# CHARACTERIZATION, POPULATION GENETICS AND MANAGEMENT OF *PYTHIUM* SPP. FROM FLORICULTURE CROPS IN MICHIGAN

By

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**ABSTRACT**

CHARACTERIZATION, POPULATION GENETICS AND MANAGEMENT OF *PYTHIUM* SPP. FROM FLORICULTURE CROPS IN MICHIGAN

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Michigan ranks third in the U.S. for the wholesale value of floriculture products, with an estimated value of $375.7 million. Seedling damping-off, and root and crown rot are commonly incited by *Pythium* spp. and are important problems for greenhouse growers. *Pythium* spp. associated with Michigan's floriculture crops were characterized as a means to improve current management strategies. During 2011 and 2012, symptomatic potted poinsettias were sampled from nine greenhouses in Kent, Kalamazoo and Wayne counties. The following year, from the same three counties, symptomatic geranium and snapdragon bedding plants were sampled from 11 greenhouses. Isolates were confirmed to be *Pythium* spp. via morphology and sequencing of the ITS region. A total of 1,014 *Pythium* spp. isolates were obtained; *P. irregulare*, *P. ultimum*, and *P. aphanidermatum* were most prevalent. A subset of isolates was chosen for pathogenicity and mefenoxam sensitivity testing. Most of the *Pythium* species were virulent to germinating geranium seeds. This study suggests that mefenoxam may not be effective to control *P. ultimum* or *P. cylindrosporum*.

To understand the population dynamics of the *P. ultimum* collection simple sequence repeats (SSRs) were developed using *P. ultimum* transcriptome to address the population structure. After screening in-silico SSR markers, six SSRs were selected based on their polymorphism on a sub set of *P. ultimum* isolates. A total of 166 *P. ultimum* isolates were analyzed using the six fluorescent-labeled SSRs. The average genotypic diversity (0.938), evenness (0.56), and the recovery

of 12 major clones, out of the 64 multilocus genotypes obtained may suggest that *P. ultimum* is not a recent introduction into Michigan greenhouses. Analysis revealed a clonal population with limited differentiation among seasons, hosts and counties sampled. To develop strategies that limit Pythium root rot on geranium and snapdragon, greenhouse trials were conducted to test plant protectants and screen cultivars for resistance. Seven fungicides and two biological control agents were evaluated on plants inoculated with *P. aphanidermatum, P. irregulare* or *P. ultimum.* The AUDPC values differed significantly ($P<0.001$) among *Pythium* spp. and treatments. Mefenoxam and *Streptomyces lydicus* WYEC108 effectively controlled root rot on geranium. For snapdragon, treatment efficacy varied depending on the *Pythium* sp. When eleven geranium cultivars, and twelve snapdragon cultivars were inoculated with *P. aphanidermatum* or *P. irregulare*, none were completely resistant. However, geranium 'Nano White Hybrid' and 'Bulls Eye Cherry' and snapdragon 'Twinny White' and 'Candy Showers Yellow' were least susceptible. Integrating effective fungicides with less susceptible cultivars can limit disease caused by *Pythium* spp.

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# LITERATURE REVIEW

## *PYTHIUM* GENUS

*Pythium* is a fungal-like genus that belongs to the kingdom Straminipila, which includes brown algae and diatoms; phylum Oomycota; and class Oomycetes. The class includes Peronosporales lineage, including plant pathogens such as *Phytophthora infestans*, causal agent of the potato late blight, and *Peronospora* spp., causal agents of downy mildew in several crops are grouped (Adhikari et al., 2013, Schroeder et al., 2013). Originally, the oomycetes were considered fungi, and were classified as Phycomycetes. The organisms within this group produce mycelia characteristic of fungi, are parasites, and obtain nutrients by absorption. But several major characteristics differentiate them from true fungi and place them in the kingdom straminipila (Cavalier-Smith, 1998, Cooke et al., 2000), which results in (1) the sexual reproduction, in which fertilization of the oogonium by the anteridia results in an oospore; (2) The nuclear state of the vegetative mycelia is diploid; (3) The main components of the cell wall are cellulose and Beta-glucans; (4) The zoospores have both, a tinsel and a whiplash flagella; (5) The mycelia are aseptate(Rossman and Palm, 2006, Schroeder et al., 2013).

*Pythium* spp., in the order Pythiales, family Pithiaceae, contains approximately 150 species (Uzuhashi et al., 2010). The genus is ubiquitous and encompasses species inhabiting a wide range of ecological niches, such as marine ecosystems, undisturbed and cropped soils, and plant debris. Additionally, *Pythium* spp. have been found as facultative parasites, soil saprophytes, and parasite on fishes, crustaceans, mammals and plants in diverse environments (Martin and Loper, 1999, van der Plaats-Niterink, 1981).

As plant pathogens, *Pythium* spp. are able to affect a wide diversity of plants, causing seed, root and lower stem rot, as well as seedling damping-off. These pathogens reduce plant vigor and frequently reduce crop yield (Martin and Loper, 1999). The genus has been reported on wheat (Paulitz and Adams, 2003), soybean(Broders et al., 2007), turfgrass (Vargas Jr, 1994), on vegetable crops such as carrot (Lu et al., 2012), potato (Salas et al., 2003),cucumber (Benhamou et al., 2000), and tomato (Rafin et al., 1994)on ormanmental plants (Moorman et al., 2002)and nursery crops (Weiland et al., 2013).

**TAXONOMY AND PHYLOGENY**

Traditionally, *Pythium* species classification and identification have been based on morphological features of the reproductive structures. The sporangia can be filamentous, or globose, but in some species are absent. The oogonia can have smooth wall or ornamented with spines. The oospore can be pleriotic (if the oospore fills all the oogonia), or apleriotic (if there is a space between the walls of the oospore and the oogonia). In addition, characteristics such as the position of the anteridia in relation to oogonia and the shape of attachment are considered (Uzuhashi et al., 2010, van der Plaats-Niterink, 1981).

The genus *Pythium* was first described by Pringsheim in 1858, with *P. monospermum* Pringsh as the type species. In 1892, Fischer divided the genus in three subgenera based on sporangial morphology: Aphragmium, consisting of species with filamentous sporangia without a septum that separates it from the hypha; Nematosporagium was characterized by species with filamentous sporangia with a septum that separates the sporangia from the hypha; and Spharosporangium, which included species with globose sporangia delimited by septa from the hypha (Uzuhashi et al., 2010). Since

then, the taxonomy of the genus has been changed based on several morphological characteristics. Although the most recent taxonomic approaches have used molecular techniques, the classic monograph by van der Plaats Niterink (1981) and the keys of Dick (1990) are still being used for morphological identification(André Lévesque and De Cock, 2004, Uzuhashi et al., 2010).

Identification based on morphology alone is a limitation, because the characteristics mentioned above are often very similar among species and sometimes not all reproductive structures are formed in culture, making identification laborious and causing misidentification (André Lévesque and De Cock, 2004, Martin, 2000, Uzuhashi et al., 2010). Fortunately, the use of molecular methods such as sequencing of ribosomal DNA, the large nuclear ribosomal subunit (LSU), and the CoxI region have helped to elucidate and identify the species of *Pythium* (André Lévesque and De Cock, 2004, Martin, 2000, Uzuhashi et al., 2010).

According to the phylogeny of the genus by Lévesque and de Cock (2004), based on the ribosomal DNA internal transcribed spacer (ITS) region, and the D1-D3 domains of the large nuclear ribosomal subunit (LSU), *Pythium* was characterized as a polyphyletic genus, containing 11 major clades designated A thru K. It was found that morphological characteristics such as the shape of the sporangia is correlated with the major clades, whereas the ornamentation of the oogonia and the heterothallism are not, indicating that they could have been lost or acquired through evolution within the genus (André Lévesque and De Cock, 2004).

Besides the molecular similarity based on the DNA regions amplified that determined the clusters in the genus, it is interesting that some of the species within each cluster share similarities in terms of the niche where they were isolated. For example, *P.*

*aphanidermatum* a known plant pathogen, *P. deliense*, *P. adhaerens* from Clade A were collected from dicotyledons in warmer regions. Clade B, cluster B1a includes *P. torulosum*, and *P. catenulatum*, which were isolated from monocotyledons, bryophytes, green algae and soil. In the clusters B1d and B1e, *P. graminicola, P, inflatum, P. periilum* were isolated mostly from monocotyledons. The species *P. oligandrum, P. acanthicum* and *P. periplocum* are grouped in clade D, are pathogenic to dicotyledons, and are mycoparasites. The species that encompass clade E were mainly isolated from soil. In clade F, important plant pathogens such as *P. irregulare, P. spinosum, P. mamillatum and P. parecandrum* were grouped. Finally, the Clade I includes the plant pathogens *P. ultimum, P. splendens* and *P. heterothallicum* able to cause disease in dicotyledons (André Lévesque and De Cock, 2004). The cluster grouping obtained with the LSU and ITS markers, were similar to the phylogeny of the genus with the cytochrome oxidase II (Cox II) (Martin, 2000).

The phylogenetic relation between the genus *Pythium* and the other genera within the Peronosporales lineage, such as *Phytophthora*, still needs to be established, but according to Cooke et al. (2000), the ITS-based phylogeny in the first study mentioned suggests that *Phytophthora* evolved from *Pythium*-like ancestors (Cooke et al., 2000). Also, Villa et al. (2006) basing their study on ITS, Cox II and β-tubulin phylogeny showed that *Pythium* is a polyphyletic group, whereas *Phytophthora* is a monophyletic group, suggesting that the latter is a relatively recent genus that has no radiated as has been observed in *Pythium* (Villa et al., 2006).

**IDENTIFICATION METHODS**

In addition to morphological identification based on the reproductive structures of the genus, serological methods were among the first techniques used to identify *Pythium* spp. The detection method consisted of polyclonal antibodies binding to antigens in the fungal cell wall. Commercial ELISA kits for *Pythium* were developed during the 1990's and are still being used in greenhouses and nurseries(Schroeder et al., 2013).

Currently, DNA-based techniques for species identification are widespread tools that allow disease diagnosis (Schroeder et al., 2013). Random Fragment Length Polymorphism (RFLP) technique is based on the DNA digestion by restriction enzymes, which creates a banding pattern useful for species identification (Martin and Tooley, 2003). RFLP has been used for *Pythium* diagnostic and identification in an amplified DNA region like ITS, from sugar beet in Australia (Scott et al., 2005), and detecting *Pythium* in ginger roots (Kernaghan et al., 2008) .

In addition to pathogen identification, its quantification is important to elucidate the distribution and abundance of the organism in samples. In a study to identify and quantify the most common *Pythium* species inhabiting soil in Washington, species-specific ITS primers were designed, and each species was quantified by qPCR (Paulitz and Adams, 2003). Quantification of *Pythium ultimum* on ginseng roots has been also studied (Kernaghan et al., 2008)

**BIOLOGY AND PHYSIOLOGY**

*Pythium* survives in the soil as oospores in the absence of a possible host or organic matter, which supports its saprophytic growth. These structures are the result of the sexual cycle, which takes place by the interaction among the reproductive structures:

oogonia and antheridia. The oogonia can be spherical or limoniform, and their wall can be smooth or ornamented. The antheridia can be on the same hypha of the oogonial stalk (monoclinous) or can be originated in a different hypha (diclinous). When the antheridium reaches the oogonium, it forms a fertilization tube, penetrates the oogonium, and a zygote is formed in the oogonial content (van der Plaats-Niterink, 1981).

Oospores have a thick wall to prevent desiccation, allowing them to survive in the soil for long periods of time. The dormancy of the oospores varies depending on the *Pythium* species. For example, it has been reported that *P. ultimum, P. aphanidermatum* and *P. graminicola* oospores were viable after eight to eleven months in the soil. Another factor that differs between species is the thickness of the oospore wall; for *P. ultimum* there is a reduction in thickness of the oospore wall, while for *P. aphanidermatum* there is not change in wall thickness when the oospore is during the maturation process.

Oospore germination is affected by environmental factors and by the presence of minerals and nutrients such as calcium and carbohydrate sources. Germination starts by the absorption of the endospore, followed by the formation of a germ tube to release the zoospore (van der Plaats-Niterink, 1981). Light and humidity also, stimulate germination of *P. ultimum, P. aphanidermatum, P. splendens*, and *P. sylvaticum.* According to Martin and Loper (1999), temperature does not have effect on oospore germination, depending on the optimal temperature for each species.

Sporangia are the asexual structures of *Pythium,* as well as the oospores, sporangia can survive in the soil for long periods of time; and they can be the inoculum to attack a

susceptible plant or for saprophytic colonization (Martin and Loper, 1999). Among the genus, there are two types of sporangia: filamentous and globose, being *P. aphanidermatum* and *P. ultimum* respective examples of these two types of sporangia. Morphologically it is difficult to differentiate between *Pythium* species, because the sporangia of the genus share very similar characteristics (André Lévesque and De Cock, 2004, Uzuhashi et al., 2010, Villa et al., 2006).

The sporangium is separated from the rest of the mycelium by a cross wall. Inside sporangia there is an undifferentiated content that moves through a discharge tube until forms a vesicle at its ends, being the place where the zoospores are formed (van der Plaats-Niterink, 1981).

The zoospores of *Pythium sp*. have a thin wall, are bean or pear shaped, and have anterior and posterior flagella. The anterior flagella called mastigomata, consist of a flagellum with hair-like rod lets attached to it, and the posterior flagella just has an ending axis (van der Plaats-Niterink, 1981) that helps zoospores motility after germination.

Sporangia and Oospore germination is stimulated by chemical compounds such as $Ca^{+2}$ (Van West et al., 2002), electric cues and soil moisture produced by the plant, seed exudates, or organic matter (Martin and Loper, 1999, Raftoyannis and Dick, 2006). The zoospores produced, are considered the most susceptible phase of the *Pythium* life cycle (Raftoyannis and Dick, 2006), when zoospores are released in the soil, they stay viable for a short period of time, or infect plant tissue (Agrios, 2005, Martin and Loper, 1999).

**DISEASE CYCLE**

The infection starts when the zoospores encyst to the roots surface due to the secretion of adhesive proteins such as glycoproteins, the presence of high humidity, concentration of nutrients, peptides, and Calcium (Van West et al., 2002). Once the zoopores are attached to the root surface, they form a germ tube to penetrate the root, and secrete wall degrading enzymes: endocellulases, 1.3 β-glucanases, β gluclosidades, cutinases, pectin-esterases, galactanaes and endopolygalacturonases (van West et al., 2003), in order to colonize the plant tissue, breaking down the protoplasm of the plant cells and causing collapse and disintegration of the host cell walls (Agrios, 2005).

The plant as well produces carbohydrates on the root surface that stimulate zoospore attachment. Polysaccharides as polyunorates alginate, polygalacturonic acid and gum arabic produced on plant roots, as well as the exudates released by seeds, enhance the germ tube growth of *Pythium* spp. (Martin and Loper, 1999).

When the infection is only caused in the cortex of the root or the stem of the seedling, the plant may live until the lesion extends above the soil, and then collapses because the infected tissue cannot support the seedlings. If *Pythium* spp. infect mature plants, the invasion is limited to the cortex. *Pythium* enters the root tips and proliferates, causing cell dead of the rootlet (Agrios, 2005).

**EPIDEMIOLOGY**

The major environmental factor that favors the development of the disease is high soil moisture, because the mobility of the zoospores is enhanced by water (Martin and Loper, 1999). For example disease severity in barley increases when *P. graminicola, P. irregulare* and *P. volutum* are in soils with high moisture (Bratoloveanu and Wallace, 1985). Similar results were obtained for *P. ultimum* in poinsettia (Bateman, 1961) and soybean (Schlub and Lockwood, 1981) showed the same trend.

Ideal temperature conditions for disease development varies among species and their host (Martin and Loper, 1999). Several studies during the 70's and 80's (Martin and Loper, 1999), show that disease severity caused by *P. ultimum* and *P. irregulare* increased at cool temperatures from 12 to 25°C. However, Ingram and Cook (1990), showed that the temperature changes depending on the host. For example *P. ultimum* caused higher disease incidence in wheat from 15 to 25°C, lentil from 10-25°C, and pea from 5 to 25°C (Ingram and Cook, 1990).

The optimum temperatures for other species such as *P. aphanidermatum* and *P. myriotylum* are in the range of 25 to 35°C (Martin and Loper, 1999). On turf grass, *P. aphanidermatum* is more destructive when temperatures are between 29 and 35°C (Abad et al., 1994, Vargas Jr, 1994).

**PYTHIUM ON ORNAMENTALS**

Michigan ranks the third in the U.S. for the wholesale value of floriculture products, with an estimated wholesale value of $375.744 millions (USDA, 2013b) leading the nation in value of sales for nine floriculture crops (Table 1.1). At a regional level, floriculture is the fourth commodity in cash receipts for Michigan (USDA, 2013a) Besides meeting market demands for quality (i.e. flowering and plant architecture), ornamental growers have to deal with adequate disease management. Diseases such as root and crown rot caused by *Pythium* spp. are considered important problems for greenhouse facilities (Daughtrey and Benson, 2005).

*Pythium* species affecting ornamentals are not lethal to mature plants, but on seeds and seedlings, they cause seed rot, root rot, seedling damping-off, and rot of lower stems

(Martin and Loper, 1999, Moorman et al., 2002) reducing plant vigor and subsequently affecting plant growth and marketability.

*Pythium spp*. can infect a wide variety of ornamental plants (Kucharek and Mitchell, 2000, Stephens and Powell, 1982), as shown in the latest survey of *Pythium spp.* on ornamental crops, by Moorman *et al*. (2002) in Pennsylvania greenhouses. In this study *P*. *irregulare, P. aphanidermatum* and *P. ultimum* were the most frequently detected species with a prevalence of 45%, 29% and 10% respectively of the plants tested. Geranium and poinsettia were the common hosts for all three species, as well as snapdragons, begonias and chrysanthemum for *P. irregulare* and *P. u.ltimum* (Moorman et al., 2002).

Studies of *Pythium* species causing damping-off of seedling bedding plants in Ohio (Stephens and Powell, 1982) also showed the wide host range of this genus. This study was found that *P. aphanidermatum, P.ultimum, P. spinosum,* and *P. debaryanum* caused damping off in impatiens, celosia and vinca. Also Stephens and Powell (1982) reported that *P. ultimum* was the species most often associated with seedling disease in Ohio bedding-plant greenhouses(Stephens and Powell, 1982).

Among the annual flowering plant crops most affected by *Pythium* species are poinsettia, geranium, chrysanthemum and snapdragon. They all show similar symptoms of root rot, ending in wilting and plant stunting (Hendrix and Campbell, 1973).

In commercial poinsettia (*Euphorbia pulcherrima*) production, root and stem rot are caused most commonly by *P. aphanidermatum, P. iregulare* and *P. ultimum* (Benson et al., 2002). They cause stunting, leaf drop, and the base of cuttings turn brown with a

water-soaking appearance until the entire root rots and the plant wilts (Benson et al., 2002, Kaye et al., 1984).

Geranium (*Pelagorium x hortorum*), the most important commercial variety of propagated seed geraniums in Europe and the United States (Hausbeck et al., 1989), is affected by *P. ultimum*, which causes crop loss (Chagnon and Belanger, 1991). The symptoms are seedling damping off, chlorosis on lower leaves, stunting, stem rot developed at the surface of soilless media, and root rot on young and some mature plants, until the entire plant collapses and dies (Hausbeck et al., 1989).

*Chysanthemum morifolium* is the variety most cultivated over the world (Tsukiboshi et al., 2007) and since 1950, *Pythium* spp. have been reported to cause basal rot on cuttings (Tompkins and Middleton, 1950a). The symptoms start to appear as dark lesions at the base of the cutting, followed by discoloration of the stem tissue, the lower leaves turn yellow, and finally the entire plant wilts and collapses (Tompkins and Middleton, 1950a, Tsukiboshi et al., 2007). In Japan, *P. ultimum, P. sylvaticum, P. dissotocum, and P. dochilum* were isolated from chrysanthemum showing root rot (Tsukiboshi et al., 2007).

**OTHER HOSTS**

*Wheat***.** *Pythium* spp. cause the disease known as common cold of wheat, affecting yield due to reduction in seedling emergence, plant stunting, reduced tillering, and loss of feeder roots (Agrios, 2005).

In a survey of *Pythium* communities from wheat fields on different soils in eastern Washington, Paulitz et al. (2003) recovered a total of 532 isolates from 80 fields

sampled. The ITS region of 200 isolates were sequenced and 14 *Pythium* species were identified, including *P. abappressorium* sp. nov. (50%), *P. rostratum* (40%), *P. debaryanum* (37.5%), *P. heterothallicum* (33.7%), *P. oligandrum* (31.2%), an unidentified *Pythium* sp. (aff. *echinulatum*) (25%), and *P. ultimum*(18%). In terms of community composition, depending on the type of soil sampled, it was found that *P. abappressorium, P. oligandrum* and *P. ultimum* were prevalent in coarser-textured soils, in areas with lower precipitation, whereas *P. debaryanum* and *P. heterothallicum* were more prevalent in finer-textured soils, in areas with higher precipitation (Paulitz and Adams, 2003).

