zoospores. Since *P. ultimum* var. *ultimum* was isolated from symptomatic stems and pods, this work proves that *P. ultimum* var. *ultimum* propagules other than zoospores are capable of causing infection in the field.

New information was determined on the host range and distribution of two species, *P. catenulatum* and *P. deliense*. *P. catenulatum* was isolated from water-soaked lesions on lima bean (*Phaseolus lunatus* L.) stems in New Jersey in 2009. This species is widespread in distribution internationally as a pathogen causing damping-off and root rots, and has also been recognized as a pathogen on lettuce (*Lactuca sativa* L.) in Arizona and snap bean in Wisconsin (Kobriger and Hagedorn, 1984; McLeod et al., 2009; Misra and Hall, 1996; Stanghellini, and Kronland, 1986). Although this species has been documented as a root rot pathogen on snap bean and cowpea (*Vigna sinensis* L.) (Kobriger and Hagedorn, 1984; Yu, 1998), it has not been isolated from aerial parts of a lima bean plant. *P. catenulatum* has been reported as the causal agent of leaf blight symptoms on turfgrass (Kim and Park, 1999), but to our knowledge, this is first report of this species causing aerial blight on a vegetable crop. To our knowledge, this is also the first report of *P. catenulatum* on lima bean and the first report of this species in New Jersey. This species also has the potential to cause Pythium blight on snap bean, as shown by pathogenicity testing. *P. deliense* is also widespread in distribution internationally, especially in warmer regions, where it is a problematic pathogen on several legume, cucurbit, and solaneceous crops (de Cara et al., 2008; Lévesque et al., 1998; Liu, 1977). Within the U.S., it has been documented only twice, in Arizona and Texas, both as the causal agent of a root rot of sugar beet (*Beta vulgaris* L.) (Rush, 1987; Stanghellini et al., 1982). In June of 2009, several isolates of *P. deliense* were collected from snap beans and squash (*Cucurbita pepo* L.) in southern Georgia. Most isolates were recovered from water-soaked lesions on the snap bean stems, and the remaining isolates were recovered from bean pod and squash fruit. This research is the first report of *P. deliense* as a pathogen of snap bean and squash, the first report in Georgia, and the third report of this species in North America. Other reports and this research support the possibility that *P. deliense* may be indigenous to the soils of regions with warmer temperatures, and that optimal environmental conditions are necessary for its manifestation as a serious pathogen. Although both *P. catenulatum* and *P. deliense* are usually reported as the causal agent of damping-off and root rot diseases, these species are also capable of causing blight on aerial plant parts.

Homogeneity of ITS sequences within many *Pythium* spp., including *P. aphanidermatum,* is often observed (Levesque and de Cock, 2004). This research supports these observations for, not only the multiple isolates of *P. aphanidermatum*, but also for isolates of *P. ultimum*, *P. myriotylum*, and *P. deliense*. Multiple isolates of the same sp. examined in this work all had 100% similarity in sequence. Therefore, isolates, except for *P. deliense,* were successfully identified to species by performing BLAST and comparing to other ITS sequence entries in GenBank. *P. aphanidermatum* and *P. deliense* only differ 2-3% in their ITS sequences, and cannot be conclusively differentiated using the ITS region (Levesque and de Cock, 2004). Slightly differing morphological characteristics described by van der Plaats-Niterink (1981) were observed that allowed for identification of *P. deliense*. The PCR assays using *P. deliense*-specific primers further confirms the morphological identification of *P. deliense.*

Previous research suggests that different spp. of *Pythium* vary in their sensitivity to certain fungicides; this supports the importance of this work. Understanding which species pose a serious threat (i.e. cause Pythium blight) and their sensitivity to fungicides can be critical for constructing disease management strategies. Fungicide sensitivity differences may not be solely due to fungicide resistance development within populations, because, in previous studies, sensitivity levels were consistent within isolates of the same species and different from isolates of other species (Broders et al., 2007; Kato et al., 1990). Therefore, future research is needed to determine if *Pythium* spp. causing snap bean Pythium blight vary in their sensitivity to fungicides commonly used in vegetable production.

In conclusion, Pythium blight in eastern U.S. snap bean production is caused by several previously documented species, as well as this first report of *P. deliense* and *P. catenulatum*. These isolates were collected from agricultural systems, and they provide insight into the abundance and geographic distribution of pathogen populations capable of economic disease damage. This information will aid in selecting the spp. of *Pythium* that should be screened for sensitivity to fungicides used for control of Pythium blight, and later, determining best management strategies in snap bean production.

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Table 2.1. Soil samples collected during summer 2008 and 2009 in Virginia and Georgia.

Code	Location	Collection date	Current crop
Yey	Pungoteague, VA	5-2008	Snap bean
TaT	Capeville, VA	5-2008	Snap bean
HoF	Tifton, GA	6-2009	Fallow

Table 2.2. PCR primers used in this study.

Name	Sequence (5’ to 3’)	Reference
For ITS sequencing		White et al. 1990
ITS 4	TCC TCC GCT TAT TGA TAT GC	
ITS 6	GAA GGT GAA GTC GTA ACA AGG	
P. deliense specific		Arif et al., 2010
PD1F	TCC TTG AGG TGT ACC TGA T	
PD1R	AAA ATT GTC TGC TAA AAG C	

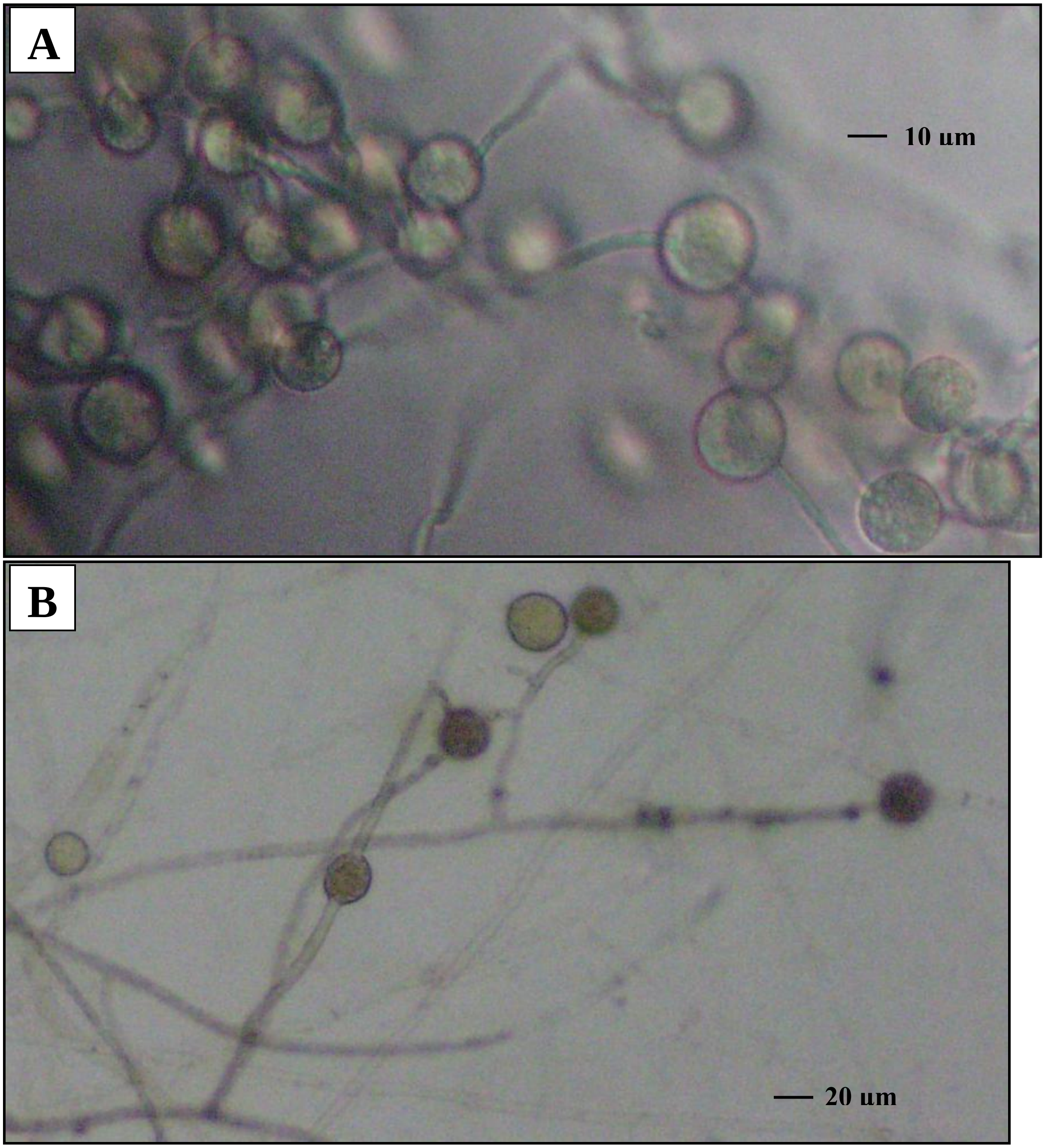

Fig. 2.1. Spherical hyphal swellings of **A)** *Pythium catenulatum* and **B)** *Pythium ultimum*.

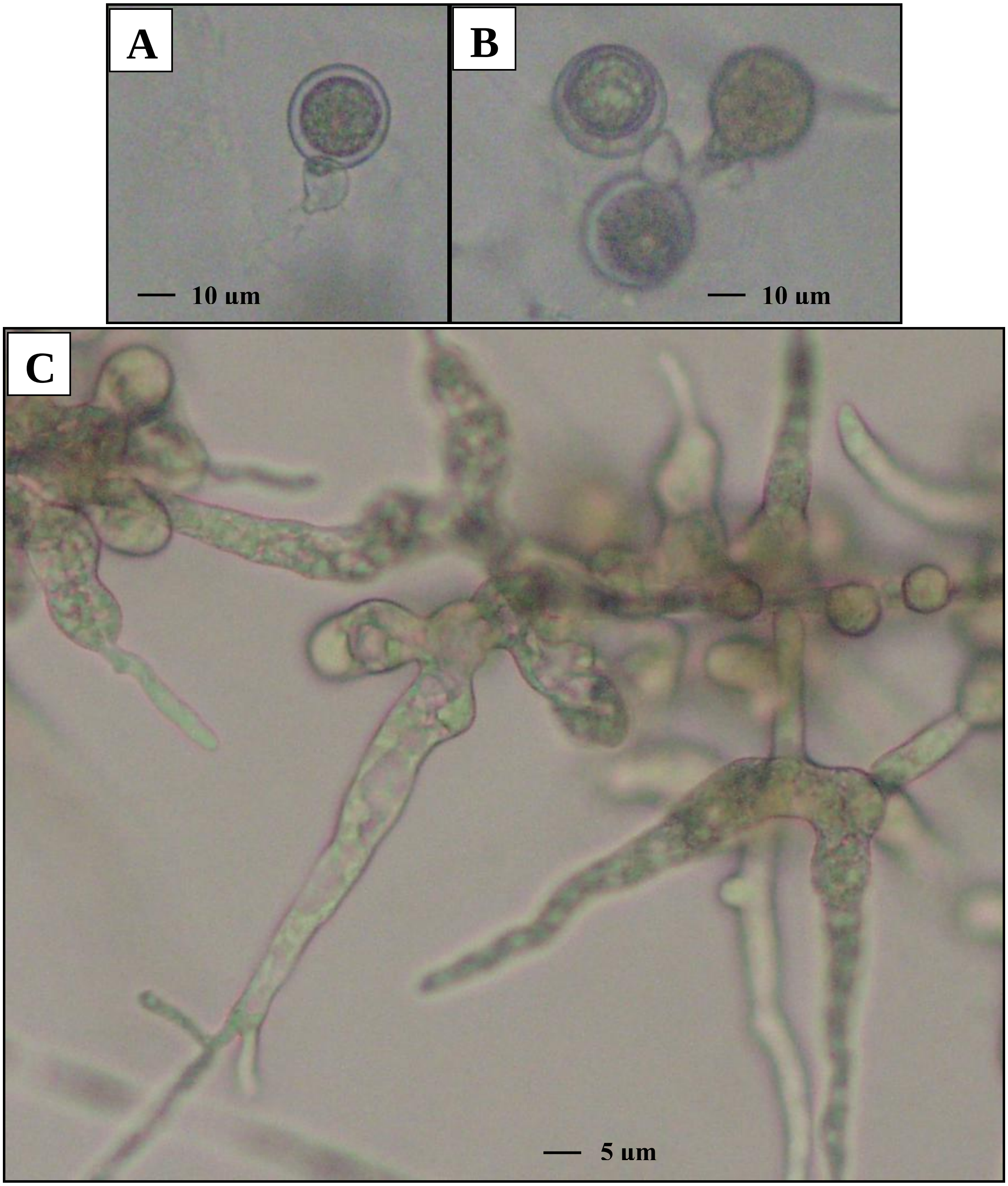

Fig. 2.2. *P. aphanidermatum* morphological characters: **A and B)** sexual structures and **C)** terminal, unbranched, lobate sporangia.

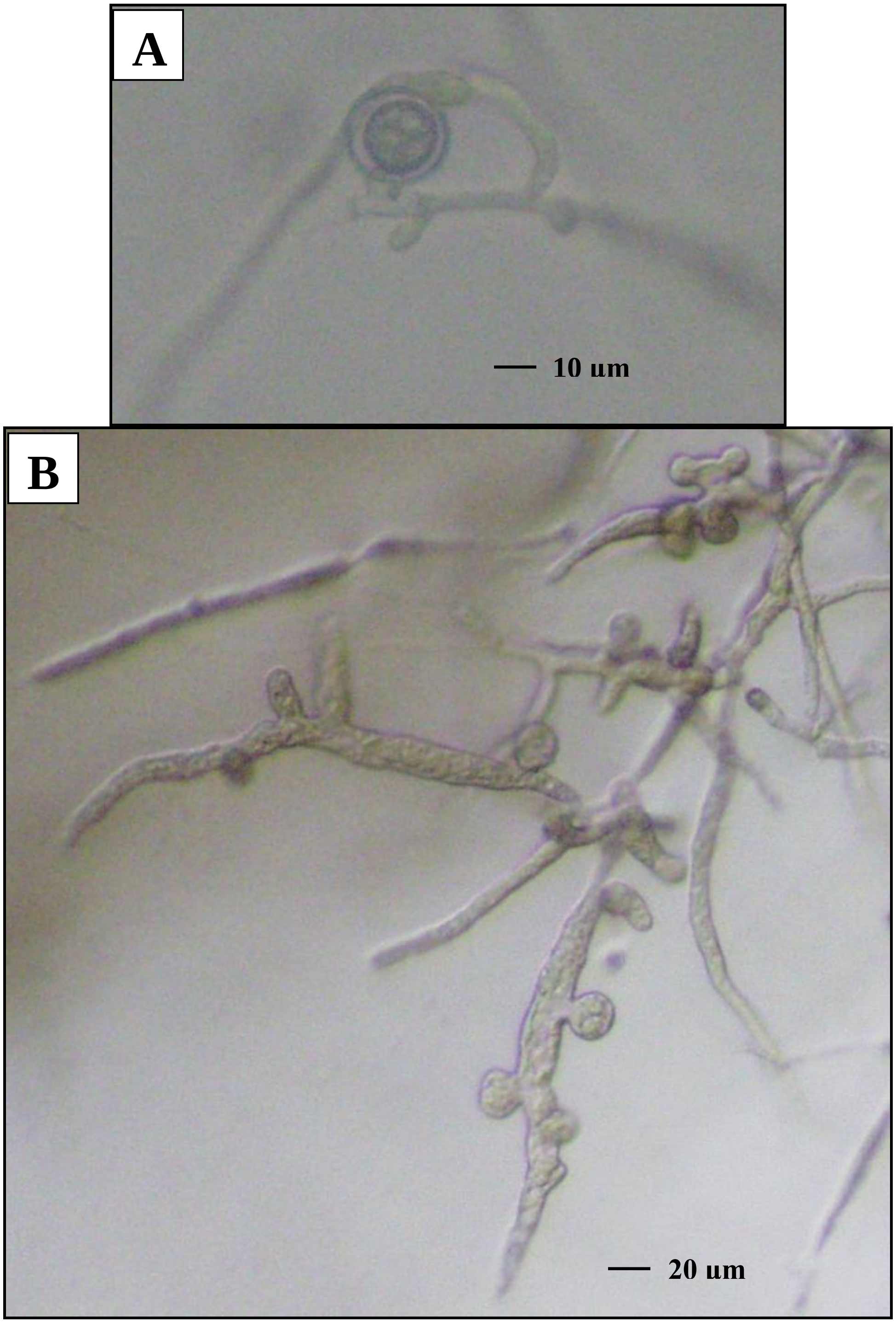

Fig. 2.3. *Pythium deliense* morphological characters: **A)** curved oogonial stalk and oospore, and **B)** unbranched, lobate sporangia.