*Corn and soybean.* Seeds and seedlings of *Zea mays* L. and *Glycine max* (L.) are most susceptible to *Pythium*. The pathogen has been isolated from these crops during their early growth, when *Pythium* causes damping-off, reduces root and shoot growth, reducing plant vigor and eventually affecting yield (Griffin, 1990). In Ohio, the planting dates for corn and soybean have recently began earlier than previous years; this cultural shift may be unfavorable for seed germination, and may prolong the seedlings stage which might favor the establishment of *Pythium* (Broders et al., 2007). Broders et al. (2007), identified *Pythium* isolates from corn and soybean seedlings, and the pathogenicity and the sensitivity of the isolates to mefenoxam, trifloxystrobin and azoxystrobin were tested. A total of 19 *Pythium* species were identified, with *P. dissotocum* (23%), *P. sylvaticum* (20%), *P. irregulare* (15%), *P. torolosum* (12%), *P. ultimum* (8%), *P. inflatum* (8%) and *P. attrantheridum* (7%) the dominant species in both crops. Overall, pathogenicity test showed that the reduction of germination was more pronounce in soybean than in corn. *Pythium graminicola* and *P. ultimum* were highly pathogenic to both crops, whereas *P. sylvaticum* and *P. dissotocum* were highly

pathogenic to soybean only. In terms of fungicide sensitivity, species with filamentous sporangia (*P. graminicola, P. dissotocum, P. inflatum and P. torulosum*) were statistically less sensitive than species with globose sporangia (*P. irregulare, P. ultimum, P. sylvaticum*, and *P. attrantheridium*). The species with globose sporangia were also less sensitive to azoxystrobin and trifloxystrobin (Broders et al., 2007).

*Turfgrass. Pythium* blight can be a devastating disease on perennial ryegrass, when high temperature (29-35°C) and humidity favor pathogen germination and plant colonization (Abad et al., 1994). The symptoms start as circular reddish brown spots in the turf. The infected leaves appear water-soaked and dark, and mycelial growth can be seen on the leaf blades (Vargas Jr, 1994). Outbreaks of Pythium blight as well as Pythium root rot caused by other species of the genus, have been reported from golf courses in Japan, Australia, Finland and the United States (Abad et al., 1994, Vargas Jr, 1994).

**DISEASE MANAGEMENT IN THE GREENHOUSE**

Greenhouses are protected from external contamination, but *Pythium* spp. can be introduced by irrigation water, potting media, tools, or vegetative cuttings from other greenhouses (Daughtrey and Benson, 2005, Hausbeck et al., 1988).

Sanitation measures are very important for limiting root rot diseases. Preseason cleanup, such as cleaning floors, walkways, benches, and disinfesting surfaces, pots, and flats with a label product like chlorine bleach are recommended to avoid the pathogen (Hausbeck M., 2013, Cornell, 2012). The use of a well-drained soilless media, or pasteurization of soil mix are also preventive measures.

Water management is an important component for the overall disease control and prevention. Because this pathogen can spread through water, it is important to avoid

over-head and excess watering, which can be controlled with good drainage system, soil aeration, space between plants and good ventilation in the greenhouse(Cornell, 2012, Daughtrey and Benson, 2005).

The most common strategies to control Pythium root rot include the implementation of cultural practices like the use of soilless medium, that prevents greenhouses from inoculum of soilborne pathogens; disinfestations of irrigation water, for greenhouses using recirculating irrigation systems (Daughtrey and Benson, 2005); and the application of preventive measures such as eradication by scouting symptomatic plant material, and the use of fungicides and biopesticides (Moorman and Kim, 2004, Moorman et al., 2002). Nevertheless, the number of registered products for ornamental use against *Pythium* spp. is limited (Garzón et al., 2011).

Fungicides including mefenoxam, azoxystrobin, etridiazole, fenamidone are used in greenhouse facilities to manage Pythium root rot (Cornell, 2012, Moorman and Kim, 2004, Hausbeck M., 2013). Mefenoxam, has been widely use to control oomycetes population in greenhouse facilities (Moorman et al., 2002, Olson and Benson, 2011, Hausbeck M., 2013, Daughtrey and Benson, 2005), and the repeated use of this product has resulted in resistance of *Pythium* spp. isolates (Garzón et al., 2011, Moorman and Kim, 2004, Taylor et al., 2002). Recently, the integrated use of fungicides and biological controls such as *Trichoderma harzianum* and *Streptomyces lydicus* have shown to be effective at controlling Pythium root rot (Harman, 2000, Little et al., 2003, Daughtrey and Benson, 2005, Moorman and Kim, 2004).

**POPULATION STUDIES**

The study of population genetics attempts to investigate how gene frequencies change in populations to shape their genetic structure; these changes in gene frequencies can

be caused by mutation, migration, genetic drift, selection or recombination (Linde, 2010, McDonald and Linde, 2002a). Molecular markers such as Amplified Fragment Length Polymorphims (AFLP), Single sequence repeat (SSR), and Random amplified polymorphic DNA (RAPD) have been used to study populations of *P. aphanidermatum, P. irregulare,* and *P. cryptoirregulare* (Garzón et al., 2005b, Lee et al., 2010, Lee and Moorman, 2008).

One of the first studies was an AFLP fingerprint analysis of *Pythium* spp. present in greenhouse crops in Pennsylvania (Garzón et al., 2005b). Using one set of AFLP primers, nine *Pythium* spp. were characterized. The fingerprint obtained revealed a monomorphic band pattern for each species, distributing the isolates into nine groups: *P. aphanidermatum, P. irregulare, P. vexan, P. spinosum, P. dissotocum, P. myriotylum, P. torulosum, P. ultimum* and *P. heterothallicum*.

In addition, AFLP fingerprints were evaluated at the intraspecific level in *P. aphanidermatum*, *P. irregulare* and *P. ultimum.* The *P. aphanidermatum* isolates were subdivided into four groups, depending on the fingerprint obtained, but the grouping was not associated with host specificity or geographic distribution. For *P. irregulare*, two groups were obtained, without correlation in geographic origin; and in *P. ultimum* two subdivisions were found, one of them grouping isolates obtained from geranium, and the other group with isolates form different hosts and locations (Garzón et al., 2005b).

Later, Lee and Moorman (2008) developed SSR markers for *P. aphanidermatum, P. cryptoirregulare* and *P. irregulare* using genomic-rich libraries. They found a total of 106, 82 and 73 SSRs primers for the species mentioned above. The markers were

tested on eight isolates of each species in order to select the most polymorphic. A subset of four SSRs was selected for *P. aphanidermatum*, generating a total of six genotypes in the species, three markers for *P. irregulare* generating 17 genotypes, and four for *P. cryptoirregulare* with 15 genotypes. The high number of genotypes found in *P. irregulare* and *P. cryptoirregulare* can be attributed to the complex of cryptic species found in *P.* irregulare (Lee and Moorman, 2008).

In this study, the transferability of the SSRs across related *Pythium* species was also tested. It was found that all SSR markers from *P. irregulare* and *P. cryptoirregulare* were transferred between species, and most of the markers from both species were transferrable to species that belong to clade B (e.g. *P. cylindrosporum* and *P. spinosum*). For the case of *P. aphanidermatum*, SSRs were transferable among some species included in Clade A. This result suggests that the transferability of SSR markers depends on the phylogenetic relationship among the species (Lee and Moorman, 2008). Lee et al. (2010) performed more population studies related to the genus in 2010. They determined the genetic variation of *P. aphanidermatum* isolates from Pennsylvania greenhouses using SSR and AFLP markers. Six AFLP and four SSR markers were tested in 123 *P. aphanidermatum* isolates. The AFLP and SSR genotypes revealed three genetic groups, with sampling location instead of host being the main factor that contributed to genetic diversity. Overall, with AFLP a high level of polymorphism was found in the population, and the four SSRs showed a high level of heterozigocity, suggesting a high rate of random mating in the species (Lee et al., 2010).

**GENOMICS**

The first genome available for the genus was that of *P. ultimum var. ultimum,* sequenced by Levésque et al (2010). The size of the genome is 42.8 Mb; according to its

annotation it encompasses 15,297 genes, enconding 15.324 transcripts (Lévesque et al., 2010). Compared with the genome sizes of other plant pathogens in the peronosporales lineage sequenced to date, *P. ultimum* has a small genome size. *Phytophthora infestans* genome is 240 Mb (Haas et al., 2009), that of *Ph. sojae* is 95 Mb (Tyler et al., 2006), *H. arabidopsis* has 100 Mb (Baxter et al., 2010).

Recently, Adhikari et al. (2013) published the sequence and annotation of six more *Pythium* genomes, *P. aphanidermatum* (35.9 Mb), *P. arrhenomanes* (44.7 Mb), *P. irregulare* (42.9 Mb), *P. iwayamai* (43.3 Mb*), P. ultimum* var. *sporangiiferum* (37.7 Mb) and *P. venax* (33.9 Mb). The numbers of genes in these genomes varies from 11,9757 to 14,875. For all the species of *Pythium* studied, it was found that many of the genes involved in pathogenicity and signaling process such as elicitin, necrosis inducing peptidase, protease inhibitor and ubiquitin ligase (Adhikari et al., 2013) are conserved. These genes are potentially involved in degradation of host tissue and in the infection process. In terms of the proteins secreted by *Pythium* spp. during infection, the most common secretome gene families are the polysaccharide lyase, protease inhibitors, cellulose-binding elicitor lectine and expanded families of cell wall degrading enzymes (Adhikari et al., 2013).

Effector proteins are characteristic elements secreted toward the plant cell during infection by oomycetes (Bozkurt et al., 2012). A well-known family, the RXLR effector motifs present in *Phytophthora* spp. and *Hyaloperonospora* spp. genomes (Baxter et al., 2010, Bos et al., 2006) are absent in all *Pythium* species (Adhikari et al., 2013). This find may be related to the necrotrophic lifestyle of *Pythium*, which does not require RxLR effector for colonization and infection in the plant (Adhikari et al., 2013). However, Léveque et al. (2010) and Adhikari et al. (2013) revealed novel families of

secreted proteins named YxSL[RK] that were found in the genomes of all *Pythium* spp. studied. These effectors potentially can contribute to pathogenicity. Other effectors that are present in oomycetes and that were found in *Pythium* spp. are the crinkling and necrosis proteins (CRN), which affect the host cytoplasm and elicit necrosis (Bozkurt et al., 2012). Adhikari et al. (2013) identified 45 CRN proteins in the six *Pythium* spp studied. Interestingly, an interspecific variation in the numbers of CRN effectors was found, indicating that *Pythium* spp. have species-specific strategies for interaction with the host during the infection process (Adhikari et al., 2013).

**APPENDIX**

**TABLE 1.1.** Top nine ornamental crops in Michigan according to their sales value in 2012 (USDA, 2013b).

| Crops | Units sold (million) | Sales Value (million dollars) |
|---|---|---|
| Impatiens (flats) | 1.6 | 12.60 |
| Begonia (flat) | 0.76 | 6.30 |
| Begonia hanging baskets | 0.52 | 3.20 |
| Geranium hanging baskets | 0.65 | 4.90 |
| Impatiens hanging baskets | 0.62 | 3.10 |
| Petunia hanging baskets | 1.2 | 6.50 |
| Potted Easter lilies | 1.2 | 5.00 |
| Potted geranium | 9.0 | 7.10 |
| Potted petunias | 4.5 | 9.00 |
| **Total** | **20.1** | **57.70** |

**LITERATURE CITED**

## LITERATURE CITED


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## CHAPTER I: *Pythium* spp. Associated With Greenhouse Floriculture Crops in Michigan


### ABSTRACT

Michigan ranks third in the U.S. for the wholesale value of floriculture products, with an estimated value of $375.7 million. Seedling damping-off, and root and crown rot are commonly incited by *Pythium* spp. and are important problems for greenhouse growers. *Pythium* spp. associated with Michigan's floriculture crops were characterized as a means to improve current management strategies. During 2011 and 2012, potted poinsettias with root rot symptoms were sampled from nine greenhouses located in Kent, Kalamazoo and Wayne counties. The following year, from the same three counties, symptomatic geranium and snapdragon bedding plants were sampled from 11 greenhouses. Isolates were confirmed to be *Pythium* spp. via morphology and sequencing of the ITS region. A total of 288 *Pythium* spp. isolates were obtained from poinsettias and 726 isolates from geranium and snapdragon. Seven *Pythium* spp. were identified, with *P. irregulare*, *P. ultimum*, and *P. aphanidermatum* being the most prevalent species. A subset of isolates was chosen for pathogenicity and mefenoxam sensitivity testing. Six of the species were virulent to germinating geranium seeds. Most *P. ultimum* and *P. cylindrosporum* isolates tested were intermediate to highly resistant to mefenoxam, whereas most *P. aphanidermatum* isolates were sensitive. This study suggests that *Pythium* spp. recovered from the same Michigan greenhouses may vary depending on the host, and that mefenoxam may not be effective to control *P. ultimum* or *P. cylindrosporum*.

**INTRODUCTION**

Michigan ranks third in the U.S. for the wholesale value of floriculture products, with an estimated wholesale value of $375,744 millions and leads the nation in value of sales for nine floriculture crops (USDA, 2013a). In Michigan, floriculture ranks fourth in cash receipts (USDA, 2013b). Growers of floriculture crops must meet market demands for quality and limiting disease is critical to optimize plant growth and flowering. Root rot caused by *Pythium* spp. is considered an important problem for greenhouse-grown crops (Daughtrey and Benson, 2005). *Pythium* spp. associated with ornamentals may cause seed rot, root rot, seedling damping-off, black leg, and rot of lower stems. Seeds and seedlings tend to be more affected than mature plants (Martin and Loper, 1999, Moorman et al., 2002) and exhibit reduced plant vigor that subsequently affects plant growth, marketability and overall production(Garzón et al., 2011). *Pythium spp*. infect a wide variety of ornamental plants (Kucharek and Mitchell, 2000, Stephens and Powell, 1982). Moorman et al. (2002) assessed ornamental crops for *Pythium* spp. in Pennsylvania greenhouses and detected *P. irregulare, P. aphanidermatum* and *P. ultimum* most frequently (45%, 29%, and 10%, respectively). Geranium and poinsettia were common hosts for all three species; snapdragons, begonias and chrysanthemum were often infected by *P. irregulare* and *P. ultimum* (Moorman et al., 2002). Earlier studies of *Pythium* spp. associated with bedding plants in Ohio (Stephens and Powell, 1982) found that *P. aphanidermatum, P. ultimum, P. spinosum,* and *P. debaryanum* caused damping-off of impatiens, celosia, and vinca; *P. ultimum* was the most prevalent species.

Traditionally, classification and identification of *Pythium* spp. have been based on morphological features of the reproductive structures (Uzuhashi et al., 2010, van der

Plaats-Niterink, 1981). However, microscopic characteristics can appear similar among species and reproductive structures may not form in culture making identification laborious and prone to mistakes (André Lévesque and De Cock, 2004, Martin, 2000, Uzuhashi et al., 2010, Schroeder et al., 2013, Lee and Moorman, 2008). Molecular methods such as sequencing the ribosomal DNA internal transcribed spacer (ITS) region, the large nuclear ribosomal subunit (LSU), and the Cox I region can now be used to accurately identify *Pythium* spp. (André Lévesque and De Cock, 2004, Martin, 2000, Uzuhashi et al., 2010).

The ITS region is the most commonly-used marker for identifying oomycetes at the species level (Robideau et al., 2011, Weiland, 2011). The ITS region, as an identification marker, has been used to elucidate *Pythium* spp. from forest nursery soil in Oregon and Washington (Weiland, 2011), corn and soybean plants in Ohio (Broders et al., 2007), wheat soils in Washington (Paulitz and Adams, 2003) , and ornamental crops in Pennsylvania (Moorman et al., 2002). Phenotypic characterization of *Pythium* spp. populations is based on pathogenicity, virulence and sensitivity to the fungicide mefenoxam (Al‚Äl-Sa'di et al., 2007, Broders et al., 2007, Moorman et al., 2002, Olson and Benson, 2011, Weiland et al., 2013).

The objectives of this study were to identify the *Pythium* spp. associated with greenhouse floral crops in Michigan, and characterize the population for pathogenicity and fungicide sensitivity.

**MATERIALS AND METHODS**

**Isolate collection**

Potted poinsettias (*Euphorbia pulcherrima*) with root rot symptoms were sampled from nine greenhouses located in Kent (3), Kalamazoo (3), and Wayne (3) Counties during the months of October and November of 2011 and 2012. Symptomatic geranium (*Pelargonium* x *hortorum*) and snapdragon (*Antirrhinum majus*) bedding plants were sampled from 12 greenhouses located in Kent (4), Kalamazoo (5) and Wayne (3) counties during the months of March and April in 2012. In 2013, symptomatic hibiscus (*hibiscus* sp.) and lantana (*Lantana camara*) plants were also sampled from one location in Kent County. Roots of symptomatic plants were washed under running tap water, cut, air-dried, and placed on three plates per sample of corn meal agar (CMA) amended with ampicillin (0.25 mg/L), rifampicin (0.01 g/L), pentachloronitrobenzene (0.1 g/L) and benomyl (0.05 g/L). Suspected isolates of *Pythium* spp. were initially confirmed via microscopic observation of oogonia and sporangia following the van der Plaats-Niterink identification key (1981), and mycelial growth was transferred and purified in amended CMA. After 48h an actively growing hyphal tip of each isolate was transferred to CMA to establish a single culture. For long-term storage, three small agar blocks (0.49 $cm^2$) from one-week-old culture were placed in 1.5 mL micro-tubes containing sterile distilled water and hemp seeds, and stored at 20°C.

***Pythium* spp. identification**

Plugs of actively growing mycelia from pure cultures were placed into petri dishes with V8 broth for one week at 20°C. Mycelia were harvested with sterile tongue depressors, placed in 1.5 mL micro-tubes, stored at -20°C and lyophilized. DNA extraction were performed in the Research Technology Support Facility (RTSF) at Michigan State

University, East Lansing, MI using the Autogen 850 robot. DNA was quantified with a NanoDrop Spectrophotometer ND-1000 (Thermo scientific; Wilmington, DE). Amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA was performed with the ITS4 and ITS5 (White et al., 1990) primers, and amplification of the mitochondrially-encoded cytochrome oxidase II *cox* II gene was performed with the FM58 and FM66 primers (Martin, 2000) for a group of isolates identified as *Pythium* spp. with the ITS sequence . Reactions consisted of 2 mM $MgCl_2$, 1X Buffer, 0.2 µM dNTPs, 0.2 µM of primers ITS5, ITS4, and FM58 and FM66 respectively, 1 U of Taq polymerase, and 1µL of DNA (50 ng) in a 25 µL reaction volume. Amplifications were performed on a Mastercycler thermal cycler (Eppendorf, Westbury, NY) with initial denaturation at 96°C for 3 minutes, followed by 35 cycles of 96°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. Samples were visualized by 1% agarose gel electrophoresis using Quantity One Software (Biorad, Hercules, CA), and sequenced. Resulting sequences were assembled in CLC Main Workbench 6 (CLCbio, Aarhus, Denmark) and compared with a local library, built with curated sequences from Robideau et al. (2011).

**Pathogenicity and virulence assay**

A subset of 202 isolates was tested for pathogenicity using a petri dish assay similar to that used to evaluate *Pythium* spp. from soybean and corn seed (Broders et al., 2007). Isolates of *P. aphanidermatum, P. irregulare*, *P. ultimum*, *P. cylindrosporum*, *P. segnitium*, *Pythium* spp., *P. coloratum* and *P. sylvaticum* were grown for four days on CMA and a 5 mm plug of actively-growing mycelia was transferred to the center of a 8-cm plate of water agar. The isolates colonized the plate for three days, then 10 'Pinto Premium White' geranium seeds were placed on the plate 2 cm from the edge and equidistantly

from each other. The control consisted of geranium seeds placed onto non-infested water agar plates. Plates were incubated at 20 °C in the dark for seven days. Pathogenicity was evaluated, and a virulence scale from 0 to 3 was established: 0 = 100% germination with a healthy appearance; 1= > 70% germination, small brown lesions on the radicle or hypocotyl; 2 = > 30% germination, large brown lesions on the hypocotyl and radicle; 3 = < 30% germination, coalesced lesions covering the hypocotyl and radicle (Broders et al., 2007). The experiment was arranged as an incomplete block design with three replicate plates per isolate. Each experiment was conducted twice. Data were analyzed in SAS 9.3 (SAS Institute Inc., Cary, NC), with the proc glimmix procedure using a multinomial distribution and compared by contrasts.

A set of 60 *Pythium* spp. isolates tested using the *in vitro* pathogenicity test was selected for an additional pathogenicity assay in the greenhouse. The selection of these isolates was based on greenhouse location and host of origin. Inoculum was produced by adding 5 plugs (1 cm diameter) of actively grown mycelia in CMA culture to a flask containing 20 g of millet seeds mixed with asparagine (0.016 g) and water (15 mL) that was autoclaved twice(Quesada-Ocampo et al., 2009). The inoculated millet seeds were incubated at 20°C under constant fluorescent light for 10 days, and flasks were shaken to disperse the inoculum evenly. Inocolum of each isolate (3 g) was transferred to 5.5 x 7.0 cm plastic pots containing soilless media (Suremix MI Grower Products Inc, Galesburg MI), where 4-week-old geranium 'Pinto Premium White' seedlings were transplanted. Each inoculated plant was placed on a saucer and grown on a raise plant bench in the MSU Plant Sciences Research Greenhouses for four weeks. Plants were irrigated from the bottom as needed or every second day. After four weeks, plants were visually assessed for the presence of lesions on the roots and lower stems using

the image analysis software Asses 2.0 (Lamari, 2008), that calculated the percentage of tissue area infected. To complete Koch's postulates, re-isolation of the pathogen from symptomatic plant tissue was achieved using amended CMA. The isolates were identified as previously described. The experiment was arranged as a complete randomized designed, with three replicate plants per isolate. Each experiment was conducted twice. Data analysis was performed with SAS 9.3 (SAS, Institute Inc., Cary, NC), using a proc mixed procedure.