Table 2.3. Summary of the morphological characteristics of the *Pythium* spp. collected from each location, as described by van der Plaats-Niterink (1981) and Waterhouse (1968).

Species	**State**	**Sporangia**	**Hyphal swellings**	**Oogonial surface**	**Filling oogonium**	**Number of antheridia**	**Antheridial[a] attachment**	**Oospore[b] size (µm)**
P. aphanidermatum	VA	Branched/unbranched lobate, intercalary/mostly terminal	—	Smooth	Aplerotic	1-2	M-d	19-25
	GA	Branched/unbranched lobate, intercalary/mostly terminal	—	Smooth	Aplerotic	1-2	M-d	19-23
	NJ	Branched/unbranched lobate, terminal	—	Smooth	Aplerotic	1-2	M-d	19-22
P. ultimum	VA	n/a	Spherical	Smooth	Aplerotic	1-2	M-d	15-22
	GA	n/a	Spherical	Smooth	Aplerotic	1-2	M-d	16-21
	NJ	n/a	Spherical	Smooth	Aplerotic	1-2	M-d	15-21
P. deliense	GA	Branched/ mostly unbranched lobate, terminal/intercalary	—	Smooth	Aplerotic	1-2	M-d	14-19
P. myriotylum	VA	Filamentous, terminal/intercalary	—	Smooth	Aplerotic	2-7	D-m	20-26
P. catenulatum	NJ	Irregular mycelial swellings, catenulate	Spherical	Smooth	Plerotic	5-10	D	22-27
Pythium sp.	GA	Spherical	—	Smooth	Aplerotic	1	M	14-16

[a] M= monoclinous, D= diclinous. Capitalized letters represent the predominant form of antheridia attachment observed.

[b] Range of oospore size measurements of 20 randomly selected oogonia per isolate.

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P051   CCACACCATAAAA-CTTTCCACGTGAACCGTTGAAATCATGTTCTGTGCTCTCTCTCGGG
P080   CCACACCATAAAAACTTTCCACGTGAACCGTTGAAATCATGTTCTGTGCTCTCTTTCGGG
       ************* **************************************** *****

P051   AGGGCTGAACGAAGGTGGGCTGCTTAATTGTGGTCTGCCGATGTATTTTTCAAACCCATT
P080   AGGGCTGAACGAAGGTGGGCTGCTTAATTGTAGTCTGCCGATGTATTTTTCAAACCCATT
       ******************************* ****************************

P051   TACCTAATACTGATCTATACTCCAAAAACGAAAGTTTCTGGTTTTAATCCATAACAACTT
P080   TACCTAATACTGATCTATACTCCAAAAACGAAAGTTTATGGTTTTAATCTATAACAACTT
       ************************************* *********** **********

P051   TCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAA
P080   TCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAA
       ************************************************************

P051   TGCGAATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCACATTGCACTTTCGGGTT
P080   TGCGAATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCACATTGCACTTTCGGGTT
       ************************************************************

P051   ATGCCTGGAAGTATGCCTGTATCAGTGTCCGTACATCAAACTTGCCTTTCTTTTTCTGTG
P080   ATGCCTGGAAGTATGCCTGTATCAGTGTCCGTACATCAAACTTGCCTTTCTTTTTCTGTG
       ************************************************************

P051   TAGTCAGGGAGAGAGATGGCAGAATGTGAGGTGTCTCGTTGACTCCCTTTTCGGAGGAGA
P080   TAGTCAGGGAGAGAGATGGCAGAATGTGAGGTGTCTCGCTGGCTCCCTTTTCGGAGGAGA
       ************************************** ** ******************

P051   AGACGCGAGTCCCTTTAAATGTACGTTCGCTCTTTCTTGTGTCTAAGATGAAGTGTGATT
P080   AGACGCGAGTCCCTTTAAATGTACGTTCGCTCTTTCTTGTGTCTAAGATGAAGTGTGATT
       ************************************************************

P051   CTCGAATCGCAGTGATCTGTTTGGATCGCTTTGCGCATTTGGGCGACTTCGGTTAGGACA
P080   CTCGAATCGCGGTGATCTGTTTGGATCGCTTTGCGCATTTGGGCGACTTCGGTTAGGACA
       ********** *************************************************

P051   TTAAAGGAAGCAACCTCTATTGGCGGTATGTTAGGCTTCGGCCCGACGTTGCAGCTGACG
P080   TTAAAGGAAGCAACCTCTATTGGCGGTATGTTAGGCTTCGGCCCGACGTTGCAGCTGACA
       *********************************************************** 

P051   GAGTGTGGTTTTCTGTTCTTTCCTTGAGGTGTACCTGATTTGTGTGAGGCAATGGTCTGG
P080   GAGTGTGGTTTTCTGTTCTTTCCTTGAGGTGTACCTGAATTGTGTGAGGCAATGGTCTGG
       ************************************** *********************

P051   GCAAATGGTTGCTGTGTAGTAGGGTTTTGCTGCTCTTGGGCGCCCTGTTTTCGGATAGGG
P080   GCAAATGGTTGCTGTGTAGTAGGGTTTTGCTGCTCTTGGACGCCCTGTTTTCGGATAGGG
       *************************************** ********************

P051   TAAAGAAGGCAACACCAATTTGGGACTGTTTGC---TTTTAGCAGACAATTTTCTAA
P080   TAAAGGAGGCAACACCAATTTGGGACTGTTTGCAATTTATTGTGAACAACTTTCTAA
       ***** ***************************   ** * *   **** *******
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Fig. 2.4. Alignment of the complete sequence of the ITS1, 5.8S, and ITS2 of a *P. deliense* isolate (P051) and a *P. aphanidermatum* isolate, using Clustal W (Higgins et al., 1992).

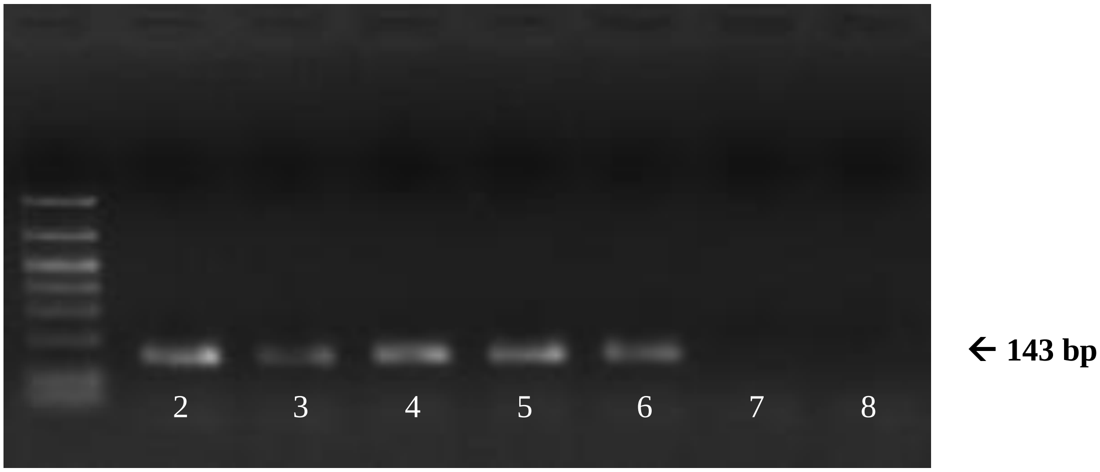

Fig. 2.5. Agarose gel with DNA amplicons of putative *P. deliense* isolates and control isolates. These amplicons were produced by using *P. deliense* specific PCR primers PD1F and PD1R (Arif et al., 2010): lane 1) DNA ladder, 2) *P. deliense* reference isolate (P165), 3-6) putative isolates(P022, P027, P029, P042), 7) *P. aphanidermatum* isolate (P090), 8) water.

Table 2.4. Isolates of *Pythium* spp. recovered from symptomatic plants and baited from soil during 2007, 2008, 2009, and 2010 in Virginia, New Jersey, and Georgia.

Code	***Pythium* spp.**	**Host/substrate**	**Location & date isolated**
P001	*P. aphanidermatum*	Snap bean	Tifton, GA Oct. 2007
P002	*P. ultimum*	Snap bean	Painter, VA Nov. 2007
P003	*P. aphanidermatum*	Snap bean	New Church, VA May 2008
P004	*P. aphanidermatum*	Eggplant	Painter, VA July 2008
P007	*P. aphanidermatum*	Snap bean	Painter, VA June 2008
P008	*P. aphanidermatum*	Potato	Painter, VA June 08
P009	*P. aphanidermatum*	TaT soil	Capeville, VA May 2008
P010	*P. aphanidermatum*	Tomato	Painter, VA June 2008
P011	*P. aphanidermatum*	Yey soil	Pungoteague, VA June 2008
P012	*P. aphanidermatum*	Yey soil	Pungoteague, VA June 2008
P013	*P. aphanidermatum*	Yey soil	Pungoteague, VA June 2008
P014	*P. aphanidermatum*	Cucumber	Virginia Beach, VA July ‘08
P015	*P. aphanidermatum*	Cucumber	Virginia Beach, VA July ‘08
P016	*P. aphanidermatum*	Potato	Painter, VA June 2008
Ref.[a]	*P. ultimum*	Geranium	PA (P17)
Ref.[a]	*P. myriotylum*	Geranium	PA (P44)
Ref.[a]	*P. aphanidermatum*	Chrysanthemum	PA (P12)
Ref.[a]	*P. deliense*	Poinsettia	PA (P165)
P021	*P. aphanidermatum*	Zucchini	Tifton, GA June 2009
P022	*P. deliense*	Zucchini	Tifton, GA June 2009
P023	*P. deliense*	Summer squash	Tifton, GA June 2009
P024	*P. deliense*	Zucchini	Tifton, GA June 2009
P025	*P. deliense*	Summer squash	Tifton, GA June 2009
P026	*P. aphanidermatum*	Summer squash	Tifton, GA June 2009
P027	*P. deliense*	Snap bean	Americus, GA June 2009
P028	*P. deliense*	Snap bean	Americus, GA June 2009
P029	*P. deliense*	Snap bean	Americus, GA June 2009
P030	*P. deliense*	Snap bean	Americus, GA June 2009
P031	*P. deliense*	Snap bean	Americus, GA June 2009
P032	*P. ultimum*	Snap bean	Americus, GA June 2009
P033	*P. deliense*	Snap bean	Americus, GA June 2009
P034	*P. deliense*	Snap bean	Chula, GA June 2009
P035	*P. deliense*	Snap bean	Chula, GA June 2009
P036	*P. deliense*	Snap bean	Chula, GA June 2009
P037	*P. deliense*	Snap bean	Chula, GA June 2009
P038	*P. deliense*	Snap bean	Chula, GA June 2009
P039	*P. deliense*	Snap bean	Chula, GA June 2009
P040	*P. deliense*	Snap bean	Chula, GA June 2009
P041	*P. deliense*	Snap bean	Chula, GA June 2009
P042	*P. deliense*	Snap bean	Chula, GA June 2009

[a] Ref.= previously characterized isolates obtained from G. Moorman (Pennsylvania State Univ.).

Table 2.4 cont. Isolates of *Pythium* spp. recovered from symptomatic plants and baited from soil during 2007, 2008, 2009, and 2010 in Virginia, New Jersey, and Georgia.

Code	***Pythium* spp.**	**Host/substrate**	**Location & date isolated**
P043	*P. deliense*	Snap bean	Chula, GA June 2009
P044	*P. deliense*	Snap bean	Chula, GA June 2009
P045	*P. deliense*	Snap bean	Chula, GA June 2009
P046	*P. deliense*	Snap bean	Chula, GA June 2009
P047	*P. deliense*	Snap bean	Chula, GA June 2009
P048	*P. deliense*	Snap bean	Chula, GA June 2009
P049	*P. deliense*	Snap bean	Chula, GA June 2009
P050	*P. deliense*	Snap bean	Chula, GA June 2009
P051	*P. deliense*	Snap bean	Chula, GA June 2009
P052	*P. deliense*	Snap bean	Chula, GA June 2009
P053	*P. deliense*	Snap bean	Chula, GA June 2009
P054	*P. deliense*	Snap bean	Chula, GA June 2009
P055	*P. aphanidermatum*	Summer squash	Manor, GA June 2009
P056	*Pythium* sp.	HoF soil	Tifton, GA June 2009
P057	*P. ultimum*	HoF soil	Tifton, GA June 2009
P059	*P. aphanidermatum*	Snap bean	New Church, VA June 2009
P060	*P. myriotylum*	Snap bean	Cape Charles, VA June 2009
P061	*P. aphanidermatum*	Cucumber	Painter, VA August 2009
P062	*P. aphanidermatum*	Acorn squash	Painter, VA August 2009
P063	*P. aphanidermatum*	Pumpkin	Painter, VA August 2009
P064	*P. aphanidermatum*	Snap bean	Painter, VA August 2009
P065	*P. aphanidermatum*	Cucumber	Suffolk, VA August 2009
P066	*P. aphanidermatum*	Tomato	Onley, VA August 2009
P067	*P. aphanidermatum*	Tomato	Painter, VA August 2009
P068	*P. ultimum*	Snap bean	Norma, NJ Sept. 2009
P069	*P. ultimum*	Snap bean	Norma, NJ Sept. 2009
P070	*P.catenulatum*	Lima bean	Vineland, NJ Sept. 2009
P071	*P. aphanidermatum*	Zucchini	Vineland, NJ Sept. 2009
P072	*P. ultimum*	Eggplant	Vineland, NJ Sept. 2009
P073	*P. ultimum*	Eggplant	Vineland, NJ Sept. 2009
P074	*P. ultimum*	Zucchini	Vineland, NJ Sept. 2009
P075	*P. ultimum*	Snap bean	Bainbridge, GA Oct. 2009
P076	*P. aphanidermatum*	Snap bean	Tifton, GA Oct. 2009
P077	*P. ultimum*	Snap bean	Painter, VA Oct. 2009
P078	*P. myriotylum*	Tobacco	Suffolk, VA Mar. 2010
P079	*P. aphanidermatum*	Snap bean	Painter, VA Oct. 2009
P080	*P. aphanidermatum*	Snap bean	New Church, VA June 2010
P081	*P. aphanidermatum*	Snap bean	New Church, VA June 2010
P082	*P. ultimum*	Snap bean	New Church, VA June 2010
P083	*P. aphanidermatum*	Snap bean	New Church, VA June 2010
P084	*P. aphanidermatum*	Snap bean	New Church, VA June 2010

Table 2.4 cont. Isolates of *Pythium* spp. recovered from symptomatic plants and baited from soil during 2007, 2008, 2009, and 2010 in Virginia, New Jersey, and Georgia.

Code	***Pythium* spp.**	**Host/substrate**	**Location & date isolated**
P085	*P. aphanidermatum*	Snap bean	New Church, VA June 2010
P086	*P. ultimum*	Snap bean	New Church, VA June 2010
P087	*P. aphanidermatum*	Snap bean	Hammonton, NJ June 2010
P088	*P. aphanidermatum*	Snap bean	Hammonton, NJ June 2010
P089	*P. aphanidermatum*	Snap bean	Hammonton, NJ June 2010
P090	*P. aphanidermatum*	Snap bean	Hammonton, NJ June 2010
P091	*P. aphanidermatum*	Snap bean	Painter, VA July 2010
P092	*P. aphanidermatum*	Summer squash	Painter, VA July 2010
P093	*P. aphanidermatum*	Summer squash	Painter, VA July 2010
P094	*P. aphanidermatum*	Summer squash	Painter, VA July 2010
P095	*P. aphanidermatum*	Summer squash	Painter, VA July 2010
P096	*P. aphanidermatum*	Summer squash	Painter, VA July 2010
P097	*P. aphanidermatum*	Acorn squash	Painter, VA Aug. 2010
P098	*P. aphanidermatum*	Summer squash	Painter, VA Aug. 2010
P099	*P. aphanidermatum*	Summer squash	Painter, VA Aug. 2010
P100	*P. aphanidermatum*	Summer squash	Painter, VA Aug. 2010
P101	*P. aphanidermatum*	Zucchini	Painter, VA Sept. 2010
P102	*P. aphanidermatum*	Zucchini	Painter, VA Sept. 2010
P103	*P. aphanidermatum*	Zucchini	Painter, VA Sept. 2010
P104	*P. aphanidermatum*	Zucchini	Painter, VA Sept. 2010
P105	*P. aphanidermatum*	Zucchini	Painter, VA Sept. 2010
P106	*P. aphanidermatum*	Acorn squash	Painter, VA Sept. 2010
P107	*P. aphanidermatum*	Acorn squash	Painter, VA Oct. 2010

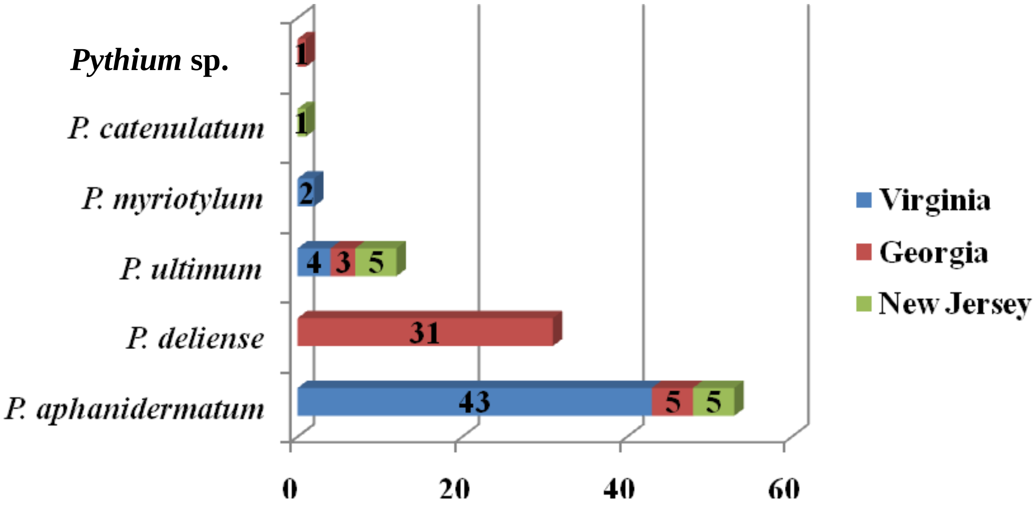

Figure 2.6. Geographic distribution of the *Pythium* spp. causing Pythium blight of snap bean and other hosts collected from Pythium blight symptomatic crops or field soil in Virginia, Georgia, and New Jersey during 2007, 2008, 2009, and 2010.

CHAPTER 3: DEVELOPMENT OF AN EFFECTIVE FIELD INOCULATION METHOD OF THE PYTHIUM BLIGHT PATHOGEN ON SNAP BEAN

Abstract:

Pythium blight has become a severe disease threatening snap beans production in important growing areas, such as the Eastern Shore of Virginia (ESV). However, Prophyt 6.7F (potassium phosphite; Helena Chemical Company, Collierville, TN) is the only effective foliar fungicide that currently has a Section 3 Federal label for control of Pythium blight. Labeling of fungicides for control is hindered by the difficulty associated with conducting successful trials with the pathogen(s), which occurs in patches in the field and sporadically from year to year. Different inoculum substrates and concentrations were evaluated in order to develop an inoculation technique that produces more uniform disease in field trials. Over 3 summers, substrates inoculated with an ESV *Pythium aphanidermatum* isolate were evaluated in the field, including sterilized soil/oatmeal (2% by weight), vermiculite/V8 juice (5:3 weight to volume), and long grain rice/water (5:3.6 weight to volume). Each inoculum substrate was applied at rates of 0, 2,500, 5,000, and 10,000 propagules per gram (ppg)/0.3 m at plant flowering. Disease incidence was recorded as percentage of diseased 0.3 m segments of row (foot rows). The vermiculite inoculum substrate (10,000 ppg/0.3 m) consistently caused at least 50% disease in all 3 field trials, and this research recommends this method for providing reliable disease pressure for cultivar selection and/or fungicide efficacy trials.

Introduction:

The Eastern Shore of Virginia's (ESV) temperate climate is conducive for successful snap bean (*Phaseolus vulgaris* L.) production, and this region produces the majority of snap beans grown in the state. During both spring and fall snap bean crops the ESV usually experiences periods of rainfall and warm temperatures that are optimal for Pythium blight (Drechsler, 1952; Harter and Zaumeyer, 1931). Pythium blight generally occurs from the onset of flowering until harvest and is most destructive when disease onset occurs on mature pods just prior to harvest. Succulent tissues, like bean pods near maturity, are most susceptible to infection by *Pythium* spp., but infection may occur at any plant age under optimal growth conditions for the pathogen (Adegbola and Hagedorn, 1969). The potential for Pythium blight is further enhanced when mature bean pods are in contact with the soil surface.

Several species have been associated with Pythium blight in snap beans, including *P. aphanidermatum, P. ultimum, P. deliense, P. debaryanum, P. dissoctocum,* and *P. myriotylum* (Adegbola and Hagedorn, 1969; Dominiak and Damicone, 2006; Drechsler, 1952; Harrison and Rideout, 2010; Harter and Zaumyer, 1931). However, the species predominately reported in the United States and the ESV to cause Pythium blight is *P. aphanidermatum* (Edson) Fitzp., (Harrison and Rideout, 2010). This species is ubiquitous in soil worldwide, possesses a wide host range, and can cause disease during periods of warm temperatures (28 to 32°C) and high humidity (95 to 100% RH) (Adegbola and Hagedorn, 1969, van der Plaats-Niterink, 1981). Under laboratory conditions, *P. aphanidermatum* caused Pythium blight on snap beans at 19°C, but a positive relationship was recorded between symptom severity and temperature increases between 19°C and 38°C (Kim, 1972). Kim (1972) observed the highest Pythium blight damage on snap beans in Maryland occurred when the minimum daily temperatures were ≥ 28°C and the maximum daily temperatures were ≥ 33°C. Harter and Zaumeyer (1931) also noted that disease symptoms were most severe when the daytime temperatures ranged between 32 to 41°C. They compared the occurrence of Pythium blight in both Colorado and Virginia in 1930 and the role of high RH and temperature. They stated that hygrothermograph readings in Virginia were near saturation for a prolonged period of time prior to symptom development and similar readings were recorded near the soil line in the flood-irrigated field in Colorado prior to symptom development. In the presence of a susceptible host and adequate pathogen populations in the soil, optimal environmental conditions have an important impact on Pythium blight severity.

Snap bean producers rely primarily upon cultural practices, such as avoiding planting at high densities and planting into poorly drained soils, deep plowing crop residue, and crop rotation, to avoid significant yield losses. Although resistance to Pythium blight is not yet well understood, cultivar selection can decrease disease incidence, especially in combination with fungicide applications (Damicone et al., 2008). The only fungicide currently possessing a Section 3 Federal label for Pythium blight control is Prophyt 6.7F (potassium phosphite; Helena Chemical Company, Collierville, TN), which effectively managed Pythium blight on snap bean caused by *P. aphanidermatum* in one field efficacy trial (Rideout et al., 2010). In 2008, Ridomil Gold Copper 65WP (mefenoxam and copper hydroxide; Syngenta Crop Protection, Greensboro, NC), which is effective against many oomycetes, received a section 24(c) label for use on snap beans in Delaware, Georgia, Maryland, and Virginia (Damicone and Trent, 2004; Rideout et al., 2010).

One aspect that hinders the assessment of efficacy and subsequent labeling of fungicides for control of Pythium blight is the difficulty associated with conducting successful field trials with the pathogen(s). The sporadic and clustered nature of this disease in the field has led to inconsistent efficacy results and, in many cases, failed trials. Previous research has documented the variable nature of this pathosystem. Rideout et al. (2008) determined that fungicide applications did not reduce disease incidence or postharvest pod rot associated with *Pythium* spp. when compared to the non-treated control. In a study by Damicone and Trent (2004), applications of Reason 500SC (fenamidone; Bayer CropScience, RTP, NC), Ranman 400F (cyazofamid, FMC Corporation, Philadelphia, PA), Ridomil Copper 70W (metalaxyl and copper hydroxide; Syngenta Crop Protection, Greensboro, NC), Kocide 2000DF (53.8% copper hydroxide; DuPont Crop Protection, Wilmington, DE), and Phostrol 6.7S (mono- and dibasic sodium, potassium and ammonium phosphites; NuFarm Americas, Inc., Burr Ridge, IL) reduced levels of Pythium blight. Although statistically significant differences were observed in their study, yields were not significantly impacted due to low disease pressure. A repeat of this study conducted by Damicone (2004) determined no significant differences in yield or disease incidence between the nontreated control and fungicide treatments. The development of a reliable and repeatable field inoculation technique will aid in producing more consistent field efficacy trials. As a result, researchers and industry personnel will better understand which fungicides are most likely to suppress levels of Pythium blight in field settings.

Techniques have been developed for field inoculation of *Pythium* spp. causing disease in other cropping systems; these involve spreading a pathogen-infested substrate onto the soil surface or incorporation into the soil (Cummings, 2007; Feng et al., 1999; Yin and Hoy, 1998). Prior research demonstrates successful inoculation techniques promote production of the pathogen's sexual propagules, oospores, within the substrate. Oospores are resilient structures that serve as *Pythium* spp. primary mechanism for longterm survival, since the mycelium of the pathogen quickly lyses as its food source is depleted (Stanghellini and Hancock, 1971). During a rain or irrigation event, either oospores or zoosporangia of *P. aphanidermatum* can liberate zoospores (Drechsler, 1947; Kim, 1972). Successful initiation of disease in the field using inoculum substrates containing oospores is dependent on a high water potential (at or near field capacity) and a suitable temperature to promote oospore germination (Lumsden and Ayers, 1975; Stanghellini and Burr, 1973). Stanghellini and Burr (1973) also showed that high soil moisture increased nutrient availability, which is necessary for *P. aphanidermatum* oospore germination. In previous field trials substrates containing oospores were only successful when plots were irrigated the day before and immediately following inoculation (Feng et al., 1999). Regardless of the inoculum substrate, adequate soil moisture is necessary for initial establishment of *Pythium* spp. in the soil, as well as its propagule production and ability to cause disease.

One of the most challenging aspects of performing field trials for Pythium blight on snap bean is achieving reliable disease pressure in the field. Improved field inoculation techniques that would provide consistent disease incidence and severity are necessary to identify cultural practices, resistant cultivars or efficacious fungicides that can suppress levels of this disease. The objective of this research is to determine effective rates of inoculum and the environmental conditions necessary to achieve consistent Pythium blight disease pressure under field conditions.

Materials and Methods:

Design of field trials

Three field trials examining selected inoculation substrates and rates were conducted in 2008, 2009, and 2010 at Virginia Tech’s Eastern Shore Agricultural Research and Extension Center (ESAREC) in Painter, VA where the field soil is classified as Bojac sandy loam.

Nitrogen was broadcast-incorporated at the rate of 60 lb/A using 30% UAN (urea and ammonium nitrate) and conventionally-tilled before each planting. Planting dates for the trials were August 5, 2008; June 17, 2009; and June 19, 2010. Snap bean seeds of the cultivar 'Bronco' (Seminis Vegetable Seeds, Inc., Oxnard, CA) were planted in the 2008 trial, and seeds of the cultivar 'Festina' (Seminis Vegetable Seeds, Inc.) were used for the trials in 2009 and 2010. Festina replaced Bronco due to observations indicating it was more susceptible to Pythium blight in previous field trials (personal communication, Ken Kmiecik, Seminis Vegetable Seeds, Inc.). For all trials, plots were 2 rows wide (1.8 m) by 7.6 m long and arranged in a randomized complete block design with 5 replications per treatment. Each plot was bordered by a non-treated row. Weather data was collected as atmospheric values at the Eastern Shore AREC. Maximum and minimum air temperature data was recorded continuously each day using the Maximum/Minimum Temperature System (RMS OmegaTechnology, Inc., White Marsh, MD). Rainfall was manually recorded twice a day using an 8-inch non-recording gauge (unknown manufacturer, issued by the National Weather Service). Percentage of relative humidity was collected every minute (values average to 1-hour intervals) using the S-12 Console instrument (Rainwise, Inc., Harbor, ME), and the data was viewed using Weather View 32 software (version 7.0; Weather Information Systems, Amity, OR).

Inoculum preparation and inoculation

The inoculum substrates examined included 1) a sterilized soil and oatmeal mix (2% by weight) (modified Cummings, 2007), 2) a vermiculite and V8 juice mix (5:3 weight to volume) (Yin and Hoy, 1998), and 3) long grain rice and water (5:3.6 weight to volume) (Holmes and Benson, 1994). For all 3 trials, a virulent *P. aphanidermatum* isolate (P003), collected from diseased snap bean on the ESV, and was used to inoculate substrate. This isolate was identified using morphological characters and sequence analysis of the rDNA-ITS regions (van der Plaats-Niterink, 1981).

For the sterilized soil – oatmeal substrate (treatment 1), non-sterile Bojac sandy loam field soil (National Cooperative Soil Survey, 2002) was collected from the ESAREC. The soil was allowed to air dry for 2 days and was then passed through a sieve (no. 2; Humboldt Manufacturing Co., Chicago, IL) to remove debris and disrupt clod formation. Raw oatmeal (Quaker, Chicago, IL) was added (2% by weight) to the soil and mixed thoroughly. Deionized water (15% w/w) was added and mixed with the soil/oatmeal mix. Next, a 500 g aliquot of this mixture was transfered to one liter flasks. A cotton-filled cheesecloth plug was inserted in the neck of the flasks and covered with triple - layered aluminum foil. The flasks were autoclaved for 50 min at 120°C and 15 psi. The flasks were cooled overnight at room temperature and autoclaved for an additional 50 min to kill any bacteria that may have germinated from dormant endospores following the first autoclave cycle. The flasks were stored at room temperature until they were inoculated.

To make the vermiculite inoculum (treatment 2), 450 ml of vermiculite (Horticultural grade; Industries Inc., North Bloomfield, OH), 20 grams of raw oatmeal (Quaker, Chicago, IL), and 300 ml of V8 juice solution (Campbell Soup Co., Camden, NJ; 200 ml V8 juice, 800 ml water, 2 g $CaCO_3$) was combined in a one liter flask. This mixture was shaken; plugged with a cotton-filled cheesecloth in the flask neck, and covered with triple-layered aluminum foil. The flasks were autoclaved as described for treatment 1 and stored at room temperature.

To produce the rice grain inoculum (treatment 3) 50 grams of white long-grain rice (Mahatma, Riviana Foods Inc., Houston, TX) and 36 ml deionized water was combined in a 500 ml flask. The flasks were shaken, covered with triple-layered aluminum foil, and autoclaved 40 min at 120°C and 15 psi. After the flasks cooled, the matted rice was loosened with a sterile glass rod and autoclaved an additional 40 minutes. The flasks were stored at room temperature.

After the substrates cooled, each flask was inoculated with 4 to 5 agar plugs (5 mm^2) from the leading edge of 2-day-old CMA cultures of the *P. aphanidermatum* isolate (P003). Flasks were hand shaken every 2 days to ensure uniform colonization and to keep the fungus from matting the substrate together. The soil cultures (treatment 1) were allowed to grow for 4 to 6 weeks; the vermiculite cultures (treatment 2) were allowed to grow for 4 to 5 weeks; and the rice cultures (treatment 3) were allowed to grow for 5 to 7 days. When thorough colonization of each flask was observed (i.e., mycelia were visible throughout the medium), the flasks were stored at 4°C until the following procedures were done: 1) Two days before inoculation, the vermiculite and soil inocula (treatments 1 and 2) were allowed to dry in the greenhouse. 2) On the day of inoculation, the nondried rice (treatment 3) was pulverized with a 14-speed Osterizer blender (Sunbeam Corp., Boca Raton, FL) using the gentle setting.

Inocula in the flasks were quantified using serial dilution plating. Ten grams of each inoculum substrate was added to a 200 ml flask containing 90 ml 0.1% WA to aid suspension of the inoculum, and hand-shaken for 12 min. Ten-fold dilutions of each suspension were carried out to 10^{-4} onto the *Pythium*-selective medium PARF. This medium is similar to PARP selective medium (pimaricin, ampicillin, rifampicin, and PCNB) (Eckert and Tsao, 1962), except PCNB was replaced with fludioxonil (Maxim 4FS, Syngenta Crop Protection, Greensboro, NC), which provides selection against *Rhizoctonia* and *Fusarium* spp. PARF was made by preparing corn meal agar (CMA) (BD Diagnostics, Sparks, MD; 17 g/L dH_2O) and adding antibiotic suspensions after the agar cooled to 45-50°C. Pimaricin (>95% a. i., Sigma Chemical Co., St.