**Mefenoxam sensitivity test**

A subset of 202 *Pythium* spp. isolates was grown for three days on CMA and a 5-mm plug of actively growing mycelia was transferred to the center of an 8-cm plate of CMA amended with mefenoxam at 0, 10, and 100 µg a.i./mL (Ridomil gold, Syngenta, NC). The isolates that correspond to *P. irregulare, P. ultimum, P. aphanidermatum, P. cylindrosporum, P. coloratum* and *P. sylvaticum* were incubated for two days, and *P. segnitium* and *Pythium* spp. group isolates were incubated for four days. Growth rate, and the diameter of the hyphal growth were measured. The growth of an isolate (%) at each fungicide concentration was calculated by measuring the hyphal growth diameter, then dividing it by the hyphal growth on the non-amended media and multiplying by 100 (Olson and Benson, 2011, Broders et al., 2007, Moorman et al., 2002). Isolates were characterized as follows: i) sensitive if hyphal growth < 30% of growth on an unamended plate, ii) intermediate if hyphal growth > 30% but < 50% of growth on the unamended plate, and iii) resistant if hyphal growth was $\geq$ 50% of growth on the unamended plate (Olson and Benson, 2011). The experiment was arranged as a randomized design, with three replicate plates per isolate at each concentration. Each experiment was conducted twice. Data analysis was performed with SAS 9.3 (SAS,

Institute Inc., Cary, NC), using a proc mixed procedure.

To determine the effective concentration causing 50% inhibition of mycelial growth ($EC_{50}$) of mefenoxam, a group of resistant and susceptible isolates from different *Pythium* spp. collected at different greenhouses were selected. Culture plugs of actively growing *Pythium* spp. were transferred to CMA plates amended at 0.1, 0.3, 0.5 and 1 μg a.i./mL; and 100, 200, 300, 500 μg a.i./mL for susceptible and resistant isolates respectively. Plates were incubated, and hyphal growth was measured as described above. Growth inhibition (%) was calculated, and the $EC_{50}$ was obtained by the regression analysis of the $\log_{10}$ of the concentrations against the probit of the inhibition percentage. The regression equations were used to interpolate the $EC_{50}$ value for each isolate (Lu et al., 2012, Olson and Benson, 2011).

## RESULTS

### *Pythium* spp. collection and identification

A total of 288 isolates were obtained from poinsettia collected during the fall of 2011 and 2012 from greenhouses in Kent, Kalamazoo and Wayne counties. From the spring sampling conducted in 2012 and 2013, a total of 634 isolates were obtained from geranium, 54 from snapdragon, 25 from hibiscus, and 13 from lantana (Table 2.1). In all, seven *Pythium* spp. were identified by sequencing the ITS region and subsequent local BLAST analysis with the curated sequences from Robideau et al. (2011). Sequences were identified to species based on an identity higher than 98% to ITS sequences deposited in the curated database. The most common species identified included *P. irregulare* (50%), *P. ultimum* (25%) and *P. aphanidermatum* (18%)(Fig. 2.1). Additional species were also identified but were found in a lower proportion and

included *P. cylindrosporum, P. segnitium*, *P. sylvaticum,* and *P. coloratum*. A group of isolates was identified as *Pythium* spp., obtaining an identity >98% to ITS region and *cox* II of *Pythium* spp. sequences deposited in the curated database and in GenBank. In this manuscript, these isolates are referred to as the *Pythium* spp. group. The species most frequently isolated from poinsettia included *P. ultimum* (57%), *P. aphanidermatum* (22%), and *P. irregulare* (18%). *Pythium* spp. commonly associated with geranium included *P. irregulare* (68%) and *P. aphanidermatum* (18%); *P. ultimum, P. cylindrosporum, P. segnitium*, *P. coloratum* and the *Pythium* spp. group were also detected but in low (5%) proportions (Fig. 2.1). Most of the isolates (97%) recovered from snapdragon were identified as *P. irregulare*. *Pythium* spp. isolated from hibiscus included *P. segnitium* (60%) and *P. irregulare* (40%). Most of the lantana (83%) that were sampled were infected by *P. irregulare*.

**Pathogenicity and virulence test**

All of the *Pythium* spp. tested were pathogenic, and caused disease symptoms on geranium seedlings. Virulence varied significantly among the species ($P$ <0.001) but not among the isolates within each species. *Pythium aphanidermatum, P. irregulare, P. ultimum, P. cylindrosporum, P. coloratum, P. silvaticum*, and the *Pythium* spp. group were highly virulent (average disease severity 3), and inhibited germination of 90% of the geranium seeds with all seedling tissue covered with coalesced lesions. The *P. segnitium* isolates exhibited low virulence (average disease severity 0.64) and inhibited germination of only 6% of the seeds with small lesions on the seedling. Seed germination (%) was significantly reduced by all *Pythium* spp., with the exception of *P. segnitium*. There were statistical differences in the virulence of all species tested, and for the isolates within *P. segnitium* and the *Pythium* spp. group (Table 2.2).

All of the *Pythium* spp. used to inoculate geranium plants infected the 4-week-old seedlings. The root area infected (%) of the inoculated plants was significantly different from the control plants. There were significant differences in the percentage of root area infected among all the *Pythium* spp. tested (Table 2.2), and also there were differences within the species ($P$<0.001). *Pythium aphanidermatum*, *P. ultimum, P. irregulare, P. coloratum*, and *P. cylindrosporum* were most virulent on geranium, causing large brown lesions on the roots, chlorosis of lower leaves, plant wilting and in some instances, plant death. Inoculation of geranium with isolates from the *Pythium* spp. group, *P. sylvaticum*, and *P. segnitium* had the lowest percentage of area infected (Table 2.2), with only small brown root lesions; the plant foliage remained symptomless. All of the species used as inoculum were successfully re-isolated from the infected tissue, and identified by sequencing the ITS region, obtaining an identity greater than 98% to ITS sequences deposited in the curated database for each isolate.

*Mefenoxam sensitivity test.* A sub-set of *Pythium* spp. isolates (202) selected based on host and location was tested for mefenoxam sensitivity. The mycelial growth (%) at 10 and 100 ppm of mefenoxam was significantly different ($P$<0.001) than the growth on control unamended plates among the *Pythium* spp. tested (Fig. 2.2). The most fungicide resistant species was *P. cylindrosporum* and all ten isolates tested were resistant to mefenoxam. Most (62%) of the 53 *P. ultimum* isolates tested were resistant to mefenoxam; the majority of the resistant *P. ultimum* isolates were obtained from poinsettia at two greenhouses in Kalamazoo County, and one greenhouse in Wayne County. For *P. irregulare*, 43% of the 55 isolates tested were resistant to mefenoxam; 50% of the resistant isolates were obtained from geranium collected from greenhouses in Kent (2), Kalamazoo (3) and Wayne (2) Counties. Most (84%) of the 50 *P.*

*aphanidermatum* isolates tested were sensitive to mefenoxam; intermediate and resistant isolates were recovered from geranium at one greenhouse in Kalamazoo County and one in Wayne County where *P. ultimum* resistant isolates were also recovered. *Pythium segnitium* isolates (18) recovered from hibiscus at one greenhouse in Kent County were mostly (67%) sensitive to mefenoxam; one isolate was intermediate and five were resistant to mefenoxam. Isolates of *P. coloratum* (1), *P. sylvaticum* (1), and the *Pythium* spp. group (14) were sensitive to mefenoxam.

The $EC_{50}$ values for the resistant isolates of *Pythium* spp. varied among the species. *P. ultimum* ranged from 197 up to >500 µg a.i./mL, *P. cylindrosporum* and *P. irregulare* from 190 up to 500 µg a.i./mL. Only a few *P. aphanidermatum* isolates were resistant and ranged from 197 to 396 µg a.i./mL. The resistant *P. segnitium* isolates were among 130 and 256 µg a.i./mL (Table 2.3; Fig 2.3a). For the sensitive isolates, the $EC_{50}$ ranged from 0.3 µg a.i./mL up to 1 µg a.i./mL for all of the *Pythium* spp. isolates. Exceptions were noted for two *P. ultimum* isolates ($EC_{50}$ = 2.25, 3.63 µg a.i./mL) (Table 2.3; Fig 2.3b).

**DISCUSSION**

Disease caused by *Pythium* spp. is not a new problem for greenhouse growers but remains a challenge with relatively few effective control options. This study was conducted to identify and characterize the *Pythium* spp. infecting greenhouse floriculture crops in Michigan as a means to develop improved and effective management strategies for the state's industry.

Seven *Pythium* spp. were identified from floriculture crops over the course of the three-year study with *P. irregulare*, and *P. ultimum* most frequently isolated. In a similar

study conducted in Pennsylvania more than a decade ago, *P. irregulare* and *P. aphanidermatum* were the most common species detected (Moorman et al., 2002). Another study conducted in Ohio more than 30 years ago determined that *P. ultimum* was the species most frequently associated with seedling disease in bedding plants (Stephens and Powell, 1982).

In this study, *P. irregulare* had the broadest host range and was isolated from geranium, poinsettia, snapdragon hibiscus and lantana. *P. irregulare* infecting these crops have been previously reported with the exception of Lantana: infected geranium has been reported in Michigan (Garzón et al., 2007), poinsettia in California (Tompkins and Middleton, 1950b), snapdragon in Pennsylvania (Moorman et al., 2002), and hibiscus in Australia (Shivas, 1989). Most of the *P. ultimum* isolates (89%) were obtained from poinsettia sampled across different greenhouses in three Michigan counties. Historically, *P. ultimum* has been associated with poinsettia and other ornamental crops, (Scheffer and Haney, 1956, Bateman and Dimock, 1959, Kaye et al., 1984, Benson et al., 2002, Hausbeck et al., 1989), but more recent studies did not identify *P. ultimum* as a significant pathogen of greenhouse ornamentals (Moorman and Kim, 2004, Moorman et al., 2002). One possible explanation for the prevalence of *P. ultimum* in Michigan may be the use of a different source of soilless media compared with other greenhouses in the country. Although soilless media comprised of sphagnum peat moss along with vermiculite and/or perlite, is thought to be superior to field soil in preventing the introduction of pathogens to greenhouses, soilborne pathogens may be present in soilless media (Daughtrey and Benson, 2005) and serve as a source of inoculum (Parke and Grünwald, 2012). *P. ultimum* may also be introduced to Michigan greenhouses via irrigation water. Most of the greenhouses sampled in this study use

well water, and two greenhouses in Wayne county use city water as irrigation sources. Plant pathogens can enter the water sources of greenhouse facilities at several points of the distribution path, and repeatedly inoculate plants (Hong and Moorman, 2005) . *Pythium aphanidermatum* was frequently isolated from both poinsettia and geranium. The association of *P. aphanidermatum* with poinsettia has been documented (Benson et al., 2002, Bolton, 1978), and was the most prevalent *Pythium* spp. recovered from this host in Pennsylvania (Moorman et al., 2002). *Pythium cylindrosporum* isolates were not frequently detected (2%) in this study, but were recovered from geranium and poinsettia; *P. cylindrosporum* had previously been isolated from snapdragon bedding plants (Moorman et al., 2002). In our study, *P. segnitium* was obtained from hibiscus and geranium. This *Pythium* sp. was the second most prevalent species isolated from the recent study of *Pythium* spp. in floricultural greenhouses in Long Island, NY (Sain L., 2014) but had not been previously documented in ornamental plants. It is possible that *P. segnitium* is a recent introduction to greenhouse facilities in Michigan. *Pythium segnitium* has been previously isolated from soil samples in Canary Islands (Paul, 2002) and from bean in Rwanda (Nzungize Rusagara, 2012). To the authors' knowledge, the species *P. coloratum*, and *P. sylvaticum* isolated from geranium and snapdragon respectively, have not been previously reported in ornamental bedding plants. *Pythium coloratum*, has been isolated from onion in New York (Bruckart and Lorbeer, 1982), carrot in Australia (El-Tarabily et al., 1996) and cucumber in Norway (El-Tarabily et al., 1996, Herrero et al., 2003). *Pythium sylvaticum* has been isolated from corn and soybean in Ohio (Broders et al., 2007), Douglas-Fir seedling in Oregon (Weiland, 2011), carrot in Michigan (Lu et al., 2012), lettuce in Arizona (Stanghellini and Kronland, 1986) and strawberry in Illinois (Nemec and Sanders, 1970). Interestingly, most of the

isolates (76%) placed in the *Pythium* spp. group were recovered from geranium at the same greenhouse facility. In order to identify the species of these isolates, additional morphological and genetic studies in addition to the amplification of ITS region and *cox* II, are needed. Although ITS is the most common region used for oomycete identification to the species level (Robideau et al., 2011), for some organisms the ITS region cannot designate species, due to the low level of sequence divergence among closely related species. In this case, the examination of more than two loci will be required to designate species (Schroeder et al., 2013, Villa et al., 2006, Robideau et al., 2011).

The petri dish pathogenicity test revealed that all of the species except *P. segnitium* were pathogenic and highly virulent on geranium seeds, and that *Pythium* spp. are virulent and inhibit geranium seed germination. Therefore, members from the *Pythium* spp. identified, could be the causal agent of root rot symptoms such as browning and reduction of root mass, chlorosis of lower leaves, and wilting rather than a secondary organism. All isolates of *P. aphanidermatum, P. irregulare, P. ultimum, P. cylindrosporum, Pythium* spp. group, and the single isolates of *P. sylvaticum* and *P. coloratum* were pathogenic and highly virulent on geranium seeds, confirming that geranium is a likely host and susceptible to *Pythium* spp. Pathogenicity and virulence studies of *Pythium* spp. obtained from different hosts indicate a wide range of virulence (low, moderate and high) among the species and isolates infecting corn and soybean seeds (Broders, et al., 2007), and Douglas-fir (Weiland, et al., 2013). These results contrast with our study, where all the species tested with the exception of *P. segnitium* were pathogenic and highly virulent to geranium seeds. In this study, *P. segnitium* isolates were determined as avirulent to geranium seeds. The *P. segnitium* isolates in

our study were primarily recovered from hibiscus exhibiting root rot symptoms, suggesting that the pathogenicity and virulence of this *Pythium* sp. may be restricted to hibiscus. The isolates that could not be identified to species and are included in the *Pythium* spp. group, varied in their virulence according to the geranium seed germination assay. This could be the result of the isolates corresponding to different *Pythium* spp. Since they could not be identified with the ITS and *cox* II markers it is possible that they may belong to different clades across the phylogenetic tree of the *Pythium* genus.

The pathogenicity test conducted on geraniums plants corroborated the results of the geranium seed bioassay where all the species infected 4-week-old geranium seedlings. A high degree of virulence was observed in several species including *P. aphanidermatum, P. ultimum, P. irregulare, P. coloratum* and *P. cylindrosporum*. The most prevalent species found in this study were *P. irregulare* and *P. ultimum*, and also correspond with the most virulent species as determined by the seed bioassay and the seedling screen. It is clear that these species are a significant risk to ornamental crop production. The *P. segnitium, P. sylvaticum*, and isolates of the P*ythium* spp. group were reduced in their virulence by comparison, but caused lesions on geranium roots. The relatively low virulence of these species on the geranium seedlings used in this study may be explained by possible host preference of these pathogens. *P. sylvaticum* has been predominantly found on alfalfa (Altier and Thies, 1995)corn, and soybean (Broders et al., 2007). The isolates included in the *Pythium* spp. group may be weak pathogens of geranium seedlings.

Of the 202 *Pythium* spp. isolates tested for mefenoxam susceptibility in this study, 39% were resistant to mefenoxam at 10 and 100 µg a.i./mL. The first report of *Pythium*

isolates resistant to metalaxyl an analog of mefenoxam, in the US (Sanders, 1984), found that metalaxyl was unable to control Pythium turf blight in Pennsylvania, with more than 60% of *P. aphanidermatum* isolates found resistant. Also resistance to metalaxyl was reported from *P. irregulare* and *P. torulosum* isolates from wheat (Cook and Zhang, 1985), *P. dissoctocum* and *P. sulcatum* isolates from carrot (White et al., 1988). Later, mefenoxam resistance was found on 4% of *P. ultimum* isolates from potato collected in Minnesota, Washington and Idaho (Taylor et al., 2002); and 5% of *Pythium* spp. isolates from carrot in Michigan (Lu et al., 2012). Specifically in ornamentals, 32% of *Pythium* isolates collected from ornamental crops in Pennsylvania were found to be resistant to mefenoxam (Moorman et al., 2002, Moorman and Kim, 2004, Garzón et al., 2011).

The ornamental industry is segmented among various types of producers and infected plant material including cuttings, prefinished and finished plants infected with fungicide resistant *Pythium* spp. isolates may be transported and shared among greenhouse facilities within the state and country. The movement of infected plant material can increase the probability of introducing resistant isolates to greenhouses. Early symptoms of *Pythium* infection can go unnoticed until the disease becomes severe and aboveground symptoms become evident.

The range of $EC_{50}$ values of mefenoxam (0.3 to 1.0 µg a.i./mL) observed in our study for the sensitive isolates are consistent with $EC_{50}$ values previously reported for *Pythium* spp. Taylor et al. (2002) reported $EC_{50}$ values of mefenoxam in the range of 0.1 to 1.0 µg a.i./mL for *P. ultimum* isolates from potato collected in Minnesota, North Dakota, Washington and Oregon (Taylor et al., 2002). The *P. irregulare, P. sylvaticum* and *P. ultimum* isolates from Douglas-fir in Oregon had an average $EC_{50}$ values of 0.20, and 0.06 µg a.i./mL, respectively (Weiland et al., 2014). The resistant *P. ultimum, P.*

*irregulare* and *P. cylindrosporum* isolates had $EC_{50}$ values ranging from 100 to 500 µg a.i./mL. For oomycetes, high $EC_{50}$ values of mefenoxam have been previously reported (Olson and Benson, 2011). *Phytophthora* spp. infecting ornamental crops in North Carolina were characterized (Olson and Benson, 2011) with the $EC_{50}$ values of mefenoxam for resistant isolates of *Phytophthora nicotianae* ranging from 246 to 435 µg a.i./mL; *Ph. drechsleri* isolates had $EC_{50}$ values over 700 µg a.i./mL.

*Pythium cylindrosporum* and *P. ultimum* had the greatest number of mefenoxam-resistant isolates. Most of these isolates were recovered from poinsettia from two greenhouse facilities sampled in Kalamazoo and one greenhouse in Wayne County. Resistant *Pythium* spp. may be established at these locations and an improved management, that combines the use of different fungicides and biological controls to control Pythium root rot could be helpful. The highest $EC_{50}$ values (>500 µg a.i./mL) obtained for isolates within *P. irregulare* (1), *P. ultimum* (3), and *P. cylindrosporum* (1) were collected from poinsettia in two greenhouses in Kalamazoo (1,2) and one in Wayne (1) Counties. The high percentage of mefenoxam resistant *P. ultimum* isolates found in this study, suggest that mefenoxam may not be effective to control this pathogen in Michigan greenhouses. Other chemistries that include azoxystrobin, fosetyl-aluminium, and etridiazole, that have been used to protect plants from *Pythium* spp. (Cohen and Coffey, 1986, Moorman and Kim, 2004), and biological control agents containing *Trichoderma harzianum*(Sivan et al., 1984), *T. virens* (Howell, 2002) *Bacillus subtilis* (Jacobsen et al., 2004), *Streptomyces griseoviridis* (Paulitz and Bélanger, 2001)and *S. lydicus* (Yuan and Crawford, 1995) could be implemented to manage Pythium root rot.

**APPENDIX**

**TABLE 2.1.** Total number of *Pythium* spp. isolates obtained from symptomatic host plants sampled in different greenhouses in Kalamazoo, Kent and Wayne counties during fall of 2011, 2012 and spring of 2012, 2013.

| | | **Fall 2011-2012** | | **Spring 2012-2013** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Poinsettia** | | **Geranium** | | **Snapdragon** | | **Hibiscus** | | **Lantana** | |
| **Area** | **Green-house** | **Plants**[y] | **Isolates**[z] | **Plants** | **Isolates** | **Plants** | **Isolates** | **Plants** | **Isolates** | **Plants** | **Isolates** |
| Kalamazoo | 1 | 31 | 71 | 6 | 7 | ..... | ..... | ..... | ..... | ..... | ..... |
| | 2 | 34 | 80 | 2 | 3 | ..... | ..... | ..... | ..... | ..... | ..... |
| | 3 | ..... | ..... | 22 | 53 | 2 | 2 | ..... | ..... | ..... | ..... |
| | 4 | ..... | ..... | 27 | 52 | ..... | ..... | ..... | ..... | ..... | ..... |
| | 5 | 2 | 3 | 96 | 223 | ..... | ..... | ..... | ..... | ..... | ..... |
| Kent | 1 | 6 | 18 | 11 | 22 | ..... | ..... | 11 | 25 | 5 | 13 |
| | 2 | 5 | 6 | 13 | 25 | 6 | 11 | ..... | ..... | ..... | ..... |
| | 3 | 19 | 42 | 48 | 121 | 16 | 34 | ..... | ..... | ..... | ..... |
| | 4 | ..... | ..... | 7 | 14 | ..... | ..... | ..... | ..... | ..... | ..... |
| Wayne | 1 | 15 | 29 | 3 | 5 | 5 | 7 | ..... | ..... | ..... | ..... |
| | 2 | 1 | 2 | 20 | 49 | ..... | ..... | ..... | ..... | ..... | ..... |
| | 3 | ..... | ..... | 25 | 60 | ..... | ..... | ..... | ..... | ..... | ..... |
| | 4 | 17 | 36 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |

[y] Total number of symptomatic host plants : Poinsettia, geranium, snapdragon, hibiscus and lantana infected by *Pythium* spp. at the greenhouse sampled.