Louis, MO; 20 μg/ml) was added to prevent fungal growth. Rifampicin (Rifampicin SV, sodium salt, Sigma Chemical Co.; 10 μg/ml) and ampicillin (>95% a. i., Sigma Chemical Co.; 250 μg/ml) were added to prevent bacterial growth. Fludioxonil (Maxim 4FS) was added to a final concentration of 60 μg/ml. Two replicate plates were inoculated with 0.5 mL aliquots of each substrate dilution. The plates were then incubated in the dark at room temperature for 24 h and colony-forming units (cfu's) were counted. The cfu's/ g substrate was calculated, and each substrate was weighed out to provide treatment rates of 0, 2,500, 5,000, and 10,000 ppg/0.3 m of each plot. The rice substrate treatments were 0, 26,400, 52,800, and 80,000 ppg/0.3 m in the 2009 trial due to initial miscalculation of inoculum concentration.

All inoculum treatments were applied by hand next to the plant crowns of corresponding treatment plots when snap bean plants were in full flower bloom. Each summer, the trials were inoculated once: September 15, 2008; on July 16, 2009; and on July 30, 2010. The naturally occurring *Pythium* populations were not enumerated. Plots received approximately 2.54 cm of overhead-irrigation the day before inoculation, and 1.27 cm immediately following inoculation. For the duration of the study, irrigation was provided every 2 days or when needed to maintain moisture levels near soil saturation capacity.

Disease ratings and yield assessment

Plots were assessed at the initial appearance of signs/symptoms as the percentage of diseased foot rows (Damicone, 2004; Rodriguez-Kabana et al., 1975). A 0.3 m-long plastic rod was used to push back the snap bean canopy within both rows of each plot and each 0.3 m length of row was evaluated for either presence or absence of symptoms or signs of Pythium blight (Fig. 3.1). The number of 0.3 m units with presence of disease was divided by the total 0.3 m units per row to give the percentage of diseased 0.3 m segments (foot rows). In 2008, symptoms were observed on 29 September and disease development was assessed on 30 September. Due to severe disease damage, yield was not measured. The 2009 trial was rated for disease incidence on 29 July-- the same day that symptoms were first observed. Additional assessments were conducted on 31 July and 3 August and the trial was harvested on 11 August. In the 2010 trial, symptoms were first observed on 14 August, disease ratings were recorded on 16 August, and harvest occurred on 24 August. After harvest, the percentage of diseased snap beans was evaluated and the amount was negligible (<1% of yield). After the last disease rating, symptomatic tissue was randomly collected from 5 plots of each trial, surface-sterilized, and transferred to PARF medium. For each trial, *P. aphanidermatum* was cultured from diseased tissue and identified using morphological characters to verify that it was the primary causal agent.

The disease assessment and yield data were subjected to an analysis of variance (ANOVA) and comparison of means using Fisher's LSD, which was performed using Agricultural Research Manager (version 7.0.4, Gylling Data Management Inc., Brookings, SD). Analyses were only performed within the separate trials. In 2009, only 4 replicates were included in the statistical analysis due to high levels of natural pathogen infection in the 5th replicate, which was concluded by the high disease values in the non-treated control plots.

Results:

Disease and yield assessments

Pythium blight symptoms were observed approximately 2 weeks after inoculation in all 3 trials. In 2008 and 2009, the majority of the observed symptoms/signs were water-soaking and cottony mycelial growth on the snap bean pods. In 2010, water-soaking on the stems was mostly observed, but cottony mycelial growth on the pods was sporadically present.

In 2008, none of the soil inoculum plots had disease values that were statistically different from each other or the non-inoculated plots (Table 3.1). A positive numerical relationship was observed between inoculum concentration and disease values, but was not statistically significant. The percentage of diseased row segments of all 3 rice-based inoculum concentrations was not significantly different from the control, and none were significantly different from each other. In this trial, all concentrations of the vermiculite treatment had significantly higher disease than the non-treated control. All concentrations had disease values of at least 50% diseased foot rows. All concentrations of the vermiculite treatment produced disease values significantly higher than all other inoculation substrates and concentrations except for the significant similarity between the highest rice concentration and the 5,000 cfu/0.3 m vermiculite treatment plots.

In 2009, none of the soil inoculum treatment plots had disease values or yields that were statistically different from each other or the non-inoculated plots (Table 3.2). There was a positive numerical relationship between the different concentrations of rice inoculum and number of diseased row segments, but these values were not statistically significant. Disease values were significantly greater and yield was significantly decreased when compared to the non-inoculated control by all concentrations of the rice substrate. Yield from the lowest rice concentration treatment was significantly less than the highest concentration. The average number of infected row segments for vermiculite treatment plots was between 60 and 74%, and all yields were significantly lower and disease values significantly higher than the non-inoculated control. One of the 10,000 ppg/0.3 m vermiculite treatment plots demonstrated disease assessment ratings as high as 82% in 2009 (Fig. 3.2). In general, as disease values increased, yields decreased.

In 2010, none of the soil inoculum plots had yield or disease values that were statistically different from each other or the non-inoculated control (Table 3.3). The disease values and yields of all 3 rice inoculum concentrations were not significantly different from the control or significantly different from each other. None of the rice inoculum treatment plots produced yields significantly different from the control. The highest concentration of vermiculite inoculum was the only treatment that had disease values significantly greater than the control, but all the treatments produced yields significantly similar to the control.

Environmental conditions and disease development

A relationship was observed between rainfall events and disease development (Table 3.4). In all trials, rainfall of at least 2.3 cm was received 2 to 4 days before symptoms were observed. Percentage relative humidity (RH) was high (>90%) for the majority of the time during the 2 to 3 days before symptom occurrence for all trials (Figs. 3.3, 3.4, and 3.5). In 2008, the RH was continuously above or near 90% for 41 hours from 26 September to 28 September. During the 3 days before symptom development in both 2009 and 2010 trials, there were 2 time intervals of at least 14 continuous hours of >90% RH.

In 2008, temperatures reached a maximum of 31.6°C and a minimum of 11.7°C during the days between inoculation and the appearance of Pythium blight symptoms and signs, with an average of 20.7°C (Fig. 3.6). The maximum daily temperature maintained near 25°C for the majority of the time. In 2009, maximum daily temperatures maintained around 30°C during the 2 weeks following inoculation (Fig. 3.7). The maximum temperature reached was 32.2°C, the minimum was 17.8°C, and the average was 25.6°C. In 2010, temperatures reached a high of 35°C and a low of 17.8°C during the period between inoculation and the appearance of Pythium blight symptoms and signs, with an average of 26.3°C (Fig. 3.8).

Discussion:

Previous attempts have been made to evaluate the efficacy of fungicides, resistant cultivars and cultural practices against Pythium blight on snap bean, but determining the most effective materials and practices has been difficult due to inconsistent disease pressure in field trials (Damicone, 2004; Damicone and Trent, 2004; Rideout et al., 2008). In this study, a reliable field inoculation technique was proven to provide the consistent disease pressure necessary to evaluate Pythium blight management tactics. Furthermore, this research confirms that a uniform increase in pathogen populations in the soil, through proper inoculation techniques, warm temperatures and periods of high relative humidity are important to promote Pythium blight disease development on snap bean.

Historically, snap bean field trials inoculated with *Pythium* spp. have failed because of the sporadic nature of the disease and difficulty in maintaining environmental conditions optimal for disease development. A successful inoculation technique was developed by Brede (1984) for Pythium blight on creeping bentgrass (*Agrostis palustris* Huds.), which required field plots to be irrigated and covered with a moistened wood-fiber mulch blanket for several days after inoculation. Germination of oospores and saprophytic growth generally occurs under circumstances where other competitive organisms have greatly reduced activity, such as high moisture environments with poor gas exchange (Griffin, 1963). Infection by the Pythium blight pathogen on snap bean usually occurs during a rain or irrigation event when water splashes propagules (mycelial fragments, oospores, sporangia, or zoospores) onto the plant (Kim, 1972). In comparison, high soil moisture levels were also critical for Pythium blight disease development on snap beans, which likely provided the environment necessary for saprophytic growth and production of pathogen propagules of *P. aphanidermatum* in these trials.

The rice substrate inoculum used in this study was only incubated for 5 to 7 days. Since the food source was not depleted by this point, the cultures likely consisted of oospore, sporangial, and mycelial growth (Stanghellini and Hancock, 1971). In 2009, all 3 rice inoculum concentrations (ranging from 26,400 to 80,000 ppg/0.3 m) produced significantly more disease and significantly decreased yields when compared to the non-inoculated control. However, in 2008 and 2010, none of the rice substrate concentrations produced disease values significantly higher from the control with concentrations ranging from 2,500 to 10,000 ppg/0.3 m. Increased disease pressure in 2009 may be explained by the higher ppg concentrations that were applied or because adequate soil moisture was present for mycelial survival and pathogen establishment in the soil. Irrigation is important immediately following an inoculation technique of this nature. The rice substrate applied at high concentrations, as in the 2009 trial, may be useful in introducing high pathogen populations in the soil, but this work needs to be repeated to confirm this conclusion.

The vermiculite inoculum was incubated for 4 to 5 weeks, providing ample time for pathogen colonization throughout the substrate. Previous research shows that *Pythium* oospore formation is encouraged by available calcium and other cations in solution (Yang and Mitchell, 1965); therefore, ample oospore production was likely simulated by the V8 juice and calcium carbonate included in the inoculum recipe. Also, *Pythium* spp. usually produce oospores in environments with near zero or negative water potential values (Bainbridge, 1970), so several weeks of incubation and decreasing moisture levels possibly simulated production of these survival structures. The vermiculite inoculum method may be more predictable and provide more flexibility in environmental requirements than the rice substrate, since oospores serve as long term survival structures for *Pythium* spp. under periods of low soil moisture (Stanghellini and Hancock, 1971). In all 3 seasons, the data showed that the vermiculite substrate treatments were, in fact, the most reliable for providing at least 50% disease incidence. In 2010, only the two highest inoculation concentrations produced disease values that were significantly greater than the non-inoculated control. These results may be explained by the extreme hot and dry weather encountered in 2010, which made it difficult to maintain soil moisture near field capacity through irrigation alone.

None of the yield measurements in the 2010 trial significantly differed from each other, including the non-inoculated control, which may be due to hot and dry weather during the course of this trial. Due to weather conditions, plants were stunted and had minimal bean set, causing yield data to be similarly low across all treatments. In the 2009 trial, yields decreased as disease values increased demonstrating the destructive potential of this pathogen on snap bean. Disease assessments were performed following methods developed by Rodriguez-Kabana et al. (1975) and modified for Pythium blight on snap bean by Damicone (2004). This method involved counting the number of 0.3 m segments in the snap bean row that had observable symptoms or signs. This method is more time efficient than incidence ratings that are based on percentage of diseased plants, and the method still falls within the definition of disease incidence as defined by Campbell and Madden (1990). Pythium blight symptoms are observed aboveground and its disease incidence may be more accurately assessed without performing destructive sampling in contrast to disease caused by vascular diseases or disease causing below ground symptoms. The validity of the disease assessment method was suggested by its correlation with yield. Although yield was not measured in 2008 and no significant differences were observed in 2010, the disease assessments provide sufficient evidence that the 10,000 ppg/0.3 m vermiculite treatment will consistently cause at least 50% disease damage. The statistically significant yield decreases in the 2009 trial also demonstrates the economic loss incurred by high Pythium blight damage.

Infection of snap beans by the Pythium blight pathogen usually occurs during a rain or irrigation event, at which time water splashes propagules (mycelial fragments, oospores, sporangia, or zoospores) from the soil onto aerial parts of the plant (Kim, 1972). Of these propagule types, Kim (1972) found that zoospores were most effective as the primary inoculum. His research also showed that *P. aphanidermatum* zoospores required at least 8 hours of free moisture after inoculation for symptoms to develop in a laboratory setting. In a field setting, high relative humidity under the plant canopy may provide this necessary free moisture for an extended period of time. In all 3 snap bean trials, symptoms first appeared approximately 2 to 3 days after a rain event. These observations agree with work conducted by Adegbola and Hagedorn (1969), which showed Pythium blight symptoms appeared 24 to 72 hours after inoculation, depending on environmental conditions. The rain events prior to symptom appearance were also followed by at least 14 hours of relative humidity near or above 90%, which provided the necessary free moisture described by Kim (1972). Although irrigation supplies the means for the splashing of pathogen propagules onto the plant, these observations suggest that the extended time period of high relative humidity provided during and after natural rain events is essential for primary infection.

Nutter et al. (1983) developed a weather forecasting system for Pythium blight (caused by *P. aphanidermatum*) on turfgrass and reported that maximum daily temperatures over 30°C followed by at least 14 hours of >90% RH and minimum daily temperatures no lower than 20°C were required for an outbreak to occur. A similar forecasting system has not been developed on snap beans, but our research suggests similar environmental conditions are required for an outbreak in both cropping systems. The temperature conditions present in the 2009 and 2010 trials closely match the parameters outlined by Nutter et al. (1983). Symptoms were still observed in the 2008 trial when the average maximum temperatures were approximately 25°C, so infection can occur at marginally cooler weather than outlined by Nutter et al. (1983). This work suggests that the climatic parameters for *P. aphanidermatum* infection may be similar on multiple hosts.

The vermiculite inoculum method developed in this study provided disease pressure in a successful fungicide efficacy trial on snap beans, which identified significant differences among treatments (Rideout et al., 2010). The study identified cyazofamid, azoxystrobin, pyraclostrobin, and potassium phosphite (at varying rates and concentrations) to be efficacious against snap bean Pythium blight. It is recommended that similar studies utilize the vermiculite (concentration 10,000 ppg/0.3 m) inoculum method for determining other effective management strategies in the future. However, providing appropriate environmental conditions for these trials will be essential as noted previously. Further research examining more specific environmental parameters that promote Pythium blight in snap bean are warranted to ensure successful fungicide trials. In addition, such research could lead to the development of a disease forecasting advisory for this pathogen.

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Fig. 3.1. Assessment of percentage of diseased 0.3 m plants per row, as described by Rodriguez-Kabana et al. (1975) and Damicone (2004). Snap bean canopies were opened using this device in order to record Pythium blight symptoms on the stems and pods.

Table 3.1. In fall 2008, percentage of diseased segments (0.3 m) per row in snap bean plots inoculated with different *Pythium aphanidermatum*-infested (isolate P003) substrates and at various concentration rates.

Substrate Treatment[a]	**Inoculum Rate (ppg/0.3 m)**	**% diseased 0.3 m plants/ row**
Non-treated control	--	21.5 c
Rice	2,500	19.0 c
Rice	5,000	21.0 c
Rice	10,000	29.5 bc
Soil	2,500	17.0 c
Soil	5,000	20.0 c
Soil	10,000	25.5 c
Vermiculite	2,500	60.0 a
Vermiculite	5,000	50.0 ab
Vermiculite	10,000	59.5 a
LSD (p=0.05)		**21.2**
Standard deviation		**14.6**

[a] Treatments means analyzed by Fisher's least significant difference (LSD) pairwise comparisons. Means followed by the same letter do not significantly differ ($p \leq 0.05$).

Table 3.2. In summer 2009, percentage of diseased segments (0.3 m) per row in snap bean plots inoculated with different *Pythium aphanidermatum*-infested (isolate P003) substrates and at various concentration rates.

Substrate Treatment[a]	**Inoculum Rate (ppg/0.3 m)**	**% diseased 0.3 m plants/ row**	**Yield (kg/ha)**
Non-treated control	--	30.0 c	5,858.9 a
Rice	26,400	56.0 ab	3,679.3 b
Rice	52,800	75.0 a	3,254.9 bc
Rice	80,000	82.0 a	2,604.0 c
Soil	2,500	35.0 bc	5,440.2 a
Soil	5,000	35.0 bc	5,416.1 a
Soil	10,000	29.0 c	5,207.9 a
Vermiculite	2,500	67.0 a	3,901.9 b
Vermiculite	5,000	60.0 ab	3,958.8 b
Vermiculite	10,000	74.0 a	2,929.5 bc
LSD (p=0.05)		**19.2**	**2.5**
Standard deviation		**13.3**	**1.7**

[a] Treatments means analyzed by Fisher's least significant difference (LSD) pairwise comparisons. Means followed by the same letter do not significantly differ ($p \leq 0.05$).

Fig. 3.2. Comparison of snap bean plants inoculated with the vermiculite substrate at a 10,000 ppg/0.3 m rate (left row) and non-inoculated plants (right row) in the 2009 trial.

Table 3.3. In summer 2010, percentage of diseased segments (0.3 m) per row in snap bean plots inoculated with different *Pythium aphanidermatum*-infested (isolate P003) substrates and at various concentration rates.

Substrate Treatment[a]	**Inoculum Rate (ppg/0.3 m)**	**% diseased 0.3 m plants/ row**	**Yield (kg/ha)**[b]
Non-treated control	--	14.0 c	445.4
Rice	2,500	33.0 abc	650.9
Rice	5,000	32.0 abc	325.4
Rice	10,000	29.0 abc	539.9
Soil	2,500	30.0 abc	651.0
Soil	5,000	19.0 bc	976.5
Soil	10,000	32.0 abc	771.9
Vermiculite	2,500	33.0 abc	651.0
Vermiculite	5,000	37.0 ab	356.5
Vermiculite	10,000	48.0 a	461.9
LSD (p=0.05)		**14.2**	**1.5**
Standard deviation		**11.1**	**1.2**

[a] Treatments means analyzed by Fisher's least significant difference (LSD) pairwise comparisons. Means followed by the same letter do not significantly differ ($p \leq 0.05$).

[b] There were no significant differences in yield.

Table 3.4. The amount of rainfall during all rain events occurring between the days of inoculation and the appearance of Pythium blight symptoms/signs in 3 snap bean field trials comparing inoculum substrate and concentrations.

Date[a]		**Rainfall (cm)**
2008	25-Sep	4.2
	26-Sep	0.9
	27-Sep	4.5
	28-Sep	0.1
2009	27-Jul	7.7
	28-Jul	0.7
	29-Jul	0.3
	30-Jul	0.2
2010	1-Aug	0.4
	2-Aug	0.2
	6-Aug	4.3
	7-Aug	0.8
	12-Aug	2.3

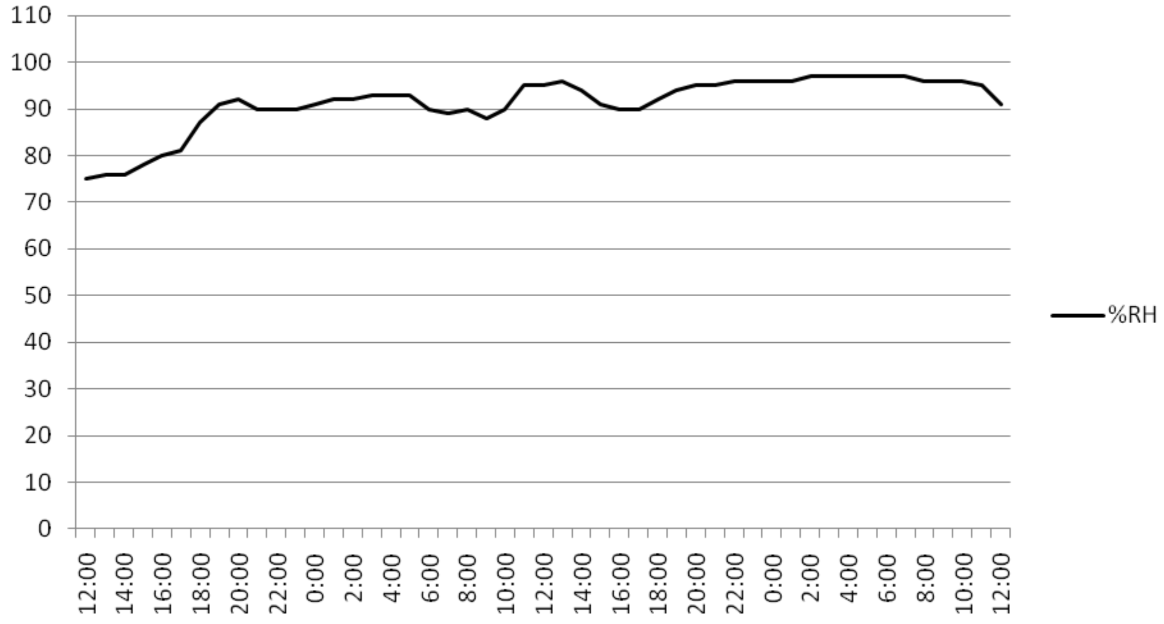

Fig. 3.3. In 2008, the percentage relative humidity between noon on 26 September and noon on 28 September. The first appearance of Pythium blight symptoms/signs was 29 September.

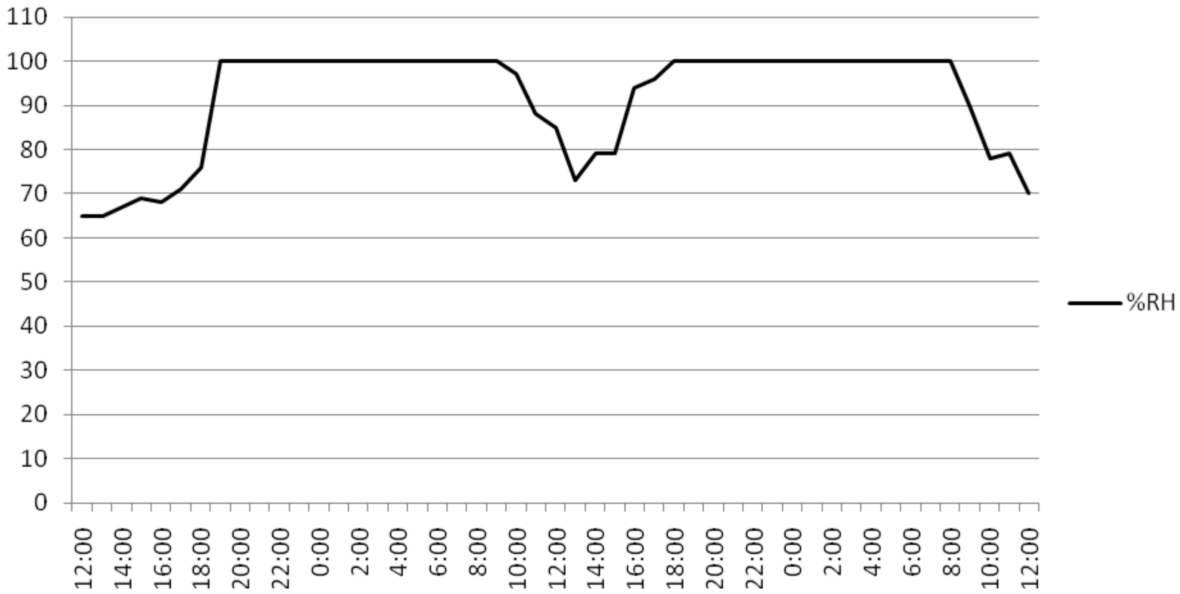

Fig. 3.4. In 2009, the percentage relative humidity between noon on 26 July and noon on 28 July. The first appearance of Pythium blight symptoms/signs was 29 July.

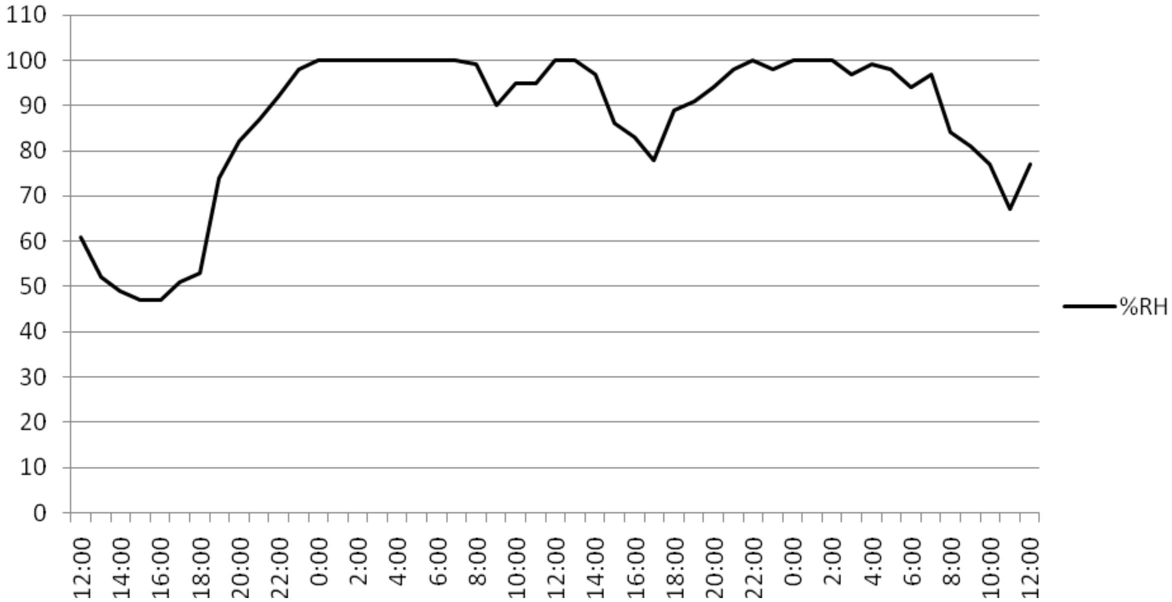

Fig. 3.5. In 2010, the percentage relative humidity between noon on 11 August and noon on 13 August. The first appearance of Pythium blight symptoms/signs was 14 August.

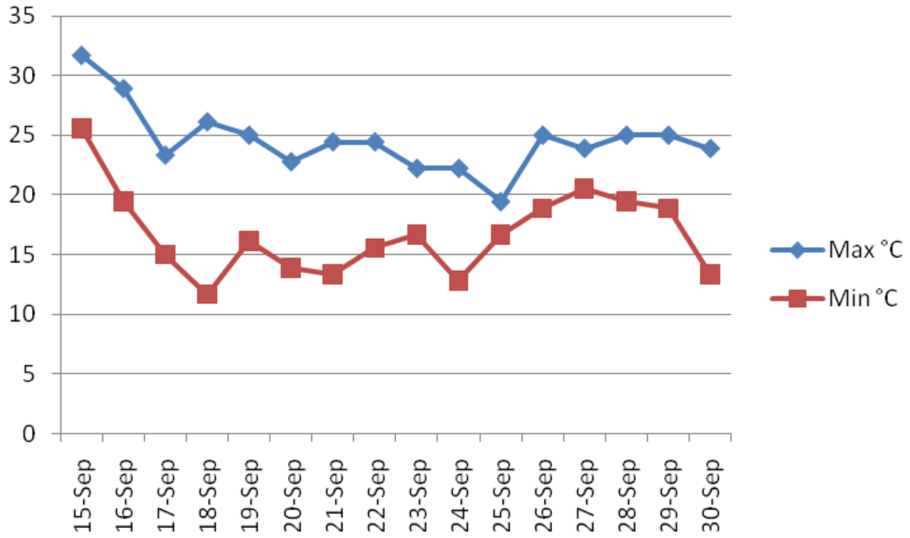

Fig. 3.6. In 2008, the maximum and minimum temperatures (°C) between the day of Pythium blight inoculation (15 September) and the first appearance of symptoms/signs (29 September).

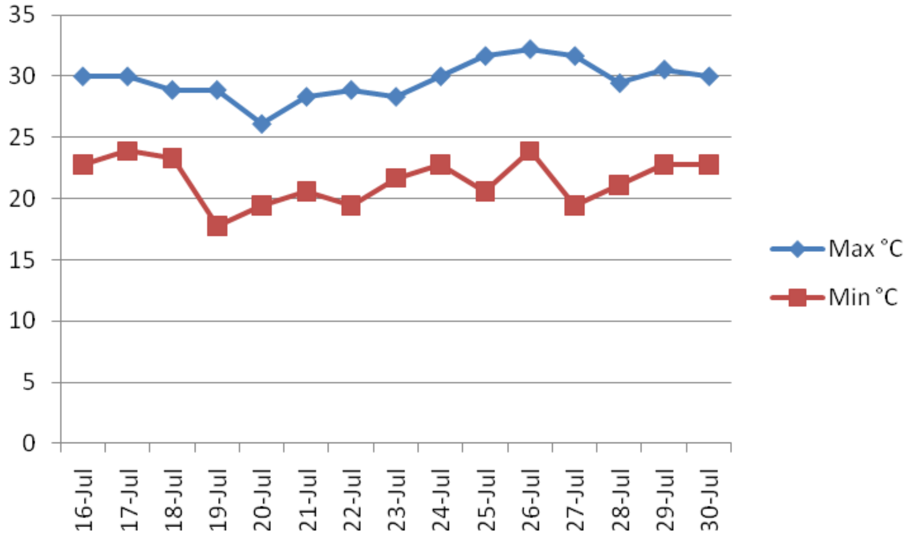

Fig. 3.7. In 2009, the maximum and minimum temperatures (°C) between the day of Pythium blight inoculation (16 July) and the first appearance of symptoms/signs (29 July).

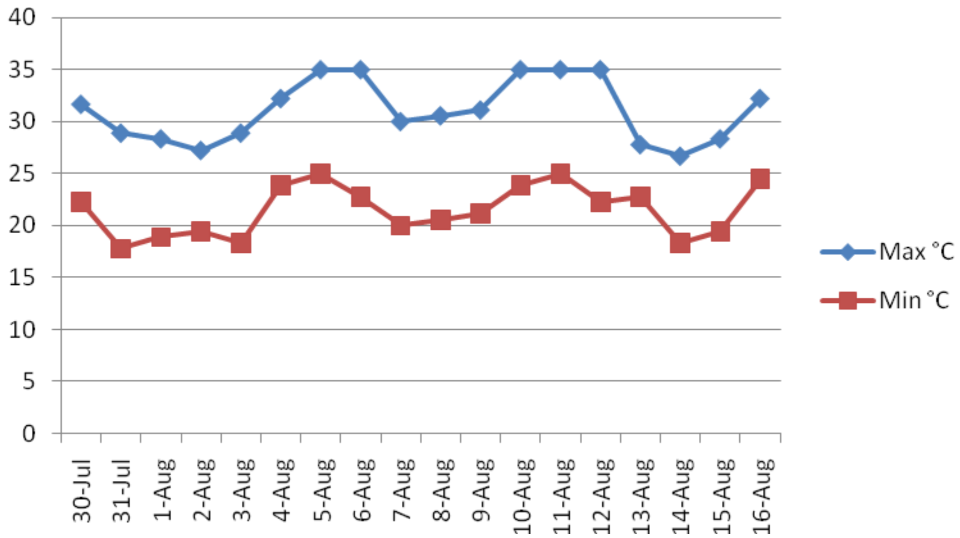

Fig. 3.8. In 2010, the maximum and minimum temperatures (°C) between the day of Pythium blight inoculation (30 July) and the first appearance of symptoms/signs (14 August).

CHAPTER 4: *IN VITRO* SENSITIVITY OF SNAP BEAN PYTHIUM BLIGHT PATHOGENS TO VARIOUS FUNGICIDES

Abstract:

Sensitivity of the pathogens causing Pythium blight on snap bean was determined *in vitro* against azoxystrobin, cyazofamid, mefenoxam, copper hydroxide, and potassium phosphite. These fungicide active ingredients are commonly used to manage Pythium blight and/or other diseases in snap bean and other vegetable production. Little research has been conducted on the sensitivity of the Pythium blight pathogen(s) to these fungicides. Twenty-two *Pythium* isolates were collected from symptomatic plants in Virginia, Georgia, and New Jersey; isolates collected include *P. aphanidermatum*, *P. deliense*, *P. ultimum*, and *P. myriotylum*. Corn meal agar was amended with four concentrations of each active ingredient: 0 µg/ml, 100 µg/ml, the µg/ml concentration equivalent to the label field recommended rate if applied on succulent beans at 187 L/ha, and the equivalent if applied at 374 L/ha. After inoculation, plates were incubated in the dark for 36 hours, and the percentage of growth reduction (GR) was determined. All isolates were completely sensitive (100% GR) to all active ingredients at the two labeled rates, except azoxystrobin. GR due to azoxystrobin showed a wide range. At the highest concentration (875 µg/ml) reductions ranged from 38.3% and 100%. At 100 µg/ml azoxystrobin concentration, one *P. deliense* isolate demonstrated 8.9% GR. All isolates were also completely sensitive to copper hydroxide at 100 µg/ml, and minimal reductions were observed on the mefenoxam-amended medium (lowest 91.9%). All *P. deliense* isolates were completely sensitive to cyazofamid, and the lowest GR at 100 µg/ml was 69.2% of a *P. aphanidermatum* isolate. At 100 µg/ml potassium phosphite, significant GR similarities were recorded within isolates of the same species, and less than 50% GR was observed in all 10 isolates of *P. deliense*. No relationship was observed

between collection location and isolate sensitivity. This research illustrates a general loss in sensitivity of Pythium blight pathogen populations to azoxystrobin, variation in *P. aphanidermatum* isolates to cyazofamid, and an inherently less sensitive reaction in *P. deliense* populations to potassium phosphite.