**TABLE 2.1 (cont'd)**

[z] A total of three isolations where done per plant sample. Roots of symptomatic plants were washed under running tap water, cut, air-dried, and placed on three plates per sample of corn meal agar (CMA) amended with ampicillin (0.25 mg/L), rifampicin (0.01 g/L), pentachloronitrobenzene (0.1 g/L) and benomyl (0.05 g/L).

**TABLE 2.2** Virulence of seven *Pythium* species on geranium seeds and seedlings.

| | Virulence in geranium seeds[w] | | | | | Virulence in geranium seedlings[w] | | |
|---|---|---|---|---|---|---|---|---|
| **Species** | **N[x]** | **Average virulence[y]** | **SE** | **% of seed germination** | **SE** | **N[x]** | **% of area infected[z]** | **SE** |
| control (media non infested) | 3 | 0 a | 0 | 99.1 c | 0.11 | 3 | 68.0 a | 3.46 |
| *P. segnitium* | 18 | 0.6 a | 0.86 | 95.9 c | 0.03 | 6 | 76.3 b | 1.49 |
| *Pythium* spp. | 14 | 2.8 b | 0.44 | 14.9 b | 0.03 | 5 | 82.4 c | 1.63 |
| *P. aphanidermatum* | 50 | 2.9 b | 0.15 | 0.9 a | 0.02 | 10 | 96.9 d | 1.15 |
| *P. ultimum* | 52 | 3.0 b | 0 | 6.5 a | 0.02 | 14 | 96.7 d | 0.97 |
| *P. cylindrosporum* | 10 | 3.0 b | 0 | 3.1 a | 0.04 | 7 | 94.3 d | 1.38 |
| *P. irregulare* | 54 | 3.0 b | 0 | 2.4 a | 0.02 | 13 | 95.7 d | 1.01 |
| *P. coloratum* | 1 | 3.0 b | 0 | 1.5 a | 0.12 | 1 | 95.5 d | 3.66 |
| *P. sylvaticum* | 1 | 3.0 b | 0 | 1.5 a | 0.12 | 1 | 82.2 bc | 3.66 |

[w] Values in a column followed by the same letter are not significantly different according to least square means significant difference ($P$<0.05). Average virulence of geranium seeds were analyzed using a proc glimmix procedure and compared by contrasts; average virulence of seedlings were analyzed using a proc mixed procedure with SAS 9.3 (SAS Institute Inc., Cary, NC).

[x] Number of isolates analyzed.

**TABLE 2.2 (cont'd)**

[y] Average virulence was determined as: 0 ) 100% seed germination with a healthy appearance; 1) > 70% seed germination, small brown lesions on the radicle or hypocotyl; 2) > 30% seed germination, large brown lesions on the hypocotyl and radicle; 3 ) < 30% seed germination, coalesced lesions covering the hypocotyl and radicle.

[z] Percentage of area infected was calculated using the image analysis software Asses 2.0 (Lamari, 2008): The threshold panel was set from 31 to 101 bits, obtaining the lowest area infected (%) for the control uninoculated plant as 65% and the highest as 100%.

**TABLE 2.3.** Distribution of $EC_{50}$ values of mefenoxam of *Pythium* spp. isolates from greenhouse floral crops in Michigan recovered during the fall of 2011 and 2012 and spring of 2012 and 2013.

| | | Distribution of isolates $EC_{50}$ values (µg/mL)[x] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Sensitive | | | | | Resistant | | | | |
| **Species** | **N** | **0.3-0.5** | **0.5-0.7** | **0.7-0.9** | **0.9-1.0** | **>1** | **100-200** | **200-300** | **300-400** | **400-500** | **>500** |
| *P. irregulare* | 13 | 1 | 4 | 1 | .... | .... | 1 | 2 | 2 | 1 | 1 |
| *P. ultimum* | 14 | 4 | .... | ..... | .... | 2 | .... | 2 | 1 | 2 | 3 |
| *P. aphanidermatum* | 10 | 0 | 2 | 4 | 1 | .... | 1 | .... | 2 | .... | ... |
| *Pythium* spp. | 5 | 4 | 1 | ... | .... | .... | .... | .... | .... | .... | .... |
| *P. cylindrosporum* | 7 | .... | .... | ... | .... | .... | 1 | 2 | 3 | .... | 1 |
| *P. segnitium* | 6 | .... | .... | ..... | 2 | 1 | 2 | 1 | .... | .... | .... |
| *P. sylvaticum* | 1 | 1 | .... | ..... | .... | .... | .... | .... | .... | .... | .... |
| *P. coloratum* | 1 | ... | 1 | ... | ... | ... | .... | ... | ... | ... | ... |

[x]Number of isolates of mefenoxam distributed across a range of $EC_{50}$ values (µg/mL). Hyphal growth was measured from CMA plates amended at 0.1, 0.3, 0.5 and 1 µg a.i./mL; and 100, 200, 300, 500 µg a.i./mL for susceptible and resistant isolates, respectively. Growth inhibition (%) was calculated, and the $EC_{50}$ was obtained by the regression analysis of the $\log_{10}$ of the concentrations against the probit of the inhibition percentage. The regression equations were used to interpolate the $EC_{50}$ value for each isolate.

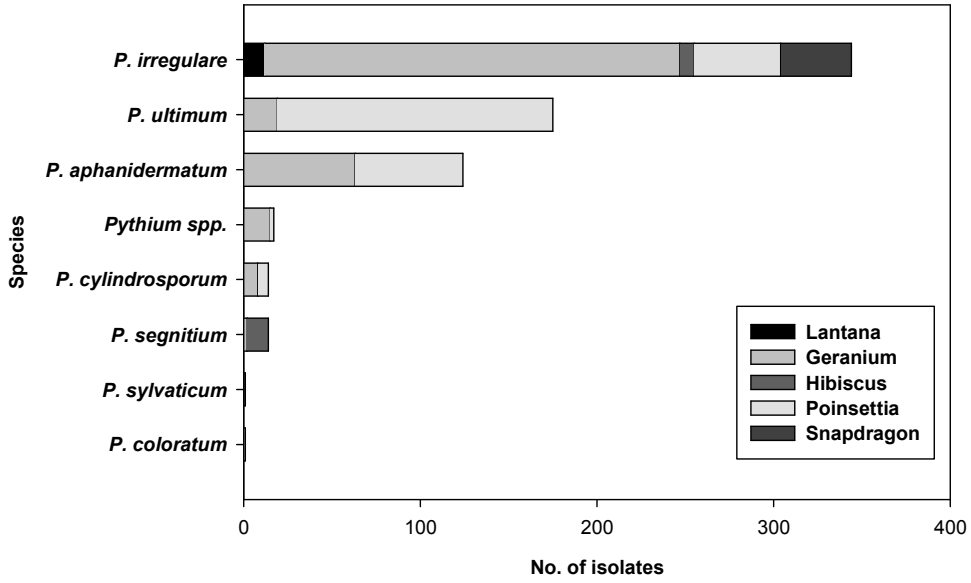


**Fig. 2.1**

**Figure 2.1**. Number of isolates of *Pythium* spp. from greenhouse floral crops in Michigan recovered during fall of 2011,2012 and spring of 2012 and 2013.

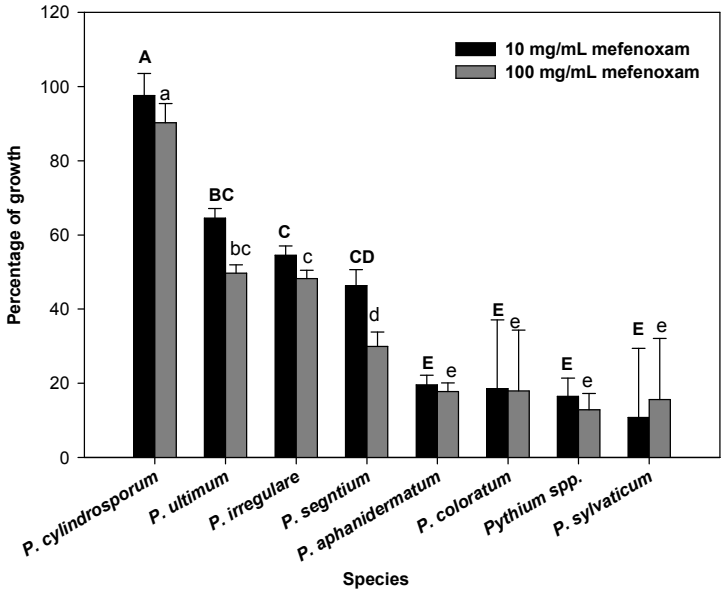


**Fig. 2.2**

**Figure 2.2.** Percentage of mycelial growth at 10 and 100 µg/ml of mefenoxam compared with mycelial growth on nonamended plates for *Pythium* spp. recovered from greenhouse floral crops in Michigan. Bars followed by the same letter are not significantly different according to least square means significant difference ($P$<0.05).

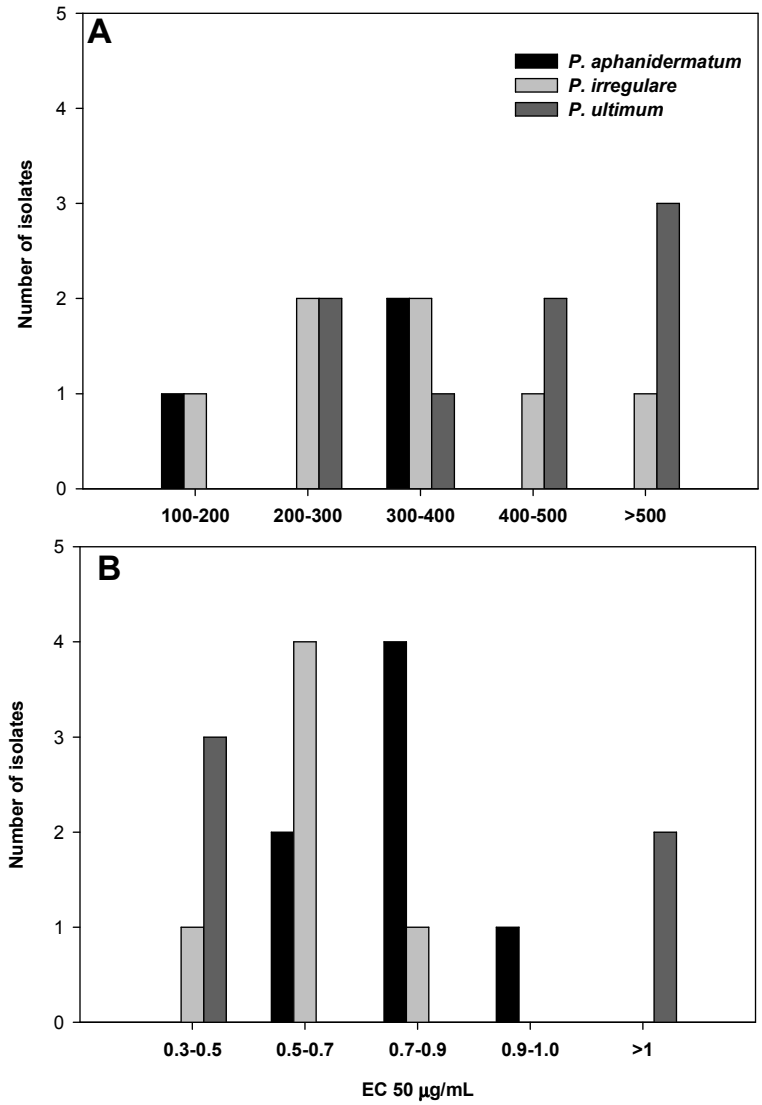


**Fig. 2.3**

**Figure 2.3.** Distribution of $EC_{50}$ of mefenoxam of *P. irregulare*, *P. ultimum* and *P. aphanidermatum*. Number of isolates with $EC_{50}$ values in the range established. **A.** Resistant isolates of *P. irregulare* (7), *P. ultimum* (8) and *P. aphanidermatum* (3). **B**. Sensitive isolates of *P. irregulare* (6), *P. ultimum* (6) and *P. aphanidermatum* (7).

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## CHAPTER II: Integrating Host Resistance and Plant Protectants to Manage Pythium Root Rot on Geranium and Snapdragon


### ABSTRACT

Root rot caused by *Pythium* spp. is a significant disease on greenhouse-grown crops and negatively affects the floriculture industry. To develop strategies that limit Pythium root rot on geranium and snapdragon, greenhouse trials were conducted to test plant protectants and cultivars. Seven fungicides and two biological control agents were evaluated on plants inoculated with *P. aphanidermatum, P. irregulare* or *P. ultimum.* Disease severity was assessed using a scale of 1 (healthy, no disease symptoms) to 5 (plant death) from 5 to 45 days post inoculation. The AUDPC values differed significantly ($P<0.0001$) among *Pythium* spp. and treatment. The interaction between plant protectants and *Pythium* spp. was also significant. In general, mefenoxam (trade name: Subdue Maxx) and *Streptomyces lydicus* WYEC108 (trade name: Actinovate) effectively controlled root rot on geranium. For snapdragon, treatment efficacy varied depending on the *Pythium* sp. When eleven geranium cultivars were inoculated with *P. aphanidermatum* or *P. irregulare*, none were completely resistant to *P. aphanidermatum*. However, when geranium cultivars 'Nano White Hybrid' and 'Bulls Eye Cherry' were inoculated with *P. irregulare* they were similar to the uninoculated control. When twelve snapdragon cultivars were inoculated with *P. aphanidermatum* or *P. irregulare*, no cultivar was completely resistant to *P. aphanidermatum* and *P. irregulare*, but 'Twinny White' and 'Candy Showers Yellow' were least susceptible. Integrating effective fungicides with less susceptible cultivars can limit disease caused by *Pythium* spp.

## INTRODUCTION

Floriculture in the United States is an important agricultural enterprise(Daughtrey and Benson, 2005) with a wholesale value of $4.40 billion (USDA, 2014). Michigan leads the nation in sales for nine floriculture crops, with an estimated wholesale value of $375.74 million (USDA, 2013a). The ornamental industry is segmented among various types of growers who produce seeds, cuttings, prefinished, and/or finished plants (Parke and Grünwald, 2012, Daughtrey and Benson, 2005). Growers of floriculture crops must meet market demands for quality and limiting disease is important to optimize plant growth and flowering.

*Pythium* spp. infect greenhouse-grown crops and negatively affect the floriculture industry (Garzón et al., 2011). *Pythium* spp. can infect a wide variety of ornamental plants (Kucharek and Mitchell, 2000, Stephens and Powell, 1982). Disease symptoms caused by *Pythium* spp. include crown and root rot, seed and seedling damping-off and rot of lower stems and stunting (Martin and Loper, 1999, Garzón et al., 2011). Root rot caused by *Pythium* spp. is a devastating disease if not detected and controlled adequately (Daughtrey and Benson, 2005, Garzón et al., 2011, Moorman and Kim, 2004). The incidence of *Pythium* spp. in floriculture crops, has been reported in Pennsylvania (Moorman et al., 2002), with *P. irregulare* and *P. aphanidermatum* as the most common species. In Michigan, it was found that *P. irregulare*, *P. ultimum* and *P. aphanidermatum* were the most frequently species isolated from floriculture crops (Del Castillo-Múnera J. and Hausbeck, 2014).

Strategies commonly used to control Pythium root rot include using soilless media, disinfesting irrigation water in recirculating systems (Daughtrey and Benson, 2005), scouting crops, rogueing symptomatic plants, and applying fungicides and/or biological

control agents (Moorman and Kim, 2004, Moorman et al., 2002). Currently, the number of plant protection products registered for ornamentals that are effective against *Pythium* spp. is limited (Garzón et al., 2011). The fungicides mefenoxam, azoxystrobin, etridiazole, and fenamidone are used in greenhouse facilities to manage Pythium root rot (Hausbeck M., 2013, Moorman and Kim, 2004, Cornell, 2012). Mefenoxam, has been widely used to control *Pythium* spp. and *Phytophthora* spp. populations in greenhouse ornamentals (Moorman et al., 2002, Olson and Benson, 2011, Hausbeck M., 2013, Daughtrey and Benson, 2005), and its repeated use has resulted in resistant isolates of *Pythium* spp. (Garzón et al., 2011, Moorman and Kim, 2004, Taylor et al., 2002). Fungicides can be integrated with biological controls such as *Trichoderma harzianum* and *Streptomyces lydicus* that have effectively demonstrated Pythium root rot control (Harman, 2000, Little et al., 2003, Daughtrey and Benson, 2005, Moorman and Kim, 2004). Choosing cultivars based on pathogen resistance is not a common control strategy for floriculture producers (Garzón et al., 2011). Few reports are available to guide growers in selecting ornamental cultivars based on host resistance to Pythium root rot (Hausbeck et al., 1987). The objectives of this study were (i) to evaluate seven fungicides and two biological control agents for their ability to limit Pythium root rot in geranium and snapdragon, and (ii) to evaluate geranium and snapdragon cultivars for their susceptibility to Pythium root rot.

## MATERIALS AND METHODS

### Isolate selection and inoculum preparation

Isolates were selected from the collection of *Pythium* spp. that had been isolated from various greenhouse floral crops in Michigan and maintained in the laboratory of M. K. Hausbeck at Michigan State University (MSU). Selected isolates were identified as *P.*

*irregulare* (9.19A), *P. ultimum* (1.59A), and *P. aphanidermatum* (21.17B) by sequencing the ITS region. The *P. irregulare* isolate (9.19A) was collected from geranium, and the *P. ultimum* (1.59A) and *P. aphanidermatum* (21.17B) isolates were obtained from poinsettia. *P. ultimum* and *P. aphanidermatum* isolates were sensitive to mefenoxam at 100 ppm, whereas the *P. irregulare* isolate was resistant to mefenoxam.

Inocolum was produced by adding 18 plugs (1.5 cm diameter) of actively growing mycelia in corn meal agar (CMA) culture to mushroom bags (RJG Sales and Supply, New Port Richey, FL) containing 600 g of millet seeds mixed with asparagine (0.48 g) and water (432 mL) that were autoclaved twice (Quesada-Ocampo et al., 2009). The inoculated millet seeds were incubated at 20°C under constant fluorescent light for two weeks and bags were shaken every other day to disperse the inoculum evenly.

**Plant Protection**

The plant protection experiment was conducted during March (trial 1) and May (trial 2) 2014 at the MSU Plant Sciences Research Greenhouses. Geranium (*Pelargonium* x *hortorum*) 'Pinto Premium Red Deep', and snapdragon (*Antirrhinum majus*) 'Liberty Classic White' seeds (Ball Horticultural Company, West Chicago, IL) were sown in 128-cell plug trays filled with soilless media (Suremix MI Grower Products Inc, Galesburg MI) and maintained on a raised plant bench. Seedlings were overhead irrigated as needed and fertilized according to standard commercial practice.

A preliminary experiment to determine the amount of inoculum needed to cause plant death on 50% of untreated inoculated plants was assessed. It was found that inoculum of 3g for *P. irregulare* and *P. ultimum*, and 2 g for *P. aphanidermatum* were needed to cause plant death in more than 50% of the plants inoculated.

Inoculum of each isolate was added to 5.5 x 7.0 cm plastic pots containing soilless media, into which 8-week-old geranium 'Pinto Premium Red Deep' and snapdragon 'Liberty Classic White' seedlings were transplanted. Pots were placed on saucers and grown on a raised plant bench in the MSU Plant Sciences Research Greenhouses for 45 days. The mean air temperature ranged from 19.4 to 27.22°C during trial 1, and from 25.4 to 27.1°C during trial 2. Plants were irrigated from the bottom as needed or approximately every second day. Biological control agents were applied three days before inoculation, and fungicide treatments were applied as a soil drench the same day as inoculation at a volume of 70ml/35 $cm^2$. Controls consisted of untreated uninoculated plants, and untreated inoculated plants. Treatments were chosen based on mode of action, and applied according to labeled rates and reapplication intervals (Table 3.1). A completely randomized experimental design with six replicates was used. Each plant was considered an experimental unit. A single fungicide + pathogen isolate combination was assigned randomly to each plant.

Disease severity was assessed at 5-day intervals from 5 to 45 days post inoculation (dpi). Plants were visually assessed using a 1-to-5 scale where 1= no symptoms; 2=lower leaves with chlorosis and slight wilting; 3=moderate chlorosis, wilting and stem discoloration; 4=wilting and stunting; and 5=plant death (Figs. 3.1 and 3. 2). The area under disease progress curve (AUDPC) values were calculated using these disease severity ratings (Shaner and Finney, 1977). Plant height and width were also measured on the same day as disease severity.

**Cultivars .**

All trials were conducted at the MSU Plant Sciences Research Greenhouses. Geranium cultivars were selected (Table 3.3) and trials conducted during May (trial 1) and July (trial 2) 2014. Snapdragon cultivars (Table 3.4) were obtained for trials that were conducted during June (trial 1) and October (trial 2) 2014. Seeds were obtained from Ball Horticultural Company (West Chicago, IL), with the exception of the geranium cultivars 'Inspire Appleblossom Hybrid' and 'Nano White Hybrid', which were obtained from Park Seed Co. (Greenwood, SC). Seeds were sown into plug trays as previously described and grown for eight weeks. Seedlings were overhead irrigated as needed and fertilized according to standard commercial practice. Plants were then transplanted into pots containing media infested with *P. irregulare* (9.19A) or *P. aphanidermatum* (21.17B) using methods similar to that described for the fungicide trials. The daily mean air temperature during the geranium cultivar trial ranged from 25.30 to 32.33°C (trial 1), and from 27.77 to 33.77°C (trial 2). The mean air temperature during the snapdragon cultivar trial ranged from 31.80 to 33.10°C (trial 1), and from 26.40 to 30.40°C (trial 2). A completely randomized experimental design with eight replicates per cultivar was used. A single pathogen isolate was assigned randomly to each plant. Disease severity was assessed at 5-day intervals from 5 to 35 days post inoculation (dpi). Plants were visually assessed using the disease scale specified above.