Introduction:

Snap bean (*Phaseolus vulgaris* L.) is susceptible to several diseases caused by *Pythium* species, including Pythium blight (Pfender and Hagedorn, 2005). The soilborne pathogen(s) can cause damage to the stems, leaves, and the bean pods, the latter being the most economically destructive phase of this disease. This disease is problematic in most snap bean-growing areas in the U.S., and several species of *Pythium* have been associated with disease symptoms or disease outbreaks (Adegbola and Hagedorn, 1969; Dominiak and Damicone, 2006; Drechsler, 1952; Harrison and Rideout, 2010; Harter and Zaumyer, 1931). Different *Pythium* spp. being reported as the causal agent may be the result of diverse climates within geographical regions, since species vary in their optimal growth temperature range (van der Plaats-Niterink, 1981). Harrison and Rideout (2010) identified *P. aphanidermatum*, *P. deliense*, *P. ultimum*, and *P. myriotylum* as pathogens causing Pythium blight of snap bean in areas of intensive vegetable production in Virginia, Georgia, and New Jersey. The Pythium blight pathogens possess a wide host range, including several economically important solanaceous and cucurbit crops, such as cucumbers (*Cucumis sativus* L.), eggplant (*Solanum melongena* L.), potato (*Solanum tuberosum* L.), tomato (*Solanum esculentum* L.), pumpkin (*Cucurbita maxima* L.), watermelon (*Citrullus lanatus* [Thunb.] Matsum. & Nakai var. *lanatus*), and melon (*Cucumis melo* L.) (Aoki et al., 2007; Blodgett, 1945; Drechsler, 1925; Dreschler, 1926; Sitterly and Keinath, 1996; Strand, 1998).

This extensive host range of the pathogen on vegetable crops contributes to population buildup within the soil resulting in severe outbreaks of Pythium blight on Eastern vegetable farms.

Snap bean and other vegetable growers rely heavily on cultural practices to manage damage caused by Pythium blight, but efficacious foliar fungicides can be an important management strategy when environmental conditions are optimal for disease (Rideout et al., 2010). Few fungicides are labeled for control of Pythium blight, and one aspect that hinders this process is the difficulty associated with conducting field trials with the pathogen(s). The sporadic and clustered nature of this disease in the field has led to inconsistent efficacy results (Damicone and Trent, 2004; Rideout et al., 2008). Potassium phosphite (ProPhyt 6.7F; Helena Chemical Company, Collierville, TN) currently possesses a Section 3 label for Pythium blight control and effectively managed Pythium blight on snap bean caused by *P. aphanidermatum* in one trial (Rideout et al., 2010). In 2008, the combination of mefenoxam and copper hydroxide (Ridomil Gold Copper 65WP; Syngenta Crop Protection, Greensboro, NC), which is effective against many oomycetes (Damicone and Trent, 2004; Rideout et al., 2010), received a section 24(c) label for use on snap beans in Delaware, Georgia, Maryland, and Virginia.

Some fungicides are effective in controlling Pythium blight but are not specifically labeled for its control on snap bean. Cyazofamid (Ranman 400SC; FMC Corporation, Philadelphia, PA) has shown efficacy in previous field trials against snap bean Pythium blight (Rideout et al., 2010), and in recent years, it has been commonly used to control downy mildew on cucurbits (Rideout et al., 2009). Cyazofamid is currently being evaluated by USDA’s IR-4 program and is expected to receive a label for use on Pythium blight of snap bean in the near future (www.csrees.usda.gov/funding/rfas/minor_crop.html). Cyazofamid belongs to the phenylimidazole fungicide class, which was introduced in 2004. Its mode of action disrupts mitochondrial electron transfer by specifically targeting the quinone-inside (Qi) site of the cytochrome *bc*$_1$ complex of Oomycetes and Plasmodiophoromycetes (Ohshima et al., 2004). Like phenylimidazoles, azoxystrobin and other strobiliruns also inhibit mitochondrial respiration and hinder spore germination and mycelial growth (Vincelli, 2002). Azoxystrobin (Quadris 2.08SC; Syngenta Crop Protection, Greensboro, NC) is labeled for use on various snap bean diseases, but, is not specifically labeled for control of Pythium blight. Since the launch of azoxystrobin in 1996, this active ingredient, as well as other strobilurins, has been heavily used to control fungal pathogens in vegetable production. All of the mentioned fungicides have provided suppression of Pythium blight caused by *P. aphanidermatum* on snap bean in limited trials (Damicone and Trent, 2004; Rideout et al., 2010), but control of other Pythium blight causal agents *P. deliense, P. ultimum,* and *P. myriotylum* has not yet been determined.

Understanding a pathogen's *in vitro* sensitivity to fungicides can be critical for constructing long-term disease and fungicide resistance management strategies. Since a fungicide's mode of action may involve preventative, curative, or vapor activity, knowledge of their direct affect on pathogen growth is necessary (Wong and Wilcox, 2001). Most fungicides are formulated to be stable under a wide range of environmental conditions, so when a product is properly applied according to the label instructions and is still not efficacious, it may be due to pathogen insensitivity (Sukul and Spiteller, 2000). In some cases, pathogen insensitivity to fungicides may not be the result of resistance development, but a result of inherent characteristics that vary among related species (Broders et al., 2007; Kato et al., 1990). Broders et al. (2007) showed that species that produce globose sporangia or hyphal swellings are generally less sensitive to azoxystrobin than those producing filamentous sporangia**.** Cook et al., (2009) showed that exposure to phosphorous acid had a slight, but significantly different, effect *in vitro* on two *Pythium* spp. causing Pythium blight on turfgrass. *P. aphanidermatum* isolates demonstrated EC_{50} values between 36 to 172 µg/mL and *P. myriotylum* isolates demonstrated EC_{50} values ranging from 124 to 221 µg/mL. These are examples of populations defined as inherently resistant or naturally insensitive (Brent and Hollomon, 2007). Some researchers define this resistance, or insensitivity, as existing in a population before even being exposed to the fungicide (Dagget et al., 1993; Kousik and Keinath, 2008).

Variations in fungicide sensitivity may result from resistance development within a population of a species. This type of resistance, or loss in sensitivity, develops after frequent use of the fungicide for control of that pathogen population. Loss in sensitivity to fixed copper bactericides has been reported for bacterial spot and speck of tomato and pepper, but this observation has not been made for *Pythium* spp. (Cuppels and Elmhirst 1999; Marco and Stall, 1983). Similarly, loss in sensitivity to azoxystrobin has been reported for several fungal pathogens (Mavroeidi and Shaw, 2005; Vinelli and Dixon, 2002; Wong and Wilcox, 2002). Pathogens within the Peronosporales have been effectively controlled by phenylamide fungicides since their introduction in the late 1970s (Morton and Urech, 1988). Fungicides within this chemistry target the organism’s ribosomal RNA polymerases, and due to this specific mode of action there is a high risk for the development of insensitive pathogen populations (Brent and Hollomon, 1998). Isolates of *Pseudoperonospora cubensis* resistant to the phenylamide metalaxyl were collected from cucumber just two years after its introduction, and resistance by a *Pythium* spp. was first reported on turfgrass in 1984 (Reuveni et al., 1980; Sanders, 1984). An isomer of metalaxyl, mefenoxam (Ridomil Gold 4SL; Syngenta Crop Protection, Greensboro, NC), has been used extensively in recent years to manage foliar oomycete pathogens. Resistance to mefenoxam has been observed in *P. aphanidermatum* on poinsettia and geranium in greenhouse production (Moorman et al., 2002; Moorman and Kim, 2004). Resistance to mefenoxam by *Phytophthora capsici* in cucurbit and pepper isolates, and *Phytophthora erythroseptica* and *Pythium ultimum* in potato isolates has been observed (Lamour and Hausbeck, 2000; Parra and Ristiano, 2001; Lamour and Hausbeck, 2003; Taylor et al., 2002; Taylor et al., 2006). Isolates of *Phytophthora capsici* that were insensitive to cyazofamid were collected from watermelon in 2008 (Kousik and Keinath, 2008). Although loss or variation in sensitivity has been reported in *Pythium* spp. causing Pythium blight against mefenoxam or cyazofamid in snap bean production, the presence of insensitivity in closely related pathogen species suggests that screening of Pythium blight isolates for sensitivity to mefenoxam, cyazofamid, and additional efficacious agrochemicals is warranted.

Materials and Methods:

Isolates

Twenty-two isolates were used in this study, which were collected from 2007 to 2010 and from 10 different agricultural fields in Virginia, Georgia, and New Jersey (Table 4.1). All isolations were taken from plant tissue showing water-soaking and/or white, cottony mycelial growth. Isolates were from recovered predominantly snap bean, but were also collected from zucchini (*Cucurbita pepo* L.), summer squash (*Cucurbita pepo* L.), cucumber (*Cucumis sativus* L.), and eggplant (*Solanum melongena* L.). This collection has four *Pythium* spp.: *P. aphanidermatum*, *P. deliense*, *P. ultimum*, and *P. myriotylum*. These species were identified as problematic pathogens causing Pythium blight of snap bean and other crops in the Eastern U.S. (Harrison and Rideout, 2010).

Symptomatic tissue was surface disinfested with a 10% sodium hypochlorite solution for 1 min and transferred to PARF. This medium is similar to PARP selective medium (pimaricin, ampicillin, rifampicin, and PCNB) (Eckert and Tsao, 1962), except PCNB was replaced with fludioxonil (Maxim 4FS, Syngenta Crop Protection, Greensboro, NC), which provides selection against *Rhizoctonia* and *Fusarium* spp. Due to the reduction in use of PCNB because of health and environmental concerns, a replacement product, fludioxonil, that controls other prevalent soil fungi was used. PARF was made by preparing corn meal agar (CMA) (BD Diagnostics, Sparks, MD; 17 g/L dH_2O) and adding antibiotic suspensions after the agar cooled to 45-50°C. Pimaricin (>95% a. i., Sigma Chemical Co., St. Louis, MO; 20 µg/ml) was added to prevent fungal growth. Rifampicin (Rifampicin SV, sodium salt, Sigma Chemical Co.; 10 µg/ml) and ampicillin (>95% a. i., Sigma Chemical Co.; 250 µg/ml) were added to prevent bacterial growth. Maxim 4FS (fludioxonil; Syngenta Crop Protection, Greensboro, NC) was added to a final concentration of 60 µg/ml).

Pathogenicity tests

Each isolate was characterized by morphology and sequence analysis of the rDNA-internal transcribed spacer (ITS) regions (Chapter 2) and pathogenicity on snaps bean was verified. Isolates used for pathogenicity testing were grown on CMA for 2 days at 22°C. Snap bean pods (cultivar ‘Bronco’) were surface sterilized in 5% sodium hypochlorite for 1 min and rinsed twice in sterile water. Agar plugs (3-mm diameter) were cut from the leading edge of the growing culture and immediately placed on the center of a non-wounded snap bean pod. Each agar plug was covered with a 0.5 ml Eppendorf™ microfuge tube cap and the edge of the cap was sealed with petroleum jelly. Pods were then placed in a plastic Ziploc™ bag lined with moistened paper towels and sealed to retain high humidity. Pods were incubated at 25°C for 4 days. Four replications of each isolate were conducted. The presence of water-soaked lesions surrounding the inoculation sites verified pathogenicity. Ten symptomatic bean pods were randomly selected, and the pathogen was re-isolated to complete Koch's postulates.

Fungicide sensitivity screening

In vitro screening of 22 isolates was performed for sensitivity to azoxystrobin, cyazofamid, mefenoxam, copper hydroxide, and potassium phosphite. CMA was amended with 4 concentrations of each fungicide after autoclaving and cooling to 50°C: 0, 100 μg/ml, the μg/ml concentration equivalent to the labeled field rate if applied on succulent beans at a 187 L/ha output, and the equivalent if applied at a 374 L/ha output (Table 4.2). Each label states the kg/L or percentage of active ingredient contained in the fungicide. This information and the recommended field application rate (also stated on the label) were used to calculate the active ingredient concentration contained in the fungicide application. Quadris 2.08SC (Syngenta Crop Protection, Greensboro, NC) was used to make the azoxystrobin-amended medium. Because Ranman 400SC (cyazofamid; FMC Corporation, Philadelphia, PA) is not currently labeled for control of snap bean Pythium blight, concentrations were calculated using rates that provided efficacy against this disease in one experimental trial based upon labeled rates on other vegetable crops (Rideout et al., 2010). The mefenoxam and copper hydroxide concentrations were determined using labeled rates of Ridomil Gold Copper 65WP. Ridomil Gold 4SL (mefenoxam; Syngenta Crop Protection, Greensboro, NC) was used to make the mefenoxam-amended medium, and copper hydroxide plates were made by using Kocide 3000DF (46.1% copper hydroxide; DuPont Crop Protection, Wilmington, DE). ProPhyt 6.7F (potassium phosphite) was used to amend the phosphorus acid medium, and the concentrations were calculated according to the equivalent (34.3%) as stated on the label. All plates were stored in the dark for 2 days at 22°C before inoculation.

Each isolate was grown on CMA agar for 2 days at 22°C. Agar plugs (5-mm diameter) were cut from the outer edge of the growing culture and placed in the center of a fungicide-amended plate and a non-amended plate. Plates were incubated in the dark at for 36 hours at 25°C, since this falls within the optimal temperature range for all tested species. The growth diameter was measured in two perpendicular directions on each culture plate, the measurements were averaged, and the diameter of the mycelia plug was subtracted. The percentage of growth reduction was then calculated using this formula: (100 – [growth of amended plate/ growth of non-amended] x 100). This test was replicated 4 times for each active ingredient concentration and isolate.

The percentage of growth reduction was subjected to an analysis of variance (ANOVA) and comparison of means using Student-Newman-Keuls (SNK), which was performed using SAS (version 9.2, SAS Institute, Cary, NC). Analyses were only performed within the same fungicide treatment.

Results:

Azoxystrobin

Only one isolate of *P. deliense* (P036) was completely sensitive (defined as 100% growth reduction, or GR) to azoxystrobin at all concentrations, and only three *P. aphanidermatum* (P003, P055, and P076) and three *P. deliense* (P029, P033, and P036) isolates were completely sensitive to the highest concentration (Table 4.3). A wide variation in sensitivity was observed at all concentrations. Isolate P025 had the lowest GR at the labeled rate concentrations, 29.7% and 38.3%. No apparent pattern was observed within isolates of the same species and their level of sensitivity, except for the majority of *P. aphanidermatum* isolates showing high to moderate sensitivity. Similarly, no apparent relationship was observed between geographic collection locations and sensitivity of isolates to azoxystrobin. The GR of the two *P. ultimum* isolates (P002 and P073) was significantly different at all concentrations. The *P. myriotylum* (P060) and the two *P. ultimum* isolates seemed to be less affected by the increase between the moderate and highest azoxystrobin concentrations compared to the other species (1.3%, 3.1%, and 6.2% respectively). P029 (*P. deliense*) had a difference of 16.2%.

Cyazofamid

No growth of any of the isolates was observed on plates amended with the two highest concentrations of cyazofamid, which are equivalent to the labeled rates (Table 4.4). Two *P. aphanidermatum* isolates (P003 and P094) from Virginia were 100% sensitive at the 100 ppm treatment, and all other *P. aphanidermatum* isolates were not as sensitive. The GR among isolates of this species ranged from 69.2% to 100%. All isolates of *P. deliense* and the one isolate of *P. myriotylum* (P060) were completely sensitive to the active ingredient. At the 100 ppm concentration, both of the *P. ultimum* isolates (P002 and P073) had a significantly lower GR than those isolates that had no GR. No pattern was observed between geographic collection locations and level of sensitivity of isolates to cyazofamid.

Mefenoxam/Copper hydroxide

Copper hydroxide is a companion material to mefenoxam in Ridomil Gold Copper 65WP (Syngenta Crop Protection, Greensboro, NC), but the active ingredients were tested separately. No growth was observed on any copper hydroxide-amended plates for all isolates.

No growth of any of the isolates was observed on plates amended with the two highest concentrations of mefenoxam, which are equivalent to the labeled rates (Table 4.5). The GR caused by the 100 ppm treatment only ranged from 91.9% to 100%, but statistical differences were still observed. Both of the *P. ultimum* isolates (P002 and P073) were completely sensitive (100% GR) to the active ingredient. *P. myriotylum* (P060) had a 95.2% GR and was significantly different than the isolates having 98% to 100% GR. *P. aphanidermatum* had the widest range of %GR, which ranged from 91.9% to 100%. All *P. deliense* isolates were at least slightly affected by mefenoxam, GR ranging from 92.4% to 98.2%. No pattern was observed between geographic collection locations and sensitivity of isolates to mefenoxam.