**Pathogen confirmation**

Upon experiment completion, approximately 15% of symptomatic inoculated plants were randomly selected for pathogen confirmation. Their roots were washed under running tap water, cut, air-dried, and placed onto CMA amended with ampicillin (0.25mg/L), rifampicin (0.01g/L), pentachloronitrobenzene (0.1g/L) and benomyl

(0.05g/L). Isolates resembling *Pythium* spp. were initially confirmed via microscopic observation of oogonia and sporangia following the van der Plaats-Niterink identification key (van der Plaats-Niterink, 1981), and mycelial growth was transferred and purified in amended CMA.

The isolates were identified by sequencing the ITS region following the procedure similar to Weiland (2011). In brief, a colony PCR was performed for each *Pythium* spp. culture. A small quantity of hyphae was taken from each culture with a sterile toothpick, transferred to a 500-µL microfuge tube containing 100 µL of double distilled sterile water and incubated at 95°C for 5 min (Weiland, 2011). Two microliters of the extract was added to a 25 µL PCR reaction that consisted of 2mM $MgCl_2$, 1X Buffer, 0.2µM dNTPs, 0.2µM of primers ITS5 and ITS4 (White et al., 1990), and 1U of Taq polymerase. Amplifications were performed on a Mastercycler thermal cycler (Eppendorf North America) with initial denaturation at 96°C for 3 minutes, followed by 35 cycles of 96°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. Samples were visualized by 1% agarose gel electrophoresis using Quantity One Software (Biorad, Hercules, CA), and sequenced. Resulting sequences were compared with a local library, built with curated sequences from Robideau et al. (Robideau et al., 2011), using CLC Main Workbench (CLCbio, Aarhus, Denmark) .

**Statistical analyses**

The AUDPC values and the days to symptom development data were analyzed by ANOVA using the Proc Mixed procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). For the fungicide experiment, the trials were considered random variables. Treatments, plant species, and *Pythium* spp. were considered fixed variables. For the cultivar experiment,

trials were also considered random variables. Cultivars and *Pythium* spp. were considered fixed variables. For the cultivar experiment, there were no significant differences among the trials for each crop, so data were pooled and analyzed together. If variances were unequal, the group option of the repeated statement was used with degrees of freedom according to Kenward-Roger. If ANOVA was significant for main effects or interaction terms, treatment means were compared using Least Square Means test at $P = 0.05$ and adjusted with Dunnett. To test simple main effects, the slice statement was used when the interactions were significant.

Disease severity data were analyzed with the Proc Glimmix procedure of SAS 9.3 (SAS Institute Inc., Cary, NC) using a multinomial distribution, and comparisons were made using contrasts.

## RESULTS

### Plant protection

The *Pythium* spp. used in this experiment caused typical root rot symptoms in geranium and snapdragon seedlings. Untreated uninoculated plants did not exhibit disease symptoms. Disease pressure in trial 2 was higher than that in trial 1. There were significant differences between the two trials, but the results revealed a similar trend of effectiveness among the plant protectants. Data are presented for trial 2 only (Table 3.2). Disease severity increased over time from 10 to 20 dpi (Fig 3.3).

Variations in AUDPC values were significant for product and *Pythium* spp. ($P < 0.0001$), and plant species ($P = 0.0024$). The product- *Pythium* spp. -plant species interaction was also significant ($P = 0.001$). In general, depending on the *Pythium* sp., there were

differences among the products for root rot control in geranium and snapdragon. Geranium and snapdragon inoculated with *P. aphanidermatum* and *P. irregulare* exhibited higher disease severity than plants inoculated with *P. ultimum* (Table 3.2, Fig. 3.3). Mefenoxam and *Streptomyces lydicus* WYEC108 limited disease on geranium inoculated with one of the three *Pythium* spp.; AUDPC values were significantly different from those of the untreated inoculated plants (Table 3.2). Cyazofamid and fluopicolide did not limit disease significantly compared with the untreated inoculated control. For snapdragons, product efficacy depended on the *Pythium* sp. Mefenoxam and fenamidone significantly reduced disease caused by *P. aphanidermatum* and *P. ultimum* compared with the untreated inoculated control. *Trichoderma harzianum* T-22, potassium phosphite and fenamidone effectively controlled *P. irregulare* (Table 3.2). Fluopicolide resulted in higher disease from *P. aphanidermatum* and *P. irregulare* compared with the untreated inoculated control possibly due to phytotoxicity. Plant height and volume for geranium and snapdragon did not differ significantly among plant protectants and *Pythium* spp.

**Geranium cultivars**

*Pythium aphanidermatum* and *P. irregulare* caused disease in all geranium cultivars tested. No cultivar was resistant. Plant death at 35 dpi was greater than 60% or 30% for the most susceptible cultivars inoculated with *P. aphanidermatum*, and *P. irregulare,* respectively. The AUDPC values were significant for geranium cultivar ($P$ =0.0007), *Pythium* spp. ($P < 0.0001$), and the interaction between them ($P = 0.0032$). In general, *P. aphanidermatum* caused higher disease severity than *P. irregulare* in the geranium cultivars tested (Fig 3.4). No cultivar was resistant to *P. aphanidermatum*, but 'Ivy Summer Shower Fuchsia', and 'Ivy Tornado Red' had the lowest AUDPC values (Table

3.3), and the lowest disease severity at the last rating date (Fig 3.4). The AUDPC values of 'Nano White Hybrid', 'Bulls Eye Cherry', and 'Horizon Red' inoculated with *P. irregulare* did not significantly differ from the uninoculated control. The average disease severity measured in the last rating date was below 2.0 for these cultivars (Fig 3.4).

Symptom appearance varied significantly within cultivar ($P = 0.0016$) for each *Pythium* species ($P < 0.0001$). In general, geranium cultivars inoculated with *P. aphanidermatum* exhibited disease symptoms sooner than cultivars inoculated with *P. irregulare* (Fig 3.5). 'Ivy Summer Shower Fuchsia' and 'Ivy Tornado Red' inoculated with *P. aphanidermatum* and *P. irregulare*, developed disease symptoms significantly later (approximately 20 dpi) than other cultivars tested (Fig 3.5). The susceptible cultivars 'Inspire Appleblossom Hybrid', 'Pinto Premium Red Deep' and 'Multibloom Salmon' inoculated with both *Pythium* spp. exhibited disease symptoms at 5 dpi.

**Snapdragon cultivars**

*Pythium aphanidermatum* and *P. irregulare* caused disease in the snapdragon cultivars tested. No cultivar was disease resistant. Plant death at 35 dpi was greater than 60% for the most susceptible cultivars inoculated with *P. aphanidermatum* or *P. irregulare*. The AUDPC values were significant for snapdragon cultivars and *Pythium* spp. ($P < 0.0001$). 'Twinny White', 'Candy Showers Yellow', 'Montego White' and 'Arrow White' were least susceptible (Table 3.4, Fig 3.6). The AUDPC value for these cultivars inoculated with *P. irregulare* was not significantly different from the uninoculated control. The cultivar most susceptible to both pathogens was 'Bells White'.

Symptom appearance among cultivars did not vary significantly ($P = 0.2362$). However, differences among the *Pythium* spp. ($P = 0.017$) and the interaction between cultivar

and *Pythium* spp. ($P = 0.05$) were significant. 'Sonnet White', 'Snapshot White' and 'Arrow White' inoculated with *P. aphanidermatum* developed symptoms significantly later than the other cultivars tested (approximately 15 dpi). 'Twinny White' and 'Liberty Classic White' were inoculated with *P. irregulare* and exhibited disease symptoms at approximately 29 dpi (Fig 3.7). The most susceptible cultivar 'Bells White, ' inoculated with both *Pythium* spp. exhibited disease symptoms at 7 dpi.

**Pathogen confirmation**

The *Pythium* spp. used for inoculum for the fungicide and cultivar experiments, were successfully re-isolated from the infected root tissue of 15% of symptomatic plants in each experiment. Cultures isolated from symptomatic plants were identified by sequencing the ITS region. The identity of the pathogen collected from the plants was confirmed by obtaining identity greater than 98% to ITS sequences of *P. aphanidermatum, P. irregulare* and *P. ultimum*, deposited in the curated database. Control plants did not exhibit disease symptoms and *Pythium* spp. were not recovered from any of the untreated uninoculated controls.

**DISCUSSION**

Plant protectants and host resistance were evaluated as a means to manage highly virulent *Pythium* spp. commonly associated with floriculture crops in Michigan (Del Castillo-Múnera J. and Hausbeck, 2014). There are relatively few effective products registered to control Pythium root rot in floriculture crops (Garzón et al., 2011) and include the fungicides mefenoxam, etridiazole, azoxystrobin and fenamidone (Hausbeck M., 2013, Moorman and Kim, 2004, Cornell, 2012). However, repeated use of

mefenoxam has resulted in resistant *Pythium* spp. populations (Garzón et al., 2011, Moorman and Kim, 2004, Taylor et al., 2002) with resistant isolates reported in ornamentals (Moorman et al., 2002), potato (Taylor et al., 2002) and carrot (Lu et al., 2012).

In our study, plants inoculated with *P. aphanidermatum* were more severely diseased than plants inoculated with *P. irregulare* and *P. ultimum*. Mefenoxam and *Streptomyces lydicus* WYEC108 were most effective in limiting disease symptoms caused by *P. aphanidermatum*, *P. irregulare* and *P. ultimum* on geranium, and *P. aphanidermatum* and *P. ultimum* on snapdragon. Mefenoxam has been widely used to control *Pythium* spp. in ornamentals, and effectively limits Pythium root rot (Moorman et al., 2002, Olson and Benson, 2011, Hausbeck M., 2013, Daughtrey and Benson, 2005, Chase, 1993, Cohen and Coffey, 1986). Similar to our results, fungicide efficacy studies conducted with bermudagrass infected with *P. aphanidermatum* concluded that mefenoxam was highly effective when applied preventively and curatively (Stiles et al., 2005). Mefenoxam was also effective in preventing post-emergence damping off of Douglas fir seedlings infected with *P. irregulare* (Linderman et al., 2008). In our study, the *P. irregulare* isolate was resistant to mefenoxam. This isolate was determined as resistant, because its mycelial growth on CMA amended with 100 ppm of mefenoxam was >50% compared with mycelial growth in unamended CMA (Del Castillo-Múnera J. and Hausbeck, 2014, Olson and Benson, 2011). However, mefenoxam limited *P. irregulare* in geranium, but not snapdragon (Table 3.2). Although the *P. irregulare* inoculum used in this study was obtained from single cultures derived of purified hyphal tip; a possible contamination source in the greenhouse with a mefenoxam sensitive *P. irregulare* isolate may explain this unexpected result. Also, the disease severity of *P. irregulare*

was higher on the untreated inoculated plants of snapdragon than geranium (Table 3.2). This higher disease pressure of *P. irregulare* isolate on snapdragon, and its resistance to mefenoxam may explain the lack of efficacy of this product compared with the results obtained from geranium. The inability of mefenoxam to limit root rot has been reported in resistant isolates of *P. myriotylum* infecting tobacco seedlings (Gutiérrez et al., 2012).

The biological control agent *Streptomyces lydicus* WYEC108 was one of the most effective treatments in limiting disease symptoms caused by *P. aphanidermatum*, *P. irregulare* and *P. ultimum* on geranium, and *P. ultimum* on snapdragon. *Streptomyces lydicus*, was first characterized by Yuan and Crawford (1995) as a potential antagonist of fungal root pathogens. *Streptomyces* spp. produce a wide range of antibiotics and fungal cell wall degrading enzymes including cellulases, hemicellulases, chitinases, amylases, and are able to colonize the rhizosphere (Yuan and Crawford, 1995). Studies on poinsettia inoculated with *P. ultimum* showed that *S. lydicus* effectively controlled root rot. Inoculuated poinsettias treated with *S. lydicus* exhibited a dry weight and number of shoots similar to those treated with mefenoxam and the control uninoculated plants (Little et al., 2003). The potential of *S. lydicus* to limit *Pythium* spp. was also determined in an in vitro assay that evaluated growth inhibition of several *Pythium* spp. isolates from Oregon nurseries (Weiland, 2014).

*Streptomyces lydicus* WYEC108 was more effective against *Pythium* spp. on geranium than snapdragon. It has been shown that the efficacy of biological control agents in controlling soilborne diseases can be variable (Leisso et al., 2009, Handelsman and Stabb, 1996, Whipps and McQuilken, 2009, Weiland, 2014, Linderman et al., 2008). Inconsistent results may be related to biotic and abiotic factors that affect the

antagonistic properties of the biocontrol agents. These factors may include the influence of the microbiome inhabiting the rhizosphere of the plants, genotypic variability of the host plant and the pathogen, and environmental conditions including temperature, relative humidity, and UV radiation(Paulitz and Bélanger, 2001, Weiland, 2014, Guetsky et al., 2002). Differences in the efficacy of the biological controls agents evaluated in this study may depend on the *Pythium* spp. Although *S. lydicus* WYEC108 effectively limited disease caused by *P. aphanidermatum*, *P. irregulare* and *P. ultimum* in geranium, it did not effectively control *P. irregulare* in snapdragon.

Although azoxystrobin was not among the most effective products in this study, disease severity was significantly reduced in geranium inoculated with *P. irregulare*, compared with the untreated inoculated control (Table 3.2). Gutiérrez et al. (2012) showed that azoxystrobin effectively controlled root rot in tobacco seedlings infected with a mefenoxam-resistant isolate.

Snapdragon plants infected with *P. aphanidermatum* or *P. ultimum* had a relatively low disease severity when treated with fenamidone. To the authors' knowledge, the effectiveness of fenamidone against crown and root rot in ornamentals, has been reported for disease incited by the oomycete *Phytophthora cryptogea* (Benson and Parker, 2011), but not by *Pythium* spp. Benson and Parker (2011) showed that fenamidone reduced root rot on daisy inoculated with *Ph. cryptogea* in three trials, with no significant difference from the untreated uninoculated control. In our study, *Trichoderma harzianum* was the most effective treatment, in limiting disease on snapdragon inoculated with *P. irregulare*. *Trichoderma harzianum* has been shown to effectively control root rot caused by *P. ultimum* on geranium (Harman, 2000), and poinsettia (Little et al., 2003) when applied preventively (Harman, 2000).

Disease pressure caused by the three *Pythium* spp. inoculated, was significantly higher for trial 2 than trial 1, and could be a result of differences in temperatures. The mean air temperature was lower in trial one (19.4 to 27.22°C) compared to trial 2 (25.4 to 27.1°C). Ideal temperatures differ among *Pythium* spp. and their hosts (Martin and Loper, 1999). *P. aphanidermatum* and *P. myriotylum* caused high disease severity at temperatures ranging from 25 to 35°C (Martin and Loper, 1999). On turfgrass, *P. aphanidermatum* is more destructive when temperatures are between 29 and 35°C (Abad et al., 1994, Vargas Jr, 1994). Several studies during the 70s and 80s (Martin and Loper, 1999) indicated that disease severity caused by *P. ultimum* and *P. irregulare* increased at cool temperatures from 12 to 25°C in various hosts.

Our results indicate that the level of susceptibility to root rot for the geranium and snapdragon cultivars screened varies depending on the *Pythium* sp. No cultivar was completely resistant to root rot caused by *P. aphanidermatum* or *P. irregulare*. These results are consistent with an earlier study that evaluated 30 geranium cultivars for resistance to *P. ultimum* (Hausbeck et al., 1987). In our study, 'Ivy Summer Shower Fuchsia', and 'Ivy Tornado Red' were the least susceptible cultivars to *P. aphanidermatum*. The cultivars 'Nano White Hybrid', 'Bulls Eye Cherry', and 'Horizon Red' had the lowest AUDPC and disease severity at 35 dpi when inoculated with *P. irregulare*. Hausbeck et al. (1987) showed that the cultivars 'Ringo Scarlet' and 'Ringo Salmon', currently available in the market, had a low percentage (10%) of plant death in soilless media infested with *P. ultimum*.

Snapdragon cultivars 'Twinny White', 'Candy Shower Yellow' 'Montego White' and 'Arrow White' were the least susceptible to *P. aphanidermatum* and *P. irregulare*.

Currently, resistant geranium and snapdragon cultivars are not available. However, growers can integrate the fungicides mefenoxam, fenamidone, azoxystrobin with biocontrol agents containing *S. lydicus* and *T. harzianum*, and with cultivars that are less susceptible to Pythium root rot, in an overall program to limit disease. Specifically, mefenoxam and *S. lydicus* were the best products protecting geranium against *P. aphanidermatum*, *P. irregulare* and *P. ultimum*; and fenamidone and mefenoxam were the best treatments against *P. aphanidermatum* and *P. ultimum* in snapdragon, whereas *T. harzianum* was the best treatment to control *P. irregulare*. The development of fast and accurate diagnostic tools to identify *Pythium* spp. infecting floriculture crops is needed, in order to better tailor management strategies to effectively limit disease.

**APPENDIX**

**TABLE 3.1**. Fungicides, rates and frequency of application for managing Pythium root rot caused by *Pythium* spp. on geranium and snapdragon in greenhouse trials.

| Trade name | Active ingredient (a.i) | Manufacturer[y] | FRAC code[z] | Rate/L | Frequency (days) |
|---|---|---|---|---|---|
| Subdue maxx | Mefenoxam | Syngenta | 4 | 0.08 ml | 30 |
| Terrazole | Etridiazole | OHP | 14 | 0.5 ml | 30 |
| Heritage | Azoxystrobin | Syngenta | 11 | 0.07 g | 14 |
| Rootshield | *Trichoderma harzianum* T-22 | BioWorks | | 0.4 g | 14 |
| Segway | Cyazofamid | FMC | 21 | 0.3 ml | 14 |
| Vital | Potassium Phosphite | Phoenix Environmental Care | 33 | 1.6 ml | 14 |
| Adorn | Fluopicolide | Valent | 43 | 0.2 ml | 14 |
| FenStop | Fenamidone | OHP | 11 | 1.1 ml | 30 |
| Actinovate SP | *Streptomyces lydicus* WYEC108 | Natural Industries | | 0.45 g | 14 |

[y] Syngenta = Syngenta Crop Protection Inc, Greensboro, NC; OHP = OHP Inc, Mainland, PA; BioWorks = BioWorks Inc, Victor, NY; FMC = FMC Corp., Agricultural Products Group, Philadelphia, PA; Phoenix Environmental Care = Phoenix environmental Care, LLC, Valdosta, GA; Valent = Valent USA Corp., Walnut Creek, CA; Natural Industries = Natural Industries, Inc., Houston, TX.

[z] FRAC= Fungicide Resistance Action Committee.

**TABLE 3.2.** Area under the disease progress curve (AUDPC) values for disease severity of geranium and snapdragon growing in greenhouse trials to evaluate plant protection products applied as a soil drench to control root rot caused by *Pythium* spp.

| | AUDPC[y] | | | | | |
|---|---|---|---|---|---|---|
| | Geranium | | | Snapdragon | | |
| **Treatment[z]** | ***P. aphanidermatum*** | ***P. irregulare*** | ***P. ultimum*** | ***P. aphanidermatum*** | ***P. irregulare*** | ***P. ultimum*** |
| Untreated uninoculated | 40.0 aA | 40.0 aA | 40.0 aA | 40.0 aA | 40.0 aA | 40.0 aA |
| Actinovate | 52.0 aA | 54.5 aA | 62.7 aAB | 124.91 dD | 156.9 cdDE | 82.6 cBC |
| Subdue Maxx | 66.7 aAB | 91.5 bB | 45.2 aA | 85.36 bB | 126.4 bcBC | 42.3 aA |
| Rootshield | 67.6 abBC | 102.0 b-dC | 89.04bcC | 94.67 bcBC | 67.9 abB | 89.9 cBC |
| Fenstop | 98.5 bcBC | 94.9 bcBC | 96.4 cC | 83.17 abB | 110.7 bBC | 62.5 a-cB |
| Heritage | 103.9 c-eC | 93.1 bBC | 72.3 bBC | 145.02 dD | 138.9 cD | 86.9 cB |
| Vital | 105.1 c-eCD | 100.1 b-dCD | 83.9 bcC | 121.37 dD | 95.1 bC | 109.5 cCD |
| Terrazole | 107.5 c-e C | 94.1 bc BC | 64.2 abB | 92.24 bcC | 167.7 cdD | 51.9 abB |
| Adorn | 107.7 c-eCD | 108.2 cdCD | 89.1 cC | 99.12 cdBC | 156.1 cdD | 42.7 aA |
| Segway | 128.6 deD | 138.0 dD | 66.4 bBC | 121.82 dD | 135.7 cD | 90.5 cD |
| Untreated inoculated | 151.1 eE | 126.3 dE | 65.5 bC | 98.32 cdD | 147.9 cdDE | 90.5 cD |

[y] AUDPC values were calculated by using disease severity ratings at 5-day intervals for 45 days post inoculation. AUDPC means within columns followed by the same lowercase letter are not significantly different within each product treatment, and means within rows followed by the same upper case letter are not significantly different within each *Pythium* spp. inoculated (Least Square Means Significant Difference $P = 0.05$).

[z] Plant protection products were applied as a soil drench following inoculation with *P. aphanidermatum*, *P. irregulare* or *P. ultimum*. Biological control agents were applied as soil drench three days before *Pythium* spp. inoculation.