Potassium phosphite

No growth of any of the isolates was observed on plates amended with the two highest concentrations, which are equivalent to the labeled rates (Table 4.6). At the 100 ppm concentration, potassium phosphite caused a wide range of GR, from 35.3% to 86.1%. Similar GR was observed within species, and a statistical difference was usually observed between species. *P. aphanidermatum* was most sensitive, and the lowest observed GR was 75.2% (P084). This species was significantly more sensitive than the other screened species. The *P. myriotylum* isolate (P060) had the closest GR at 58.6%, which was statistically similar to GR of both *P. ultimum* isolates (P002 and P073). *P. deliense* was the least sensitive species to the 100 ppm concentration. Except for one case (isolates P040 and P050), all *P. deliense* GR were statistically similar to each other and statistically different from other species. No pattern was observed between geographic collection locations and sensitivity of isolates to potassium phosphite.

Discussion:

Repeated use of fungicides possessing the same mode of action may lead to the development of insensitive pathogen populations (Brent and Hollomon, 2007; www.frac.info/). Immediately following fungicide applications, less sensitive individuals exist at low frequencies. Subsequent fungicide applications containing the same mode of action causes selection for less sensitive isolates and results in increasingly insensitive populations (Gisi et al., 2000). In this study, 22 isolates of Pythium spp. were screened for sensitivity to five fungicides.

In general, each *Pythium* sp. reacted differently to each active ingredient. This outcome was expected, since the fungicide modes of action examined in this experiment were different. Also, if pathogens develop resistance to a fungicide, the process may be gradual or distinct. The detection of fungicide insensitivity may be gradual, caused by polygenic mutations occurring within the population, or distinctly and caused by a single gene mutation (Brent and Hollomon, 2007). This gradual development is also known as quantitative resistance, in which subpopulations are somewhat less sensitive but may still be controlled by higher labeled rates of the fungicide (Vincelli, 2002). Subpopulations with distinct, or qualitative resistance, are significantly less sensitive than the wild type population and cannot be controlled by higher labeled rates (Vincelli, 2002). In either case, the mutation(s) usually exist within the site of action of the fungicide in the pathogen population. Although mefenoxam and cyazofamid are single-site inhibitors and one mutation within the pathogen could render insensitivity, complete insensitivity or resistance development was not observed in any isolates to these fungicides at the field recommended rate. However, one *P. deliense* isolate (P025) demonstrated 8.9% GR at 100 µg/ml azoxystrobin, and this isolate may represent a mutant subpopulation that has developed resistance to the fungicide.

In-season foliar fungicide applications are an important management strategy against Pythium blight on snap bean and other vegetable crops. Frequent evaluation of fungicide efficacy in the field and laboratory to monitor population sensitivity is warranted. The active ingredients currently labeled for control of Pythium blight on snap beans in Virginia and other snap bean producing states (mefenoxam plus copper hydroxide, and potassium phosphite) provided complete *in vitro* efficacy when tested at labeled concentrations against isolates representing four different *Pythium* spp. However, examining sensitivity results using the discriminatory parameter of $EC_{50} \geq 100$ µg/ml commonly used by researchers for determining pathogen resistance to a fungicide (Mondal et al., 2005), 10 *P. deliense* isolates were identified as less sensitive to potassium phosphite.

Although insensitivity to mefenoxam has been detected in several *Pythium* spp. in greenhouse and vegetable production systems (Moorman and Kim, 2004; Lamour and Hausbeck, 2000; Parra and Ristiano, 2001; Lamour and Hausbeck, 2003; Taylor et al., 2002; Taylor et al., 2006), it was not observed for any *Pythium* isolates examined in this study. However, some insensitivity was observed in isolates of *P. aphanidermatum, P. deliense,* and *P. myriotylum* at the 100 µg/ml concentration. The percent growth reduction by mefenoxam was too high for any isolates to be considered resistant according to standards outlined by Sanders (1984), which states that isolates should be considered mefenoxam- or metalaxyl-resistant if growth is not slowed by at least 50% at 100 μg/ml ($EC_{50} \geq 100$ μg/ml). This research supports that labeled application rates of mefenoxam should provide adequate Pythium blight management.

Copper hydroxide is a companion active ingredient to mefenoxam in Ridomil Gold Copper 65WP, providing broad spectrum suppression of fungal and bacterial pathogens and theoretical resistance management. Copper hydroxide is a contact, protectant material (whereas mefenoxam is systemic). Its mode of action is not fully understood; therefore, the pathogen's mode of resistance, if it exists, is also unknown (Klittich, 2008). Copper hydroxide present on plant surfaces should provide protection against the *Pythium* spp. causing Pythium blight, since a concentration (100 ug/ml) lower than the labeled rate inhibited pathogen growth *in vitro*.

On the ProPhyt 6.7F label, Helena Chemical Company states that phosphorus acid is "effective in prevention and control of downy mildew and *Phytophthora*, by activation of the plants' natural resistance mechanism, as well as by direct activity of the fungus." (www.cdms.net/LDat/ld5KK006.pdf) Otherwise, the specific mode of action is largely unknown, although limited research provides some suggestions. Niere et al. (1994) showed that high concentrations of potassium phosphite *in vitro* caused increased accumulation of pyrophosphate and polyphosphate within cells of three *Phytophthora* spp., which inhibits phosphate metabolism. This accumulation is believed to divert energy away from other metabolic pathways, which leads to decrease in growth. Although the label does not specifically mention *Pythium*, this research indicates that potassium phosphite does have a direct effect on *Pythium* spp. *in vitro* at labeled rates. At a concentration (100 μg/ml) considerably lower than the labeled rate, significant variation was observed between species, which is supported by the work conducted by Cook et al. (2009). All 10 *P. deliense* isolates screened demonstrated GR less than 50% at 100 μg/ml, indicating that inherent insensitivity is present within populations of this species. Because the variation in sensitivity was observed at a rate much lower than the field recommended rate (18,873 μg/ml), theoretically, there is minimal risk of less sensitive individuals surviving and increasing within the pathogen population, as long as the fungicide is applied according to label directions. However, the pH of the medium was not recorded, so the 100% GR of isolates at the two highest concentrations may be a result of inhibitive acidity levels.

There is evidence for either a loss in sensitivity or variation in response to azoxystrobin from the Pythium blight pathogens tested in this study. An isolate of *P. deliense* (P025) had 8.9% GR at the discriminatory concentration of 100 μg/ml. Damicone and Trent (2004) found that Quadris 2.08SC (a.i. azoxystrobin; 0.22 kg a.i./ha) did not significantly decrease Pythium blight caused by *P. aphanidermatum.* However, in a field trial conducted by Rideout et al. (2010), Quadris 2.08SC applied at rates of 0.27 kg a.i./ha was effective at reducing disease levels. Frequent use of strobilurin fungicides (Quinone outside inhibitors-QoI), such as azoxystrobin, in vegetable production may have caused an increase of insensitivity within the Pythium blight pathogen populations, because these isolates were collected from agricultural fields presumably exposed to azoxystrobin or other QoI fungicides. Several researchers have also included salicylhydroxamic acid (SHAM) in strobilurin-amended media, which inhibits the action of the alternative oxidase respiratory pathway in *Pythium* spp. (Broders et al., 2007). Although SHAM was not used in this research, it is hypothesized that its addition may have potentiated the growth inhibition caused by the azoxystrobin amendment.

Cyazofamid belongs to a new class of fungicides introduced in 2004 (Ohshima et al., 2004). Although Ranman 400SC (a.i. cyazofamid) has been commonly used in vegetable production in recent years, only slight insensitivity was detected in Pythium blight pathogen isolates. Kousik and Keinath (2008) identified 13 isolates of *Phytophthora capsici* collected in the southeastern U.S. that were insensitive to cyazofamid. In their work, isolates characterized as insensitive had an EC_{50} of ≥ 100 µg/ml. According to this standard, none of the Pythium blight isolates tested in this study are insensitive, although three *P. aphanidermatum* isolates (two from Virginia and one from Georgia) approached this sensitivity level with growth reductions of 69%-73% at 100 µg/ml. If cyazofamid receives a federal label for control of Pythium blight in the near future, care should be taken to rotate or tank-mix this fungicide with materials possessing different modes of action to prevent the buildup of cyazofamid-resistant *Pythium* populations.

The *in vitro* fungicide effect on a pathogen may not always be an accurate indication of fungicide effect in the field. Also, fungicides may have differing effects on mycelial growth compared to spore germination. For example, spore germination of *Alternaria solani* was more sensitive to the strobiliruns than mycelial growth (Pasche et al., 2004). In the field, a *Pythium* isolate may also be identified as less sensitive to a fungicide because of superior fitness attributes, such as sporulation capacity or the ability to cause a larger lesion size (Gisi et al., 2000). Only the colony growth was used to assess fungicide sensitivity in this research, and in the future, the measurement of such attributes could provide insight to the possibility of this pathogen to cause an epidemic. *In vivo* screening in the field will also be important in detecting the degree of pathogen sensitivity in a field setting.

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Table 4.1. Origin and host of *Pythium* isolates used for *in vitro* sensitivity screening against azoxystrobin, cyazofamid, mefenoxam, copper hydroxide, and potassium phosphite.

Isolate	***Pythium* spp.**	**Host**	**Location & Year Isolated**
P001	*P. aphanidermatum*	Snap bean	Tifton, GA Oct. 2007
P002	*P. ultimum*	Snap bean	Painter, VA Nov. 2007
P003	*P. aphanidermatum*	Snap bean	New Church, VA May 2008
P021	*P. aphanidermatum*	Zucchini	Tifton, GA June 2009
P025	*P. deliense*	Summer squash	Tifton, GA June 2009
P029	*P. deliense*	Snap bean	Americus, GA June 2009
P033	*P. deliense*	Snap bean	Americus, GA June 2009
P034	*P. deliense*	Snap bean	Chula, GA June 2009
P036	*P. deliense*	Snap bean	Chula, GA June 2009
P040	*P. deliense*	Snap bean	Chula, GA June 2009
P043	*P. deliense*	Snap bean	Chula, GA June 2009
P044	*P. deliense*	Snap bean	Chula, GA June 2009
P050	*P. deliense*	Snap bean	Chula, GA June 2009
P053	*P. deliense*	Snap bean	Chula, GA June 2009
P055	*P. aphanidermatum*	Summer squash	Manor, GA June 2009
P060	*P. myriotylum*	Snap bean	Seaview, VA June 2009
P065	*P. aphanidermatum*	Cucumber	Suffolk, VA August 2009
P071	*P. aphanidermatum*	Zucchini	Vineland, NJ Sept. 2009
P073	*P. ultimum*	Eggplant	Vineland, NJ Sept. 2009
P076	*P. aphanidermatum*	Snap bean	Tifton, GA Oct. 2009
P084	*P. aphanidermatum*	Snap bean	New Church, VA June 2010
P094	*P. aphanidermatum*	Summer squash	Painter, VA July 2010

Table 4.2. Concentrations of each active ingredient used to test the sensitivity of 22 *Pythium* isolates that cause Pythium blight on snap bean. Concentrations of 0 and 100 µg/ml were also used.

Active ingredient	**Fungicide labeled field rate**[a]	**374 L/ha output equivalent (µg/ml)**[b]	**187 L/ha output equivalent (µg/ml)**[b]
Azoxystrobin	0.7 L/ha (Quadris 2.08SC)	438	875
Cyazofamid	0.2 L/ha (Ranman 400SC)	214	428
Mefenoxam	2.2 kg/ha (Ridomil Gold Cu 65WP)	300	600
Copper hydroxide	2.2 kg/ha (Ridomil Gold Cu 65WP)	3,595	7,190
Potassium phosphite	7 L/ha (ProPhyt 6.7F)	9,436	18,872

[a] The recommended field rate as stated on each fungicide label for application on succulent beans.

[b] The µg/ml concentration equivalent to the labeled field rate if applied on succulent beans at a 187 L/ha output, and the equivalent if applied at a 374 L/ha output.

Table 4.3. Mean percentage of growth reduction of four species of *Pythium* causing Pythium blight evaluated on media amended with azoxystrobin at different concentrations. Isolates were collected from multiple locations.

Isolate	Species	Location	Fungicide concentration (ppm)[a]		
			100	**438**	**875**
P036	*deliense*	GA	100 a	100 a	100 a
P055	*aphanidermatum*	GA	88.5 b	96.8 ab	100 a
P076	*aphanidermatum*	GA	87.4 b	96.5 ab	100 a
P021	*aphanidermatum*	GA	84.6 bc	93.8 abc	95.4 ab
P094	*aphanidermatum*	VA	83.3 bcd	96.8 ab	100 a
P071	*aphanidermatum*	NJ	83.0 bcd	94.3 abc	94.3 ab
P003	*aphanidermatum*	VA	81.1 bcd	94.4 abc	100 a
P002	*ultimum*	VA	79.6 bcd	82.6 cde	85.7 cd
P050	*deliense*	GA	78.5 bcde	84.2 cd	90.8 bc
P033	*deliense*	GA	73.2 cdef	90.6 abc	100 a
P084	*aphanidermatum*	VA	72.1 def	89.6 abc	93.5 ab
P065	*aphanidermatum*	VA	71.4 def	89.5 abc	96.2 ab
P053	*deliense*	GA	71.2 def	82.6 cde	90.2 cd
P029	*deliense*	GA	70.7 def	83.8 cd	100 a
P040	*deliense*	GA	66.7 ef	77.2 def	85.9 cd
P060	*myriotylum*	VA	66.6 ef	85.7 bcd	87.0 cd
P034	*deliense*	GA	66.4 ef	75.6 def	79.4 e
P001	*aphanidermatum*	GA	65.8 ef	86.4 bcd	94.2 ab
P073	*ultimum*	NJ	65.1 f	70.6 f	76.8 e
P043	*deliense*	GA	63.8 f	72.3 ef	82.5 de
P044	*deliense*	GA	60.6 f	75.1 def	82.3 de
P025	*deliense*	GA	8.9 g	29.7 g	38.3 f

[a] Values are the mean percent growth reduction compared to the non-amended control of the isolate. Values in a column followed by the same letter are not significantly different according to Student-Newman-Keuls ($P \leq 0.05$).

Table 4.4. Mean percentage of growth reduction of four species of *Pythium* causing Pythium blight evaluated on media amended with cyazofamid at different concentrations. Isolates were collected from multiple locations.

Isolate	**Species**	**Location**	**Fungicide concentration (ppm)[a]**		
			100	**214**	**428**
P003	*aphanidermatum*	VA	100 a	100 a	100 a
P094	*aphanidermatum*	VA	100 a	100 a	100 a
P025	*deliense*	GA	100 a	100 a	100 a
P029	*deliense*	GA	100 a	100 a	100 a
P033	*deliense*	GA	100 a	100 a	100 a
P034	*deliense*	GA	100 a	100 a	100 a
P036	*deliense*	GA	100 a	100 a	100 a
P040	*deliense*	GA	100 a	100 a	100 a
P043	*deliense*	GA	100 a	100 a	100 a
P044	*deliense*	GA	100 a	100 a	100 a
P050	*deliense*	GA	100 a	100 a	100 a
P053	*deliense*	GA	100 a	100 a	100 a
P060	*myriotylum*	VA	100 a	100 a	100 a
P002	*ultimum*	VA	90.3 b	100 a	100 a
P071	*aphanidermatum*	NJ	85.5 c	100 a	100 a
P021	*aphanidermatum*	GA	82.5 d	100 a	100 a
P001	*aphanidermatum*	GA	81.1 d	100 a	100 a
P076	*aphanidermatum*	GA	81.0 d	100 a	100 a
P073	*ultimum*	NJ	80.4 d	100 a	100 a
P055	*aphanidermatum*	GA	72.9 e	100 a	100 a
P084	*aphanidermatum*	VA	72.2 e	100 a	100 a
P065	*aphanidermatum*	VA	69.2 f	100 a	100 a

[a] Values are the mean percent growth reduction compared to the non-amended control of the isolate. Values in a column followed by the same letter are not significantly different according to Student-Newman-Keuls ($P \leq 0.05$).

Table 4.5. Mean percentage of growth reduction of four species of *Pythium* causing Pythium blight evaluated on media amended with mefenoxam at different concentrations. Isolates were collected from multiple locations.

Isolate	Species	Location	Fungicide concentration (ppm)[a]		
			100	**300**	**600**
P002	*ultimum*	VA	100 a	100 a	100 a
P073	*ultimum*	NJ	100 a	100 a	100 a
P055	*aphanidermatum*	GA	100 a	100 a	100 a
P084	*aphanidermatum*	VA	100 a	100 a	100 a
P071	*aphanidermatum*	NJ	98.2 ab	100 a	100 a
P025	*deliense*	GA	98.2 ab	100 a	100 a
P053	*deliense*	GA	96.0 bc	100 a	100 a
P021	*aphanidermatum*	GA	95.9 bc	100 a	100 a
P050	*deliense*	GA	95.8 bc	100 a	100 a
P029	*deliense*	GA	95.7 bc	100 a	100 a
P040	*deliense*	GA	95.5 bc	100 a	100 a
P036	*deliense*	GA	95.3 bc	100 a	100 a
P001	*aphanidermatum*	GA	95.3 bc	100 a	100 a
P060	*myriotylum*	VA	95.2 bc	100 a	100 a
P076	*aphanidermatum*	GA	95.0 bc	100 a	100 a
P033	*deliense*	GA	95.0 bc	100 a	100 a
P094	*aphanidermatum*	VA	94.6 bc	100 a	100 a
P003	*aphanidermatum*	VA	94.0 c	100 a	100 a
P034	*deliense*	GA	93.9 c	100 a	100 a
P043	*deliense*	GA	93.3 c	100 a	100 a
P044	*deliense*	GA	92.4 c	100 a	100 a
P065	*aphanidermatum*	VA	91.9 c	100 a	100 a

[a] Values are the mean percent growth reduction compared to the non-amended control of the isolate. Values in a column followed by the same letter are not significantly different according to Student-Newman-Keuls ($P \leq 0.05$).

Table 4.6. Mean percentage of growth reduction of four species of *Pythium* causing Pythium blight evaluated on media amended with potassium phosphite at different concentrations. Isolates were collected from multiple locations.

Isolate	**Species**	**Location**	**Fungicide concentration (ppm)[a]**		
			100	**9,436**	**18,873**
P021	*aphanidermatum*	GA	86.1 a	100 a	100 a
P071	*aphanidermatum*	NJ	81.0 ab	100 a	100 a
P065	*aphanidermatum*	VA	80.6 ab	100 a	100 a
P055	*aphanidermatum*	GA	79.3 ab	100 a	100 a
P076	*aphanidermatum*	GA	78.8 ab	100 a	100 a
P003	*aphanidermatum*	VA	77.3 b	100 a	100 a
P001	*aphanidermatum*	GA	76.7 b	100 a	100 a
P094	*aphanidermatum*	VA	76.5 b	100 a	100 a
P084	*aphanidermatum*	VA	75.2 b	100 a	100 a
P060	*myriotylum*	VA	58.6 c	100 a	100 a
P073	*ultimum*	NJ	56.0 c	100 a	100 a
P002	*ultimum*	VA	55.6 c	100 a	100 a
P040	*deliense*	GA	45.6 d	100 a	100 a
P043	*deliense*	GA	44.9 de	100 a	100 a
P025	*deliense*	GA	43.9 de	100 a	100 a
P044	*deliense*	GA	43.6 de	100 a	100 a
P033	*deliense*	GA	43.5 de	100 a	100 a
P053	*deliense*	GA	43.5 de	100 a	100 a
P029	*deliense*	GA	43.4 de	100 a	100 a
P034	*deliense*	GA	43.3 de	100 a	100 a
P036	*deliense*	GA	41.5 de	100 a	100 a
P050	*deliense*	GA	35.3 e	100 a	100 a

[a] Values are the mean percent growth reduction compared to the non-amended control of the isolate. Values in a column followed by the same letter are not significantly different according to Student-Newman-Keuls ($P \leq 0.05$).

APPENDIX A

Table A.1. Morphological characteristics of the *Pythium* spp. included in this study as described by van der Plaats-Niterink (1981) and Waterhouse (1968).

Isolate[a]	**Species**[b]	**Sporangia**	**Hyphal swelling**	**Oogonial surface**	**Filling oogonium**	**No. of antheridia**	**Antheridia**[b] **attachment**	**Oospore**[c] **size (µm)**
P001	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	D-m	20-23
P002	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	17-19
P003	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-D	20-22
P004	*aphanidermatum*	Branched, lobate	—	Smooth	Aplerotic	1	M-D	21-23
P007	*aphanidermatum*	Branched lobate terminal	—	Smooth	Aplerotic	1-2	D-m	19-21
P008	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1	M-d	20-23
P009	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1	M-D	19-22
P010	*aphanidermatum*	Unbranched lobate	—	Smooth	Aplerotic	1-2	D-m	19-21
P011	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-D	20-21
P012	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1	M-D	19-22
P013	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1	M-d	20-22
P014	*aphanidermatum*	Unbranched lobate terminal	—	Smooth	Aplerotic	1	M-d	20-22
P015	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-D	20-23
P016	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-D	20-22
Ref.	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	16-20

[a] Ref.= previously characterized reference isolates. *Pythium aphanidermatum* and *P. ultimum* reference isolates were obtained from Gary Moorman (Pennsylvania State Univ.).

[b] M= monoclinous, D= diclinous. Capitalized letters represent the predominant form of antheridia attachment observed; lower case letters represent occasional observations.

[c] Range of oospore size measurements of 20 randomly selected oogonia per isolate.

Table A.1 cont. Morphological characteristics of the *Pythium* spp. included in this study as described by van der Plaats-Niterink (1981) and Waterhouse (1968).

Isolate[a]	**Species**[b]	**Sporangia**	**Hyphal swelling**	**Oogonial surface**	**Filling oogonium**	**No. of antheridia**	**Antheridia**[b] **attachment**	**Oospore**[c] **size (µm)**
Ref.	*myriotylum*	Filamentous, intercalary	—	Smooth	Aplerotic	4-7	D-m	21-26
Ref.	*aphanidermatum*	Branched lobate, terminal	—	Smooth	Aplerotic	1-2	M-D	20-23
Ref.	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1		17-18
P021	*aphanidermatum*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1-2	M-d	21-23
P022	*deliense*	Branched lobate, terminal	—	Smooth	Aplerotic	1		18-19
P023	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1		16-19
P024	*deliense*	Branched, lobate terminal	—	Smooth	Aplerotic	1		18-19
P025	*deliense*	Unbranched lobate, intercalary/terminal	—	Smooth	Aplerotic	1		14-18
P026	*aphanidermatum*	Branched lobate, terminal	—	Smooth	Aplerotic	1-2	M-d	20-23
P027	*deliense*	Branched & unbranched lobate	—	Smooth	Aplerotic	1		16-19
P028	*deliense*	Branched & unbranched lobate	—	Smooth	Aplerotic	1		15-18
P029	*deliense*	Branched & unbranched lobate	—	Smooth	Aplerotic	1		16-18
P030	*deliense*	Branched & unbranched lobate	—	Smooth	Aplerotic	1		15-18
P031	*deliense*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2		15-18
P032	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1	M	16-20
P033	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1		15-18
P034	*deliense*	Unbranched lobate, intercalary	—	Smooth	Aplerotic	1		15-17

[a] Ref.= previously characterized reference isolates. *Pythium aphanidermatum* and *P. ultimum* reference isolates were obtained from Gary Moorman (Pennsylvania State Univ.).

[b] M= monoclinous, D= diclinous. Capitalized letters represent the predominant form of antheridia attachment observed; lower case letters represent occasional observations.

[c] Range of oospore size measurements of 20 randomly selected oogonia per isolate.

Table A.1 cont. Morphological characteristics of the *Pythium* spp. included in this study as described by van der Plaats-Niterink (1981) and Waterhouse (1968).

Isolate[a]	**Species[b]**	**Sporangia**	**Hyphal swelling**	**Oogonial surface**	**Filling oogonium**	**No. of antheridia**	**Antheridia[b] attachment**	**Oospore[c] size (μm)**
P035	*deliense*	Branched & unbranched lobate	—	Smooth	Aplerotic	1	M	16-17
P036	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1	M	14-17
P037	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1	M	15-18
P038	*deliense*	Branched lobate, terminal	—	Smooth	Aplerotic	1	M	15-18
P039	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1	M	14-18
P040	*deliense*	Branched & unbranched lobate	—	Smooth	Aplerotic	1	M	15-16
P041	*deliense*	Unbranched lobate, intercalary	—	Smooth	Aplerotic	1	M-d	15-18
P042	*deliense*	Unbranched lobate, intercalary	—	Smooth	Aplerotic	1-2	M	15-18
P043	*deliense*	Unbranched lobate, intercalary	—	Smooth	Aplerotic	1	M	14-18
P044	*deliense*	Branched & unbranched lobate	—	Smooth	Aplerotic	1	M	15-16
P045	*deliense*	Branched & unbranched lobate	—	Smooth	Aplerotic	1	M	15-17
P046	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1	M-d	14-17
P047	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1	M	15-17
P048	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1	M	16-18
P049	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1	M	16-18
P050	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1	M	16-18
P051	*deliense*	Unbranched lobate, terminal	—	Smooth	Aplerotic	1	M	16-19

[a] Ref.= previously characterized reference isolates. *Pythium aphanidermatum* and *P. ultimum* reference isolates were obtained from Gary Moorman (Pennsylvania State Univ.).

[b] M= monoclinous, D= diclinous. Capitalized letters represent the predominant form of antheridia attachment observed; lower case letters represent occasional observations.

[c] Range of oospore size measurements of 20 randomly selected oogonia per isolate.

Table A.1 cont. Morphological characteristics of the *Pythium* spp. included in this study as described by van der Plaats-Niterink (1981) and Waterhouse (1968).

Isolate[a]	**Species**[b]	**Sporangia**	**Hyphal swelling**	**Oogonial surface**	**Filling oogonium**	**No. of antheridia**	**Antheridia**[b] **attachment**	**Oospore**[c] **size (μm)**
P052	*deliense*	Unbranched lobate, intercalary	—	Smooth	Aplerotic	1-2	D-m	15-18
P053	*deliense*	Unbranched lobate, intercalary	—	Smooth	Aplerotic	1-2	D-m	15-18
P054	*deliense*	Unbranched lobate, intercalary	—	Smooth	Aplerotic	1-2	D-m	16-18
P055	*aphanidermatum*	Branched, lobate	—	Smooth	Aplerotic	1-2	M-d	21-23
P056	*Pythium* spp.	Spherical	—	Smooth	Aplerotic	1	M	14-16
P057	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	16-20
P059	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	19-25
P060	*myriotylum*	Filamentous, terminal/intercalary	—	Smooth	Aplerotic	3-5	D	23-26
P061	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1	M-d	20-23
P062	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	22-25
P063	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	21-24
P064	*aphanidermatum*	Unbranched lobate terminal	—	Smooth	Aplerotic	1-2	M-d	20-24
P065	*aphanidermatum*	Unranched, lobate	—	Smooth	Aplerotic	1-2	M-d	20-23
P066	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	21-24
P067	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	20-23
P068	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	16-21

[a] Ref.= previously characterized reference isolates. *Pythium aphanidermatum* and *P. ultimum* reference isolates were obtained from Gary Moorman (Pennsylvania State Univ.).

[b] M= monoclinous, D= diclinous. Capitalized letters represent the predominant form of antheridia attachment observed; lower case letters represent occasional observations.

[c] Range of oospore size measurements of 20 randomly selected oogonia per isolate.

Table A.1 cont. Morphological characteristics of the *Pythium* spp. included in this study as described by van der Plaats-Niterink (1981) and Waterhouse (1968).

Isolate[a]	Species[b]	Sporangia	Hyphal swelling	Oogonial surface	Filling oogonium	No. of antheridia	Antheridia[b] attachment	Oospore[c] size (µm)
P069	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	16-21
P070	*catenulatum*	Irregular mycelial swellings, catenulate	Spherical	Smooth	Plerotic	5-10	D	22-27
P071	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	19-25
P072	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	16-20
P073	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	15-20
P074	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	17-21
P075	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	16-21
P076	*aphanidermatum*	Unbranched lobate	—	Smooth	Aplerotic	1	D-m	19-21
P077	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	17-21
P078	*myriotylum*	Filamentous, terminal/intercalary	—	Smooth	Aplerotic	2-7	D	20-24
P079	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	20-25
P080	*aphanidermatum*	Unbranched lobate terminal	—	Smooth	Aplerotic	1-2	M-d	22-25
P081	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	20-23
P082	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	15-22
P083	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	19-24
P084	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	20-24
P085	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	21-25

[a] Ref.= previously characterized reference isolates. *Pythium aphanidermatum* and *P. ultimum* reference isolates were obtained from Gary Moorman (Pennsylvania State Univ.).

[b] M= monoclinous, D= diclinous. Capitalized letters represent the predominant form of antheridia attachment observed; lower case letters represent occasional observations.

[c] Range of oospore size measurements of 20 randomly selected oogonia per isolate.

Table A.1 cont. Morphological characteristics of the *Pythium* spp. included in this study as described by van der Plaats-Niterink (1981) and Waterhouse (1968).

Isolate[a]	**Species**[b]	**Sporangia**	**Hyphal swelling**	**Oogonial surface**	**Filling oogonium**	**No. of antheridia**	**Antheridia**[b] **attachment**	**Oospore**[c] **size (µm)**
P086	*ultimum*	n/a	Spherical	Smooth	Aplerotic	1-2	M	15-20
P087	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	20-22
P088	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1	M-D	20-22
P089	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1	M-d	21-22
P090	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	20-22
P091	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	20-23
P092	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	19-23
P093	*aphanidermatum*	Unbranched lobate	—	Smooth	Aplerotic	1-2	M	21-23
P094	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	20-21
P095	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	19-22
P096	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1	M-d	20-22
P097	*aphanidermatum*	Unbranched lobate terminal	—	Smooth	Aplerotic	1-2	M-d	20-22
P098	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	20-23
P099	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	20-22
P100	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	20-24

[a] Ref.= previously characterized reference isolates. *Pythium aphanidermatum* and *P. ultimum* reference isolates were obtained from Gary Moorman (Pennsylvania State Univ.).

[b] M= monoclinous, D= diclinous. Capitalized letters represent the predominant form of antheridia attachment observed; lower case letters represent occasional observations.

[c] Range of oospore size measurements of 20 randomly selected oogonia per isolate.

Table A.1 cont. Morphological characteristics of the *Pythium* spp. included in this study as described by van der Plaats-Niterink (1981) and Waterhouse (1968).

Isolate[a]	**Species**[b]	**Sporangia**	**Hyphal swelling**	**Oogonial surface**	**Filling oogonium**	**No. of antheridia**	**Antheridia**[b] **attachment**	**Oospore**[c] **size (µm)**
P101	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	21-24
P102	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	20-23
P103	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	20-22
P104	*aphanidermatum*	Branched, lobate	—	Smooth	Aplerotic	1-2	M-d	21-25
P105	*aphanidermatum*	Branched lobate terminal	—	Smooth	Aplerotic	1-2	M-d	21-24
P106	*aphanidermatum*	Branched & unbranched lobate	—	Smooth	Aplerotic	1-2	M-d	20-23
P107	*aphanidermatum*	Branched, lobate terminal	—	Smooth	Aplerotic	1-2	M-d	21-24

[a] Ref.= previously characterized reference isolates. *Pythium aphanidermatum* and *P. ultimum* reference isolates were obtained from Gary Moorman (Pennsylvania State Univ.).

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[c] Range of oospore size measurements of 20 randomly selected oogonia per isolate

APPENDIX B

The ITS sequence of the unidentified *Pythium* sp. (isolate P056) did not align with any existing sequences in GenBank. It was identified to the genus level by morphological characters, and the results from the BLAST search showed the highest sequence similarity with *P. orthogonon*, with only 93% max identity. The partial sequence of the 18S ribosomal RNA gene, complete sequence of the internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, and partial sequence of the 28S ribosomal RNA gene is shown in FASTA format:

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>P056
CCACACCAAAAAAACTTTCCACGTGAACCGTTCTGTCCGTTTTGTGCCGTTTGCTGTT
GTCGGCGTTCGTTTGTTCGTCGAGAAGAGCAGAGTGGCTAAACGAAGGCTGTGAGC
TTCGTGCTTGCGGTCGATTTACTCTTTCAAACCCCATACGTTAAGAACTGAAGTATAC
TGTGAGGACGAAAGTCCTTGCTTTGAACTAGATAGCAACTTTCAGCAGTGGATGTCT
AGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAG
GATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGA
AGTATGTCTGTATCAGTGTCCGTACCTCAACCTTGCCTTTCTTTTTTTGTTTCCGGTGT
AGTCCGGAGCGGAAACGAGCAGACTTGAAGTGTCTCGCCTGTTGGTTTGCGAAAGC
AATTTTGCAACACAGCTGGGCGAGTCCTTTGAAATGGACACGATCTTTCTACTGTTTT
CTACGGAGTGTGGCGCTCGAAAAGCGGCGGTTTCCTTCGGGATCGCTCGCAGTTTGG
ACGGCGACTTTGGCGAATGCATATGGGAAGCAGACTCGACTCGCGGTACGTTAGGC
GTGCTGTTGTTGTTGTTGTTGTTGTTGCTTTGTTTGCAACGGCGGCGGCGGCGGCGGC
GTGCTGAACAATGTTGCGTGTTGTGGTCTTGTTTCCTGTGTTCGCTTCGAGGTGTACT
GTCTATTGGCTGTGAGAGTGAACCTTTGTGGGA
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