**TABLE 3.3.** Area under the disease progress curve (AUDPC) values for disease severity of geranium cultivars growing in greenhouse trials evaluating host resistance to root rot caused by *Pythium aphanidermatum* and *Pythium irregulare.*

| | AUDPC [z] | |
|---|---|---|
| **Geranium cultivar** | ***P. aphanidermatum*** | ***P. irregulare*** |
| Ivy Summer Showers Fuchsia | 75.1 a * | 46.5 ab * |
| Ivy Tornado Red | 75.1 a * | 47.1 ab * |
| Inspire Appleblossom Hybrid | 88.9 ab * | 61.8 b * |
| Nano White Hybrid | 91.7 ab * | 35.5 a ns |
| Horizon Red | 102.5 b * | 37.6 a ns |
| Ringo 2000 Red Deep | 105.3 b * | 41.4 a * |
| Maverick Red | 107.1 b * | 43.4 ab * |
| Score Cherry Picotee | 107.4 b * | 43.8 ab * |
| Bullseye Cherry | 108.1 b * | 35.8 ab ns |
| Multibloom Salmon | 114.8 b * | 52.2 ab * |
| Pinto Premium Red Deep | 116.7 b * | 52.4 ab * |

[z] AUDPC values were calculated by using disease severity ratings at 5-day intervals for 35 days post inoculation. AUDPC means within columns followed by the same lowercase letter are not significantly different within each cultivar. Values followed by an asterisk are significantly different from their control; values followed by ns are not significantly different from their control. (based on Least Square Means Significant Difference $P = 0.05$).

**TABLE 3.4.** Area under the disease progress curve (AUDPC) values for disease severity of snapdragon cultivars growing in greenhouse trials evaluating host resistance to Pythium root rot caused by *Pythium aphanidermatum*, and *Pythium irregulare*.

| | AUDPC[z] | |
|---|---|---|
| **Snapdragon cultivar** | ***P. aphanidermatum*** | ***P. irregulare*** |
| Twinny White | 77.9 a * | 48.9 a ns |
| Candy Shower Yellow | 81.6 a * | 61.5 a ns |
| Montego White | 83.3 a * | 66.3 ab * |
| Arrow White | 84.3 a * | 59.1 a ns |
| Oh Snap White | 105.1 ab * | 71.7 ab * |
| Floral Shower White | 105.4 ab * | 77.5 ab * |
| Snapshot White | 109.6 ab * | 80.3 ab * |
| Sonnet White | 118.9 bc * | 113.2 bc * |
| Chimes Purple White | 123.9 bc * | 75.2 ab * |
| Solstice White | 124.9 bc * | 94.2 ab * |
| Liberty Classic White | 131.2 bc * | 64.0 a * |
| Bells White | 170.4 bc * | 139.8 c * |

[z] AUDPC values were calculated by using disease severity ratings at 5-day intervals for 35 days post inoculation. AUDPC means within columns followed by the same lowercase letter are not significantly different. Values followed by an asterisk are significantly different from their controls; values followed by ns are not significantly different from their control (based on Least Square Means Significant Difference $P$ = 0.05).


**Fig. 3.1**

**Figure 3.1.** Disease scale for geranium 'Pinto Premium Red Deep' inoculated with *Pythium* spp. Plants were visually assessed using a 1-to-5 scale where 1= no symptoms; 2=lower leaves with chlorosis and slight wilting; 3=moderate chlorosis, wilting and stem discoloration; 4=wilting and stunting; and 5=plant death.


**Fig. 3.2**

**Figure 3.2.** Disease scale for snapdragon 'Liberty Classic White' inoculated with *Pythium* spp. Plants were visually assessed using a 1-to-5 scale where 1= no symptoms; 2=lower leaves with chlorosis and slight wilting; 3=moderate chlorosis, wilting and stem discoloration; 4=wilting and stunting; and 5=plant death.

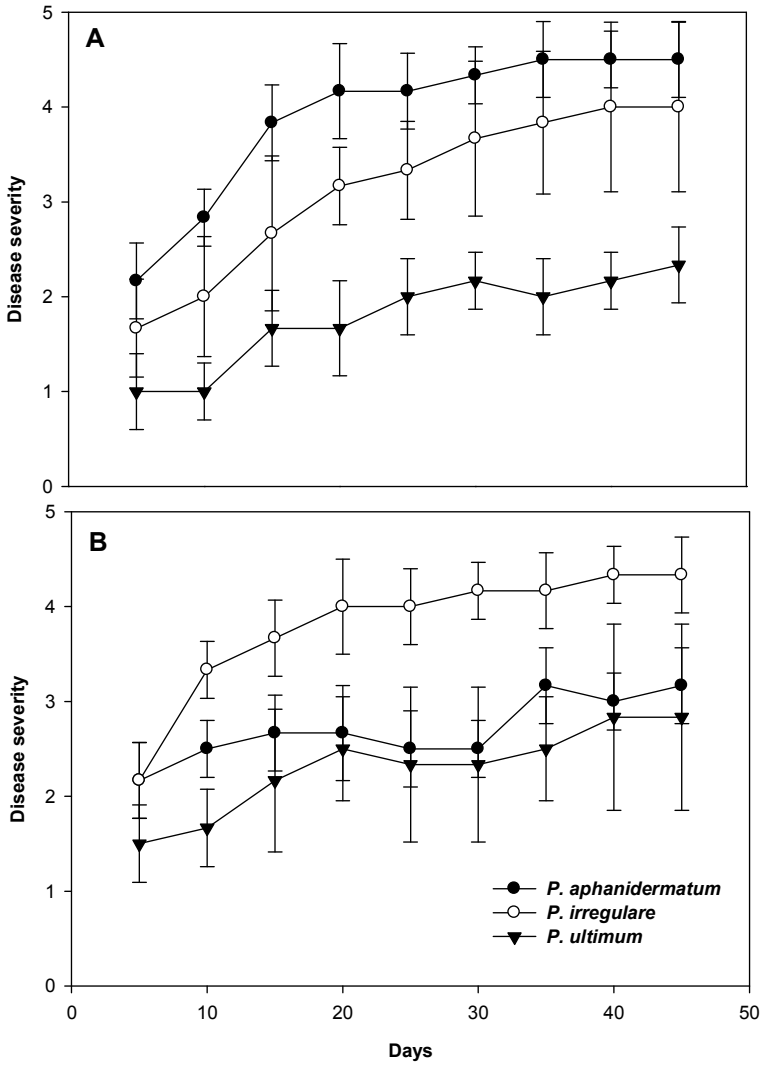


**Fig. 3.3**

**Figure 3.3.** Development of Pythium root rot on **A**, geranium and **B**, snapdragon inoculated with *P. aphanidermatum*, *P. irregulare* and *P. ultimum* in greenhouse trials evaluating the efficacy of fungicide and biopesticide treatments. Pythium root rot was rated from 5 to 45 days post inoculation on a 1-to-5 scale, where 1= no symptoms; 2=lower leaves with chlorosis and slightly wilted; 3=moderate chlorosis; wilting and stem discoloration 4=wilting and stunting of the entire plant; 5=plant death. Ratings

**Figure 3.3. (cont'd)**

are the means of the untreated inoculated plants. Error bars represent the standard error of the mean.

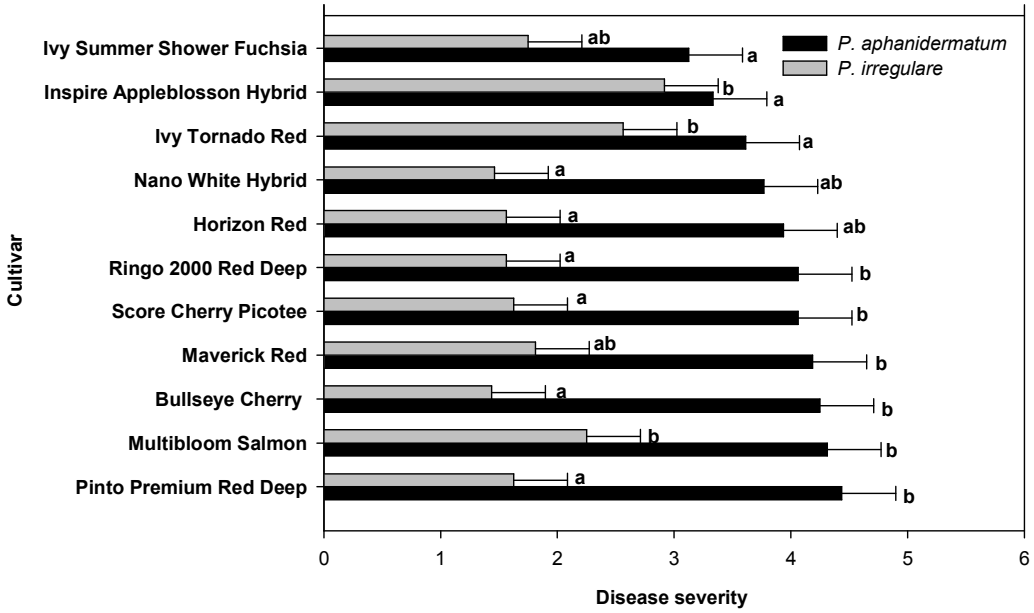


**Fig. 3.4**

**Figure 3.4.** Disease severity 35 days post inoculation in geranium cultivars growing in the greenhouse evaluated for susceptibility to root rot caused by *P. aphanidermatum* and *P. irregulare*. Values represent the means of eight replicates. Error bars represent the standard error of the mean. Bars with a letter in common are not significantly different within each cultivar based on least square means significant difference at $P < 0.05$.

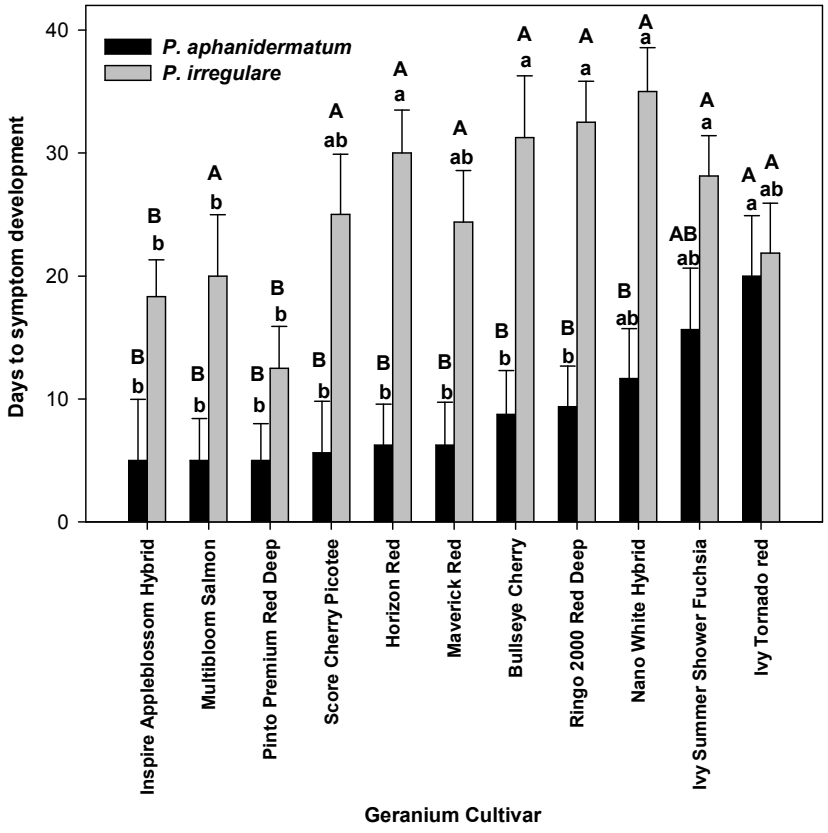


**Fig. 3.5**

**Figure 3.5.** Symptom appearance days post inoculation of geranium cultivars evaluating for susceptibility to root rot caused by *P. aphanidermatum* (21.17B) and *P. irregulare* (9.19A). Values represent the means of eight replicates. Error bars represent the standard error of the mean. Bars with a letter in common are not significantly different within each cultivar (lowercase) and among pathogen isolates (uppercase), based on least square means significant difference at $P < 0.05$.

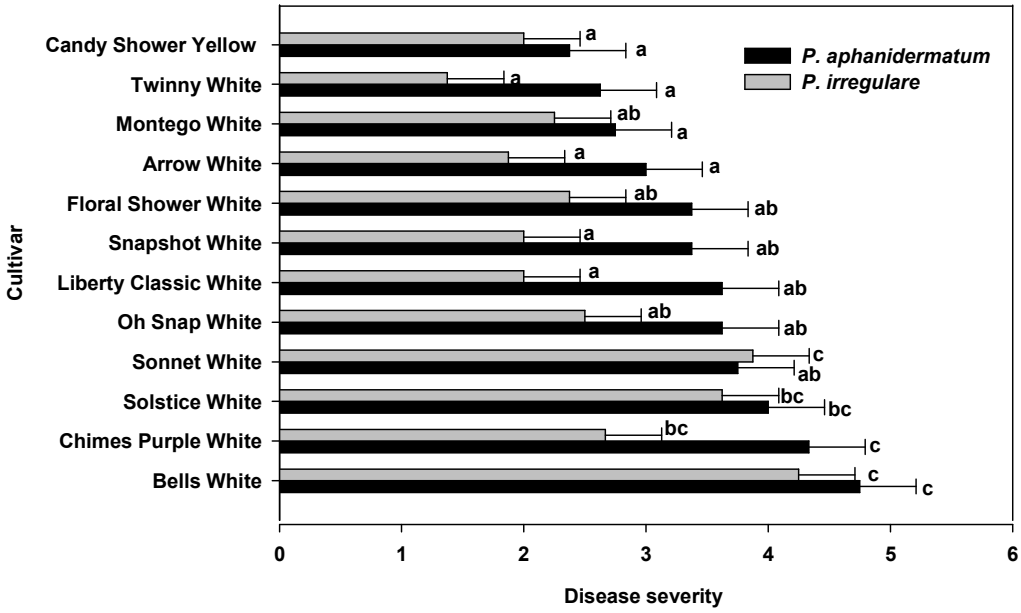


**Fig. 3.6**

**Figure 3.6.** Disease severity 35 days post inoculation in snapdragon cultivars in greenhouse evaluations for susceptibility to root rot caused by *P. aphanidermatum* and *P. irregulare*. Values represent the means of eight replicates. Error bars represent the standard error of the mean. Bars with a letter in common are not significantly different within each cultivar based on least square means significant difference at $P < 0.05$.

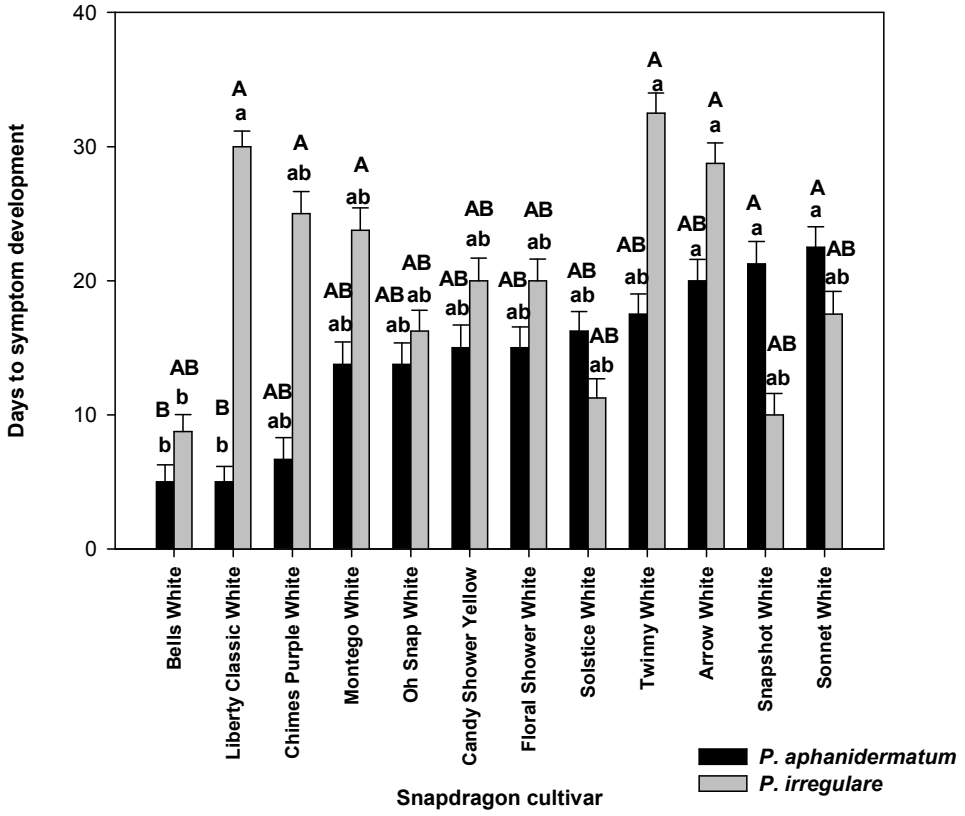


**Fig. 3.7**

**Figure 3.7.** Symptom appearance days post inoculation of snapdragon cultivars evaluating for susceptibility to root rot caused by *P. aphanidermatum* (21.17B) and *P. irregulare* (9.19A). Values represent the means of eight replicates. Error bars represent the standard error of the mean. Bars with a letter in common are not significantly different within each cultivar (lowercase) and among isolates of *Pythium* spp. (uppercase), based on least square means significant difference at $P < 0.05$.

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## CHAPTER III: Population Structure of *Pythium ultimum* from Greenhouse Floral Crops in Michigan


### ABSTRACT

*Pythium ultimum* causes seedling damping-off, and root and crown rot in greenhouse ornamental plants. In order to understand the population dynamics of *P. ultimum* in Michigan floriculture crops, simple sequence repeats (SSRs) were developed using the *P. ultimum* predicted transcriptome to assess population structure. After identifying SSR and designing flanking primers *in-silico* for marker development, six SSRs were selected for population genotyping based on their polymorphism after bulk-segregant analysis of a pool of *P. ultimum* isolates. A total of 166 isolates sampled from 2011 to 2013 from different greenhouses in Kalamazoo, Kent and Wayne counties were analyzed using the six fluorescently labeled SSRs. The average genotypic diversity (0.938), evenness (0.56), and the recovery of 12 major clones, out of the 64 multilocus genotypes obtained, may suggest that *P. ultimum* is not a recent introduction into Michigan greenhouses. Analysis revealed a clonal population, with limited differentiation among seasons, hosts and counties sampled. Despite the intensive sampling, sample size was limited for some of the locations; therefore, a monitoring program using the markers developed in this study will help to understand further the dynamics of this population in Michigan in order to propose control strategies of the pathogen. Results also indicate that sanitation could be enhanced in order to more completely eradicate resident *P. ultimum* populations. Finally, the presence of common genotypes among counties suggest that there is an exchange of infected plant material

among greenhouse facilities, or that there is a common source of inoculum coming to the region.

**INTRODUCTION**

In ornamental plants, *P. ultimum* causes seed rot, root rot, seedling damping–off, and rot of lower stems (Moorman et al., 2002, Garzón et al., 2011). In Michigan, floriculture ranks fourth in cash receipts (USDA, 2013b), and the state ranks third in the U.S. for the wholesale value of floriculture products, with an estimated wholesale value of $375,744 (USDA, 2013a). Pythium root rot is not a new disease yet it remains a challenge for greenhouse growers. In Michigan, *P. ultimum* was reported as the second most common *Pythium* sp. infecting floriculture crops; it is highly virulent on geranium seed and seedlings (Del Castillo-Múnera J. and Hausbeck, 2014, Stephens and Powell, 1982). *Pythium ultimum* is a homotallic self-fertile oomycete (van der Plaats-Niterink, 1981, Francis et al., 1994) and reproduces asexually though the formation of asexual sporangia, or hyphal swellings (Martin and Loper, 1999, Schroeder et al., 2013). Weiland et al. (2015) studied the population structure of *P. ultimum, P. irregulare,* and *P. sylvaticum* from nursery soils in Oregon using microsatellites (SSRs) and amplified fragment length polymorphisms (AFLPs). In this study, a significant differentiation among populations of *P. irregulare* and *P. sylvaticum* isolated from different nurseries was found, but geographic differentiation within the *P. ultimum* populations was not detected. Other studies have described the population structure of *P. aphanidermatum* from greenhouses in Pennsylvania using AFLP and SSRs (Lee et al., 2010). Lee and Moorman (2008) developed SSR markers for *P. aphanidermatum, P. cryptoirregulare* and *P. irregulare* and evaluated the transferability of SSRs among *Pythium* spp., but

these SSRs were evaluated among *Pythium* spp. isolates from different hosts and locations, and not among naturally occurring populations (Lee and Moorman, 2008). Population dynamics provide insight into the life history and evolutionary pattern of plant pathogens, and can be used to develop management strategies (Linde et al., 2002, McDonald and Linde, 2002a, Grünwald and Goss, 2011). Microsatellites (SSRs) are the most popular markers used for genotyping in population genetic studies (Guichoux et al., 2011, Li et al., 2013b, Li et al., 2013a). SSRs are co-dominant markers, characterized by a high degree of polymorphism and high allelic diversity and are informative for the analysis of individual isolates in a population (Ellegren, 2004, Cooke and Lees, 2004). Reduced costs in next-generation sequencing, availability of freeware for identification of many SSR loci from sequencing data, and cost-effective genotyping platforms that analyze multiple SSR loci at a time have allowed for efficient and robust analysis of pathogen populations (Li et al., 2013b, Guichoux et al., 2011, Hayden et al., 2008).
In the present study, the population structure of *P. ultimum* isolates collected from ornamental floral crops in Michigan from 2011 to 2013 was determined using microsatellite markers. Specific objectives included i) Determining the population differentiation based on location, host, or season, and ii) Assessing the degree of genotypic diversity and clonality of the *P. ultimum* population in the region.

## MATERIALS AND METHODS

### Isolate collection

Potted poinsettias (*Euphorbia pulcherrima*) with root rot symptoms were sampled from nine greenhouses located in Kent (3), Kalamazoo (3), and Wayne (3) counties during the months of October and November in 2011 and 2012. Symptomatic geranium

(*Pelargonium* x *hortorum*) plants were sampled from 12 greenhouses located in Kent (4), Kalamazoo (5), and Wayne (3) counties during March and April of 2012. Roots of symptomatic plants were washed under running tap water, cut, air-dried, and three replicates per sample were placed on plates of corn meal agar (CMA) amended with ampicillin (0.25 mg/L), rifampicin (0.01 g/L), pentachloronitrobenzene (0.1 g/L), and benomyl (0.05 g/L). Suspect isolates of *Pythium* spp. were initially confirmed via microscopic observation of oogonia and sporangia following the van der Plaats-Niterink identification key (1981) and mycelial growth was transferred and purified in amended CMA. After 48 h an actively growing hyphal tip from each isolate was transferred to CMA to establish a single culture. For long-term storage, three small blocks (0.49 $cm^2$) from a 1-week-old culture were placed into 1.5-mL micro-tubes containing sterile distilled water and hemp seeds and stored at 20°C.

### *Pythium ultimum* identification

Plugs of actively growing mycelia from pure cultures were placed in petri dishes with V8 broth for one week at 20°C. Mycelia were harvested with sterile tongue depressors, placed in 1.5-mL micro-tubes, stored at -20°C, and lyophilized. DNA extraction was performed in the Research Technology Support Facility (RSTF) at Michigan State University, East Lansing, MI using the Autogen 850 robot. DNA was quantified with a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific; Wilmington, DE). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified with the ITS4 and ITS5 (White et al., 1990) primers. Reactions consisted of 2 mM $MgCl_2$, 1X Buffer, 0.2 µM dNTPs, 0.2 µM of primers ITS5, ITS4, 1 U of Taq polymerase, and 1 µL of DNA (50 ng) in a 25 µL reaction volume. Amplifications were performed on a

Mastercycler thermal cycler (Eppendorf, Westbury, NY) with initial denaturation at 96°C for 3 minutes, followed by 35 cycles of 96°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. Samples were visualized by 1% agarose gel electrophoresis using Quantity One Software (Biorad, Hercules, CA), purified and submitted for sequence analysis at Macrogen (Macrogen, USA). Resulting sequences were assembled in CLC Main Workbench (CLCbio, Aarhus, Denmark) and compared with a local library, built with curated sequences from Robideau et al (Robideau et al., 2011).

**Characteristics of *P. ultimum* isolates**

*Pythium ultimum* was the second most common species isolated from greenhouse floriculture crops in Michigan. A total of 166 *P. ultimum* isolates maintained in the laboratory of M. K. Hausbeck at Michigan State University (MSU), collected during fall of 2011 and 2012, in October and November, and spring of 2012 and 2013, in March and April, were analyzed in this study. Approximately 89% of the isolates were collected during the fall seasons from poinsettia sampled in Kalamazoo, Wayne, and Kent counties. A smaller number (11%) of the isolates were obtained from geranium sampled in Kalamazoo and Wayne counties during the spring seasons (Table 4.5). To perform a population genetics analysis, three populations were established: i) Fall 2011: 83 poinsettia isolates, ii) Spring 2012-2013: 18 geranium isolates, and iii) Fall 2012: 65 poinsettia isolates.

**Microsatellite search and selection**

To avoid designing microsatellite markers in paralogous genomic regions, putative single copy genes were identified by performing self-blastp analysis of the *P. ultimum*

predicted proteome (Lévesque et al., 2010) using a cutoff of 1e -10 as previously described (Wallace E. C. and Quesada-Ocampo, 2014)blastp results were analyzed with custom perl scripts that retained protein sequences with only one match to the proteome and generated a fasta file with gene sequences of putative single-copy genes for SSR identification. Simple and compound SSRs consisting of mono (20 minimum repeats), di- (5 repeats), tri- (4 repeats), tetra- (3 repeats), penta- (3 repeats), and hexamers (3 repeats) were identified in the publicly available *P. ultimum* transcriptome (Lévesque et al., 2010) with the Microsatellite Identification Tool (MISA) (PGRC, 2002). A custom perl script and the output MISA files were then used to design flanking primers to amplify identified SSRs using Primer3 (Rozen and Skaletsky, 1999) as previously described (Wallace E. C. and Quesada-Ocampo, 2014). Primer3 was set to design primers between 18 and 27 bp with optimal length of 20 bp, GC content between 20 and 80 with optimal GC content of 50, and a melting temperature between 57 and 63 °C with the optimal melting temperature of 60 °C, and that would amplify products between 100-300bp. A subset of 50 SSRs was selected based on the following criteria: i) motifs with number of repeats greater than three; ii) product size between 100 and 300 bp; iii) location in different scaffolds across the *P. ultimum* genome. To select the most polymorphic markers, SSRs were screened on eight DNA samples of isolates belonging to different greenhouse locations and hosts, and on one mixture of DNA from 50 different *P. ultimum* isolates. PCR of individual microsatellites consisted of 1X Buffer, 0.2 µM dNTPs, 0.2 µM of each SSRs primer, 0.5U of Taq polymerase, and 50 ng of DNA (1 µL) in a 15-µL reaction volume. Amplification reactions were performed on a Mastercycler thermal cycler (Eppendorf, Westbury, NY), with initial denaturation at 94°C for 4 minutes, followed by 30 cycles of 94°C for 1 minute, annealing temperature

at 60°C for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72° for 10 minutes. Samples were visualized by 3% MetaPhor (Lonza, Rockland, ME) gel electrophoresis at 100V for 3 or 4 hours, using Quantity One Software (Biorad, Hercules, CA).

The amplification product of each microsatellite locus was sequenced in order to confirm that the motif of each microsatellite locus was contained in the PCR products obtained. Resulting sequences were assembled in CLC Main Workbench (CLCbio, Aarhus, Denmark) and compared with BLASTn against the *P. ultimum* genome available in the *Pythium* genome database (Lévesque et al., 2010).

**Microsatellite analyses**

A total of six markers showing polymorphism in the initial screening were selected for microsatellite analysis of 166 total *P. ultimum* isolates using fluorescently labeled primers. The forward primers were modified with the dyes HEX, FAM or NED at the 5'-end, and the reverse primers were modified with a 'PIG tail' (GTTT) at the 5'-end (Guichoux et al., 2011) (Table 1). PCR were performed as explained above using 0.5 U of Pfu DNA polymerase (Thermo Scientific, Waltham, MA). Resulting amplification products were visualized by 2% agarose gels, and combined according to different product sizes: i) PY28, PY55 and PY57, and ii) PY62, Py69 and PY30 (Table 1). Combined products were subsequently sized by fragment analysis at Macrogen (Macrogen, Korea). SSR profiles generated by fragment analysis were first analyzed using Peak Scanner 2.0 (Applied Biosystem Software) for allele size calling, and then using the Tandem software (Matschiner and Salzburger, 2009) for allele binning to convert allele sizes into discrete units. To assess if the results obtained were

reproducible, the microstalite analyses was repeated with 50% of the population evaluated.

**Population genetics analyses**

Quantitative measures of gene diversity were estimated using GenAlEx 6.5 (Peakall and Smouse, 2012). They consisted of allelic diversity; number of alleles per locus (*Na*); effective number of alleles (*Ne*); private alleles; Nei's gene diversity (*h*), the probability that two randomly selected alleles from a population are different (Nei, 1978); and the unbiased gene diversity scaled according to sample size (*uh*). To determine if the six SSRs evaluated provide enough power to discriminate among individuals of the population, a genotype accumulation curve was generated with the R package *poppr* (Kamvar et al., 2014).

Quantitative measures of genetic diversity including genotypic richness, genotypic diversity, and evenness, were estimated using the R package *poppr* (Kamvar et al., 2014). Genotypic richness (*g*) is the number of genotypes observed in the population and the expected genotypic richness (*Eg*), which measures the number of expected genotypes present at the smallest sample size ($N = 18$) based on rarefaction curves (Grünwald et al., 2003). Genotypic diversity was quantified with Stoddart and Taylor's Index (*G*) (Stoddart and Taylor, 1988), which is based on sample size. To obtain a measure of genotypic diversity that is scaled according to sample size $H_{exp}$ was calculated. $H_{exp}$ measures the probability that two randomly selected genotypes from a population are different (Nei, 1978). Genotypic evenness ($E_5$) measures the distribution of genotype abundance in the population (Ludwig, 1988, Grünwald et al., 2003).

To determine whether the populations are clonal, the index of association ($I_A$), the standardized index of association ($\check{r}_d$), and the test of significance were estimated on

non-clone-corrected and clone-corrected populations in the R package *poppr* (Kamvar et al., 2014) . $I_A$ compares the variance of pairwise distance between all individuals within the population under no linkage disequilibrium (Agapow and Burt, 2001). Since the $I_A$ value depends on the sample size of the loci, to adjust the number of loci sampled, the $\check{r}_d$ was calculated. The test of significance was calculated by randomly permuting the alleles at each locus 1000 times.

Population structure was analyzed by Analysis of Molecular Variance (AMOVA) in *poppr* (Kamvar et al., 2014). The data set was arranged into two hierarchical levels: county and season sampled. AMOVA estimates the number of differences summed over loci based on a matrix of distances between individuals. Covariance components and fixation indices (Φ) were calculated for each hierarchical level established. The significance of fixation indices was calculated by 1000 random permutations (Grünwald and Hoheisel, 2006). The level of genetic flow among populations (*Nm*) was calculated using POPGENE v.3.1(Yeh et al., 1997). Minimum spanning networks (MSN) were generated across populations by Bruvo's genetic distance, which is based on a stepwise mutation events for microsatellites (Bruvo et al., 2004). An Unweighted Pair Group Method with Arithmetic Mean (UPGM) unrooted tree was generated based on Bruvo's genetic distance. The MSN and the UPGM tree were generated using *poppr* (Kamvar et al., 2014).

## RESULTS

### Microsatellite search and selection

A total of 2060 microsatellites were found in the *P. ultimum* transcriptome. Trinucleotide repeats were the most common (76%), followed by dinucleotide repeats

(20%) with the motifs CAG and GC being the most abundant, respectively. Most of the SSR loci tested did not show variability among isolates; only six out of 50 SSRs tested were polymorphic in the subset of isolates selected for the initial bulk-segregant analysis. The sequences of the amplification product of each SSR locus, confirmed the presence of each SSR motif. These six polymorphic markers (Table 1) were selected to evaluate the 166 *P. ultimum* isolates collected from greenhouse floral crops in Michigan. The expected amplification products for all markers were consistent with the alleles observed (Table 1), with the exception of the markers Py28 and Py39, where the allele sizes obtained were greater than expected. This difference in size was likely due to the presence of an intron, given that the microsatellites were obtained from the transcriptome. The number of alleles obtained for each SSR locus, ranged from two to six (Table 1). The genotypic accumulation curve showed that with these markers a 90% power was reached in order to discriminate among the 166 individuals analyzed.

**Population genetic diversity**

In the three populations evaluated, all six loci were polymorphic. The estimated number of different alleles per locus ranged from 2.83 to 3.33, and the number of effective alleles from 1.42 to 2.49 (Table 2). The population with the highest number of isolates (N =83) was collected in fall 2011 and contained the highest number of effective alleles. The population corresponding to the isolates collected during the spring 2012-2013 did not have any private alleles, whereas the population of fall 2011 and fall 2012 had a private alleles average of 0.25 (Table 2). The average Nei's gene diversity (*h*) for the three populations was 0.44 with the highest gene diversity (0.53) observed in fall

2012 population. The unbiased gene diversity (*uh*) values were the same as the *h* values obtained.

**Population genotypic diversity and differentiation**

A total of 64 multilocus genotypes (MLG) were observed across the three populations (Fall 2011. Spring 2012/13 and Fall 2012), ranging from 14 to 39 multilocus genotypes (Table 3) per population. For the Fall 2011 population, 10 MLG were recovered more than once, grouping clonal individuals. The most frequent multilocus genotype, MLG.5, encompassed more than 15 individuals (Fig 1). For the Spring 2012-2013 population, four MLG represented two individuals per clonal group; and for the Fall 2012 population, 11 MLG grouped more than three individuals, with MLG.50, only found in this population, and representing 15 individuals (Fig 1). Of the multilocus genotypes present in the entire population, 24% were unique. For each population (Fall 2011, Spring 2012-2013, and Fall 2012), the percentage of unique genotypes was 74, 71 and 50 respectively. Forty percent of the total individuals were represented in multilocus genotypes that were commonly found among the populations established by season, and among the places of origin. A total of five multilocus genotypes (MLG 5, 20, 30, 54 and 64) were shared by Fall 2011 and Spring 2012-2013 populations, four multilocus genotypes (MLG 12, 21, 27 and 37) were shared by Fall 2011 and Fall 2012 populations, and MLG 31 was present in all three populations (Fig 2). Summarized by location, one multilocus genotype (MLG 30) was present in Kalamazoo and Kent County, and four multilocus genotypes (MLG 50, 46, 20, 31) were present in Kalamazoo and Wayne County (Fig 1). Genotypic evenness ranged from 0.49 for the Fall 2011 population to 0.93 for the Spring 2012-2013 population, showing that the genotypes were evenly distributed for the three populations. The genetic richness expected with the smallest

sample size (N= 18) ranged from 11.13 (Fall 2012) to 14.0 (Spring). The genotypic diversity scaled to sample size, $H_{exp}$, averaged 0.938, with Fall 2012 having the lowest diversity (0.91) and Spring the highest (0.97) (Table 3).

Populations were not significantly differentiated among seasons, hosts, counties, and among samples within populations based on clone-corrected data (Table 4). A total of 84% of variation was observed within samples, 7% among samples within seasons, and 5% among counties (Table 4). Fixation indices were low, being lowest among seasons $\Phi = 0.03$ ($P = 0.138$), and highest within samples $\Phi = 0.15$ ($P = 0.032$). The gene flow estimate was 3.01, suggesting genetic exchange among the populations. The lack of population differentiation also was observed in the minimum-spanning network (MSN)(Fig 2) where each node representing the most frequent MLGs or a unique genotype from Fall 2011, Spring 2012-2013, and Fall 2012 populations, were closely related, and spread across the MSN clusters. The three assigned clusters, did not group the multilocus genotypes by population (Fall 2011, Spring 2012-2013, Fall 2012). The unrooted UPGM tree, constructed based on Bruvo's distances, also revealed that the Fall 2011, Spring 2012-2013, and Fall 2012 individuals collected from greenhouses in Kalamazoo, Kent and Wayne counties did not group separately (Fig 3).

*Clonality.* Although the *P. ultimum* population appears to be genetically diverse, given the 64 multilocus genotypes obtained, it resulted to be clonal. The index of association *($I_A$)*, and the standardized index of association ($\check{r}_d$), were significantly different from zero ( $P = 0.001$) for all individuals and for the clone-corrected data, indicating that the loci within the population were linked, and rejecting the hypothesis of random mating. The $I_A$ was 1.25 for Fall 2011 and Fall 2012 populations, and 1.32 for the Spring 2012-

2013 population, including all the individuals. For the clone corrected data the $I_A$ ranged from 0.7 (Fall 2011) to 1.04 (Spring 2012-2013) (Table 3).

The index of association was also calculated grouping the isolates by county of origin (Kalamazoo, Kent, and Wayne); the population from each county was also clonal ($I_A$ = 1.12 for Kalamazoo; $I_A$ = 2.76 for Wayne; $P$= 0.01) using non-clone-corrected and clone-corrected data. The index of association for the isolates from Kent County could not be calculated due to the low number of isolates (N=4) from this location.

**DISCUSSION**

Analysis of genetic variation among *P. ultimum* isolates recovered from ornamental greenhouse crops in Michigan using six microsatellite loci revealed limited differentiation among isolates grouped by season, host, and county of collection. Data is consistent with exchange of infected plant material among greenhouse facilities, or a common source of inoculum infecting plants in greenhouses located in Kalamazoo, Kent, and Wayne counties.

Fifteen percent of multilocus genotypes that grouped 40% of the total isolates were recovered repeatedly during the different seasons sampled, but 29 of 39 multilocus genotypes obtained in the first sampling season of Fall 2011 were eradicated. The presence of common genotypes among years suggests that the control strategies that are being implemented in the greenhouse facilities are not able to eradicate *P. ultimum*. Commonly, *P. ultimum* is controlled in greenhouses with the fungicide mefenoxam (Daughtrey and Benson, 2005, Hausbeck M., 2013, Moorman et al., 2002), and other fungicides. However, resistance to mefenoxam has been reported in 60% of *P. ultimum* isolates recovered from the greenhouse facilities evaluated in this study (Del Castillo-

Múnera J. and Hausbeck, 2014). Fifty four percent of the mefenoxam resistant isolates reported in the previous study, are grouped in all the multilocus genotypes repeatedly found between Fall 2011 and Spring 2012-2013 ( MLG 5, 20, 30, 54, 64), and the common ones between Fall 2011 and Fall 2012( MLG 12, 21,27, 37,31). The prevalence of these genotypes across years may suggest an established population of *P. ultimum* that is resistant to mefenoxam in Michigan greenhouses. In order to eradicate this pathogen, effective cultural practices should be implemented, including the scouting and roguing of symptomatic plant material (Daughtrey and Benson, 2005), the use of other active ingredients such as azoxystrobin, etridiazole, fenamidone (Hausbeck M., 2013, Moorman and Kim, 2004), and the biological controls agents *Trichoderma harzianum* and *Streptomyces lydicus* (Moorman and Kim, 2004, Daughtrey and Benson, 2005, Harman, 2000, Little et al., 2003).

Historically, *P. ultimum* is a recurrent pathogen in floriculture greenhouses (Stephens and Powell, 1982, Tompkins and Middleton, 1950b, Moorman et al., 2002). However, in this study we were able to discern the introduction of new isolates versus resident isolates of the region. The recovery of new genotypes (8 from Spring and 17 from Fall 2012) in the seasons following the Fall 2011 collection suggests the recent introduction of *P. ultimum* isolates into the greenhouses. New sources of inoculum can enter through the shipment of infected cuttings, seedlings, and pre-finished plants, from other greenhouse facilities, contaminated soilless media, and/or infected tools (Parke and Grünwald, 2012, Weiland et al., 2013). Also, *P. ultimum* can enter the greenhouses via irrigation water (Hong and Moorman, 2005). Most of the greenhouses sampled, use well water, but two greenhouses in Wayne County use city water for irrigation. Interestingly, greenhouses in Kalamazoo and Kent County recirculate their water used

for irrigation. This practice may increase the likelihood that *P. ultimum* is reintroduced and may favor the presence of unique genotypes within locations. In our study, greenhouses from Kalamazoo County had the greatest number of *P. ultimum* isolates, and unique genotypes. Also, the presence of unique genotypes by location may indicate one time or recent introduction of the pathogen.

The genotypic diversity scaled to size (0.938), and evenness (0.56), the recovery of 11 major clones (groupings of more than three individuals) among the 64 multilocus genotypes obtained, and the presence of 11 genotypes repeatedly over the three years of study may suggest that there is a survival or an established population of *P. ultimum* in Michigan greenhouses. Nevertheless, to determine if the *P. ultimum* population in Michigan is established and is not a recent introduction, coalescence analysis based on sequenced data to determined the population age (Grünwald and Goss, 2011), and a comparison of the genotypic diversity of *P. ultimum* populations from a different geographic origin should be performed. Despite the intensive sampling, sample size was limited for some of the locations; therefore, a monitoring program using the markers developed in this study will help to better understand the dynamics of this population. Conversely to the population structure that we found, the study of the population structure of *P. ultimum* from nurseries in Oregon revealed that the 30 isolates evaluated are grouped in two main clones suggesting that the species was recently introduced into the nurseries evaluated (Weiland et al., 2015).

The lack of population structure or differentiation among counties is supported by the evidence of genetic exchange among locations ($Nm = 3.01$), and by the analysis of molecular variance that shows the greatest amount of variation occurs within samples, rather than among counties. The presence of five common MLG in Kalamazoo, Kent,

and Wayne counties suggest the shipment or interchange of infected plant material among greenhouses in these counties, or a common source of inoculum that is being introduced to Michigan. Interestingly, MLG 50, which was only recovered in Fall 2012, was present at high frequency in greenhouses in Kalamazoo and Wayne County. This confirms that new genotypes are being introduced into Michigan greenhouses, possibly from a common source of inoculum present in infected plant material produced elsewhere. Greenhouse growers in Michigan often import poinsettia cuttings from wholesalers located in Central America. Similar to our findings, recent research on the population structure of *Pythium* spp. from forest nursery soils in Oregon (Weiland et al., 2015) found that 30 isolates corresponding to *P. ultimum* populations were not genetically differentiated among different nurseries and the gene flow analysis showed genetic exchange. A study of the oomycete *Phytophthora ramorum* infecting oak nurseries in Oregon (Prospero et al., 2007) confirmed that migration occurred due to the presence of common *Ph. ramorum* genotypes in different locations, indicating that shipment of infected plant material among nurseries may contribute to pathogen dissemination (Prospero et al., 2007, Weiland et al., 2015). The effect of human activity on the migration of plant pathogens has been previously documented in the case of the causal agent of the potato late blight, *Ph. infestans*, from central Mexico to South America (Goss et al., 2014, Grünwald and Flier, 2005). Other studies of *Pythium* spp. population structure revealed contrasting findings related to differentiation among populations. Weiland et al. (2015) analyzed the population structure of *P. irregulare* and *P. sylvaticum*; a significant geographic pattern among nursery populations was found in these two species. The population structure analysis of 123 *P. aphanidermatum* isolates from ornamental greenhouses in Pennsylvania using six AFLP

and four SSRs markers (Lee et al., 2010) revealed that despite the genetic movement among isolates from different locations, three genetic groups were differentiated with sampling location instead of host being the main factor contributing to genetic diversity. In our study, the populations were grouped by season; this grouping also implies grouping by host (poinsettia in fall and geranium in spring). The lack of differentiation among hosts, supports the non-host-specificity (Sutton et al., 2006, Lee et al., 2010), the wide range of this pathogen within ornamentals (Kucharek and Mitchell, 2000, Moorman et al., 2002, Stephens and Powell, 1982), and the ubiquitous nature of the *Pythium* genus (Martin and Loper, 1999, van der Plaats-Niterink, 1981).

Weiland et al. (2015) reported a heterozygosity of 0.59 for *P. ultimum* from nursery soils. The average Nei's heterozygosity (0.44) for the *P. ultimum* population analyzed in our study was similar to that reported by Weiland et al. (2015). Also, a study of genetic variation among *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiiferum* revealed a low heterozygosity of 0.14 for the 22 isolates analyzed with RFLPs (Francis et al., 1994). Studies of other *Pythium* spp., such as *P. aphanidermatum* from greenhouses in Pennsylvania, reported a high genetic diversity, with an average heterozygosity of 0.54 for the SSRs loci analyzed (Lee et al., 2010). A population analysis of *P. spinosum* isolated from cucumber in Oman, showed a low genetic diversity, obtaining a low polymorphism with AFLP markers (Al‐Sa'di et al., 2008).

In our study, the genetic diversity of the Fall 2011 and 2012 populations was greater than the diversity of the Spring 2012-2013 population. Most of the *P. ultimum* isolates were recovered from poinsettia in the fall, suggesting that the genetic variation is related to population size, that introduction of new isolates is more likely to occur in the

fall, and that larger and older populations have a greater chance for mutation or mitotic recombination to occur (McDonald and Linde, 2002b).

Despite the genetic diversity detected, the index of association showed that *P. ultimum* populations in Michigan greenhouses are largely clonal. The clonality of the species supports its homothallic or self-fertile nature (van der Plaats-Niterink, 1981, Francis et al., 1994). It has been shown that *P. ultimum* var. *ultimum* is capable of outcrossing (Francis and Clair, 1993, Francis and Clair, 1997) . Francis and Clair (1993) reported that crosses between two *P. ultimum* var. *ultimum* isolates resulted in hybrid progeny under controlled laboratory conditions. In our study, we rejected the null hypothesis of random mating for the *P. ultimum* populations, due to the statistical significance of the index of association (average $I_A$ = 1,19 for all the individuals and 0.92 for clone-corrected data), and to the presence of 11 major clones (Fig. 1) that represent 50% of the entire population. This shows, that the isolates are not undergoing sexual reproduction, and reproduce clonaly via zoospores or selfing. Similarly, the population of *P. ultimum* in Oregon was determined to be clonal (Weiland et al., 2015). Despite the clonality of the *P. ultimum* population, the genetic diversity observed ( 0.44), and the presence of 64 multilocus genotypes may be explained by the introduction of new genotypes in every season, that contributes to a greater genetic diversity. Also, in the *Pythium* genus, the presence of intraspecific variation has been reported(Francis et al., 1994, Garzón et al., 2005a, Chen et al., 1992, Eggertson, 2012). Specifically in *P. ultimum*, the recent evidence that *P. ultimum* is a species complex that encompasses four genetically distinct species (Eggertson, 2012) based on multi-nuclear gene genealogies, *P. ultimum*, which encompasses the *P. ultimum* var. *ultimum*; *P. sporangiiferum*, which contains *P. ultimum* var. *sporangiiferum*; and two groups named

*Pythium* sp. nov. 1, and *Pythium* sp. nov. 2. Although our results identified three clusters within the *P. ultimum* populations, the population analysis revealed intraspecific populations.

Our study is the first to analyze a *P. ultimum* population using a pseudo-multiplexing microsatellite analysis. We combined the amplified products of six SSRs loci marked with different fluorochromes and analyzed simultaneously the alleles obtained from the SSRs loci. The cost of SSRs genotyping based on multiplex reactions is considerably reduced compared to capillary analysis with a single PCR reaction; and that the capillary analysis improves the precision of allele sizing, compared with the traditional allele scoring based on gels (Guichoux et al., 2011, Li et al., 2013b, Hayden et al., 2008). The allele sizes that we obtained were reproducible with 50% of the isolates analyzed twice in this study. Currently, advances of next generation sequencing technologies are being applied for population genetics analyses (Grünwald and Goss, 2011). Genotyping by sequencing (GBS) (Elshire et al., 2011) is a very specific and reproducible method which uses SNPs markers to analyze the population structure of organisms that are not yet sequenced (Elshire et al., 2011). In terms of cost, GBS may be more cost-effective when applied to population analysis of organisms with unknown sequence, and with a high number of loci to be analyzed. The multiplex microsatellite analyses used in this study are more cost-effective because the *P.ultimum* genome is publicly available (Lévesque et al., 2010). According to the genotype accumulation curve with the six SSRs loci used there was adequate power (90%) to discriminate between individuals. In terms of number of markers needed to study the population structure of *Pythium* spp., Weiland et al. (2015), and Lee et al. (2010), showed that five and four SSRs, respectively, provided enough genetic information to characterize a population.

The study of the population structure of *P. ultimum* from Michigan floriculture crops may impact the management strategies developed to control Pythium root rot in the region. The evidence of pathogen movement among greenhouses across the state or of a common source of inoculum coming to the region, suggests that an improved scouting of new plant material arriving to greenhouses should be performed. Fast and accurate diagnostics methods to detect *Pythium* spp. would also be helpful (Schroeder et al., 2013). The recovery of the same pathogen genotypes across the three years of study suggests that greenhouse facilities should consider more effective control strategies to reduce the *P. ultimum* population. Future studies that monitor the introduction of new genotypes based on those found in this study, and future shifts in the population dynamics of *P. ultimum* over time should be considered.

**APPENDIX**

**TABLE 4.1**. Characteristics of the SSR loci evaluated in the *Pythium ultimum* populations from greenhouse floral crops in Michigan

| | | | | Alleles[e] | |
|---|---|---|---|---|---|
| **SSR loci[a]** | **Repeat motif[b]** | **Primer sequence (5' to 3')[c]** | **Gene id.[d]** | **N** | **Size (bp)** |
| Py28 | $(CTG)_4$ | F: [HEX] ATGTCGTCAACGGTGTGGTC<br>R: GTTTGGATGTCGACGCCTGAGTAG | PYU1_T008001 | 6 | 497, 499, 501, 503, 509, 511 |
| Py30 | $(GCTCCT)_3$ | F: [HEX] AAGCGATTGTGGAGAAGCGA<br>R: GTTTTTGGAGGAGTTGCAGCTTGT | PYU1_T009522 | 3 | 418, 424, 430 |
| Py55 | $(GT)_{10}$ | F: [FAM] GCAATGCACGCTCTCTATGG<br>R: GTTTGCTGGATCATAGAGCGCTGT | PYU1_T002451 | 5 | 161, 163, 167, 171, 173 |
| Py57 | $(TCT)_7$ | F: [NED] GACTTTGAGGACGACCAGCA<br>R: GTTTCTCTCTTTCGCTGCCGTTTG | PYU1_T004435 | 3 | 195, 201, 537 |
| Py62 | $(TCG)_7$ | F: [ FAM] GTTCCTGCGCATTCATCGTC<br>R: GTTTACCATGAGTCCTGGCCATTG | PYU1_T002941 | 4 | 164, 167, 170, 173 |
| Py69 | $(CAAAGC)_3$ | F: [NED] GAACCTCGGTATCAGCTCGG<br>R: GTTTCCGATTCATCCGAGATCGCA | PYU1_T011952 | 3 | 284, 290, 296 |

[a]SSR locus ID assigned in this study.

[b]Repeat motif and the number of times it is repeated in the transcriptome sequence of *P. ultimum.*

[c]Primer sequences with the fluorophores HEX, FAM or NED used for labeling each forward primer.

[d]Gene id from the *P. ultimum* genome (Lévesque et al., 2010) of each SSR locus.

[e] Number of alleles and size of microsatellite amplicons based on results from the Peak Scanner 2.0 (Applied Biosystem Software) and Tandem (Matschiner and Salzburger, 2009).

**TABLE 4.2.** Genetic diversity of *P. ultimum* populations from floral crops in Michigan, where each population represents isolates obtained during fall and spring seasons from 2011 to 2013, analyzed with six SSR loci.

| Population[a] | N | *Na*[b] | *Ne*[c] | Private Alleles[d] | *Ho*[e] | *h*[f] | *Uh*[g] |
|---|---|---|---|---|---|---|---|
| Fall 2011 | 83 | 3.33 | 2.48 | 0.17 | 0.58 | 0.52 | 0.52 |
| Spring 2012-13 | 18 | 2.83 | 1.42 | 0 | 0.19 | 0.27 | 0.28 |
| Fall 2012 | 65 | 3.50 | 2.41 | 0.33 | 0.47 | 0.53 | 0.54 |
| Mean | 55.33 | 3.22 | 2.10 | 0.25 | 0.42 | 0.44 | 0.45 |

[a]Population denoted by sampling season: Fall 2011: 83 *P. ultimum* isolates collected from poinsettia in fall. Spring : 18 *P. ultimum* isolates collected and combined from geranium in spring of 2012 and 2013. Fall 2012: 65 *P. ultimum* isolates collected from poinsettia.

[b]*Na*. Observed number of alleles.

[c]*Ne.* Effective number of alleles.

[d]Number of alleles unique to the individual population.

[e]Observed genetic diversity (heterozygosity)

[f]*h.* Nei's gene diversity, the probability that two randomly selected alleles from a population are different (Nei, 1978).

[g]*Uh*. Unbiased gene diversity scaled to sample size.

**TABLE 4.3.** An analysis of genotypic diversity and Index of association with all individuals and with clone-corrected data of *P. ultimum* populations from floriculture crops in Michigan using six SSRs loci.

| | | All individuals | | | | | | | Clone-corrected | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Population**[q] | **N** | $g$[r] | $Eg$[s] | $G$[t] | $H_{exp}$[w] | $E_5$[x] | $I_A$[y] | $\check{r}_d$[z] | $I_A$[y] | $\check{r}_d$[z] |
| Fall 2011 | 83 | 39 | 12.127 | 11.202 | 0.921 | 0.498 | 1.255* | 0.284* | 0.775* | 0.173* |
| Spring 2012-13 | 18 | 14 | 14.0 | 12.462 | 0.973 | 0.937 | 1.320* | 0.280* | 1.047* | 0.221* |
| Fall 2012 | 65 | 22 | 11.136 | 10.536 | 0.919 | 0.680 | 1.251* | 0.260* | 0.916* | 0.189* |
| Total | 166 | 64 | 13.764 | 21.461 | 0.959 | 0.566 | 1.191* | 0.255* | 0.912* | 0.194* |

[q] Population denoted by sampling season: Fall 2011: 83 *P. ultimum* isolates collected from poinsettia in fall. Spring 2012-2013 : 18 *P. ultimum* isolates collected and combined from geranium in spring of 2012 and 2013. Fall 2012: 65 *P. ultimum* isolates collected from poinsettia.

[r] Genotypic richness: number of genotypes observed in the population.

[s]Expected genotypic riches: Number of expected genotypes present at the smallest sample size (N =18) based on rarefaction curves.

[t]Genotypic diversity calculated with the Stoddart and Taylor's Index.

[w]Genotypic diversity scaled to sample size.

[x]Genotypic evenness measures the distribution of genotype abundance in the population.

[y]Index of Association test for linkage disequilibrium for all individuals and for clone-corrected data, where an asterisk indicates statistical significance ($P$ = 0.001) compared with 1000 randomizations.

**TABLE 4.3. (cont'd)**

[z]Standardized Index of association for all individuals and for clone-corrected data, where an asterisk indicates statistical significance (*P* = 0.001) compared with 1000 randomizations.

**TABLE 4.4.** Analysis of molecular variance for SSRs data of *P. ultimum* population from floriculture crops in Michigan[a].

| Hierarchical level[a] | Variation (%) | Φ | *P* |
|---|---|---|---|
| Variation among counties | 5.093 | 0.05 | 0.909[b] |
| Variation between season within county | 2.988 | 0.031 | 0.142[c] |
| Variation between samples within season | 7.751 | 0.084 | 0.126[d] |
| Variation within samples | 84.166 | 0.158 | 0.017[e] |

[a] AMOVA estimates the number of differences summed over loci based on a matrix of distances between individuals. For this analysis, the data set was arranged into two hierarchical levels: county and season sampled.

[b]Probability of obtaining equal or lower Φ value determined by 1,000 randomizations by permuting genotypes between counties.

[c] Probability of obtaining equal or lower Φ value determined by 1,000 randomizations by permuting genotypes between seasons within counties.

[d] Probability of obtaining equal or lower Φ value determined by 1,000 randomizations by permuting genotypes between seasons.

[e] Probability of obtaining equal or lower Φ value determined by 1,000 randomizations by permuting genotypes within all the population

**Table 4.5.** Isolates of *P. ultimum* used in this study[a]

| Isolate number[z] | Host | Origin | Greenhouse | Year |
|---|---|---|---|---|
| 1.2 A | *Euphorbia pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.2 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.3 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.3 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.3 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.9 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.9 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.11A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.11 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.11 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.13 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.13 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.17 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.17 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.18 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.18 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.22 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.22 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.27 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.27 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.31 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.31 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.43 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.44 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.44 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.44 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.45 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.45 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.49 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.49 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.49 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.55 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.56 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.56 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.56 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.57 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.57 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.58 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.58 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.58 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.59 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.59 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.60 A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.61 B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 1.61 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2011 |
| 2.3 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.3 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.4 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |

**TABLE 4.5 (cont'd)**

| | | | | |
|---|---|---|---|---|
| 2.4 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.5 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.5 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.8 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.8 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.14 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.14 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.17 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.17 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.30 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.30 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.30 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.33 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.33 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.34 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.35 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.35 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.35 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.39 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.44 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.44 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.44 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.45 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.45 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.45 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.48 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.49 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.49 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.52 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.54 B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 2.54 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2011 |
| 4.2 A | *E. pulcherrima* | Kent county, MI | 1 | 2011 |
| 4.2 C | *E. pulcherrima* | Kent county, MI | 1 | 2011 |
| 4.21 B | *E. pulcherrima* | Kent county, MI | 1 | 2011 |
| 4.21 C | *E. pulcherrima* | Kent county, MI | 1 | 2011 |
| 12.3B | *Pelargorium* x *hortorum* | Wayne county, MI | 2 | 2012 |
| 12.3C | *Pelargorium* | Wayne county, MI | 2 | 2012 |
| 15.1A | *Pelargorium* | Kalamazoo county, MI | 1 | 2012 |
| 16.3 B | *Pelargorium* | Kalamazoo county, MI | 2 | 2012 |
| 16.3 C | *Pelargorium* | Kalamazoo county, MI | 2 | 2012 |
| 19.5B | *Pelargorium* | Kalamazoo county, MI | 5 | 2012 |
| 19.50B | *Pelargorium* | Kalamazoo county, MI | 5 | 2012 |
| 23.5A | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 23.5B | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 23.5C | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 23.9B | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 23.9C | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 23.15B | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 23.16 C | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 23.17A | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 23.17C | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |

**TABLE 4.5 (cont'd)**

| | | | | |
|---|---|---|---|---|
| 23.18 B | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 23.19A | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 23.19C | *E. pulcherrima* | Wayne county, MI | 4 | 2012 |
| 24.3 A | *E. pulcherrima* | Wayne county, MI | 1 | 2012 |
| 24.3B | *E. pulcherrima* | Wayne county, MI | 1 | 2012 |
| 24.3C | *E. pulcherrima* | Wayne county, MI | 1 | 2012 |
| 24.13C | *E. pulcherrima* | Wayne county, MI | 1 | 2012 |
| 24.13B | *E. pulcherrima* | Wayne county, MI | 1 | 2012 |
| 24.16C | *E. pulcherrima* | Wayne county, MI | 1 | 2012 |
| 28.13B | *E. pulcherrima* | Kalamazoo county, MI | 5 | 2012 |
| 28.13C | *E. pulcherrima* | Kalamazoo county, MI | 5 | 2012 |
| 29.1A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 29.1B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 29.1 C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 29.4A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 29.4B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 29.4C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 29.9A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 29.9B | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 29.9C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 29.10A | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 29.10C | *E. pulcherrima* | Kalamazoo county, MI | 1 | 2012 |
| 30.1 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.9A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.9B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.9C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.10A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.10B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.10C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.11B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.11C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.12B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.12C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.13A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.13 C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.15A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.15B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.15C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.16A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.16C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.18A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.18B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.18C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.19A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.19B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.19C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.23 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.23B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.23C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.24A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.24B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |

**TABLE 4.5 (cont'd)**

| | | | | |
|---|---|---|---|---|
| 30.24C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.26 A | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.34B | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 30.34C | *E. pulcherrima* | Kalamazoo county, MI | 2 | 2012 |
| 39.12 A | *Pelagorium* | Kalamazoo county, MI | 2 | 2012 |
| 40.19 A | *Pelagorium* | Kalamazoo county, MI | 2 | 2013 |
| 40.19 B | *Pelagorium* | Kalamazoo county, MI | 1 | 2013 |
| 41.6 C | *Pelagorium* | Kalamazoo county, MI | 1 | 2013 |
| 41.7 A | *Pelagorium* | Kalamazoo county, MI | 3 | 2013 |
| 41.7 B | *Pelagorium* | Kalamazoo county, MI | 3 | 2013 |
| 41.7 C | *Pelagorium* | Kalamazoo county, MI | 3 | 2013 |
| 42.26A | *Pelagorium* | Kalamazoo county, MI | 3 | 2013 |
| 42.27 A | *Pelagorium* | Kalamazoo county, MI | 4 | 2013 |
| 42.27 B | *Pelagorium* | Kalamazoo county, MI | 4 | 2013 |
| 42.27 C | *Pelagorium* | Kalamazoo county, MI | 4 | 2013 |

[a]Isolates obtained from roots of symptomatic plants were washed under running tap water, cut, air-dried, and three replicates per sample were placed on plates of corn meal agar (CMA). Poinsettias were sampled during Fall 2011 and 2012 and geraniums were sampled spring 2012 and 2013 from greenhouse floral crops in Michigan.

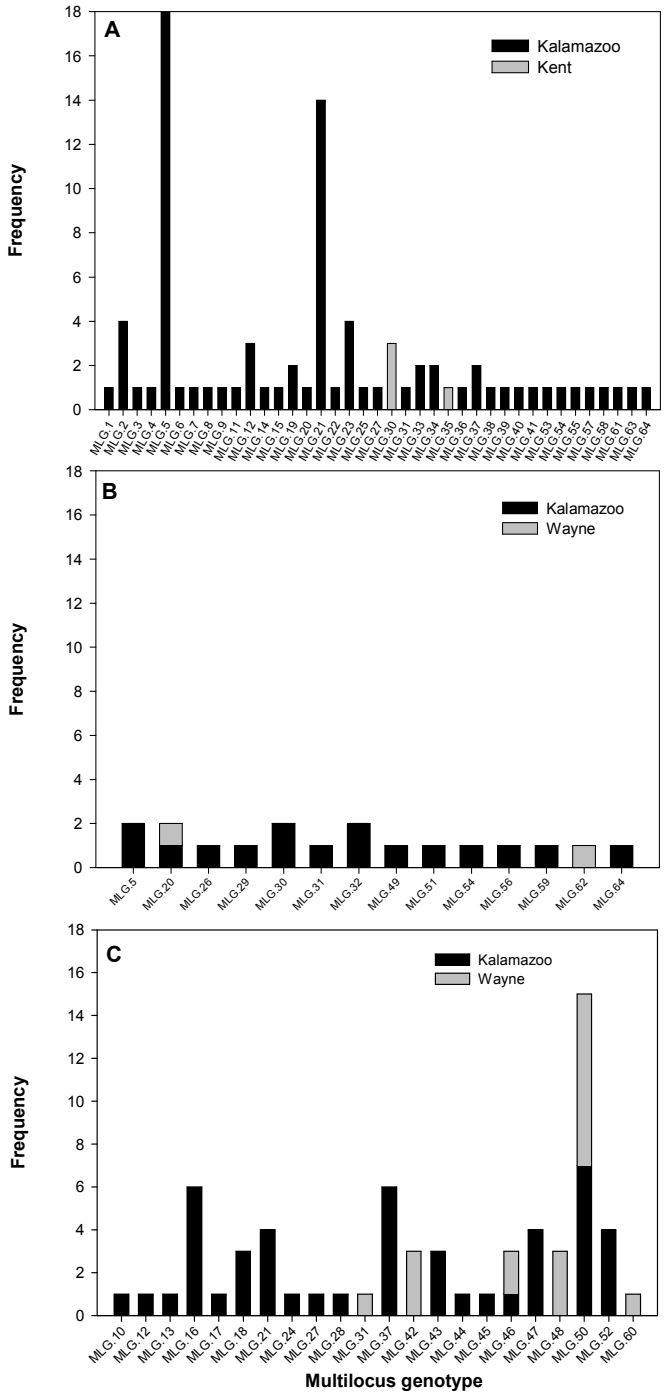


**Fig. 4.1**

**Figure 4.1.** Frequency distribution of *Pythium ultimum* genotypes detected in **A.** Fall of 2011, **B**. Spring of 2012 and 2012 and **C.** Fall of 2012 from greenhouse floral crops in Kalamazoo, Kent and Wayne Counties.

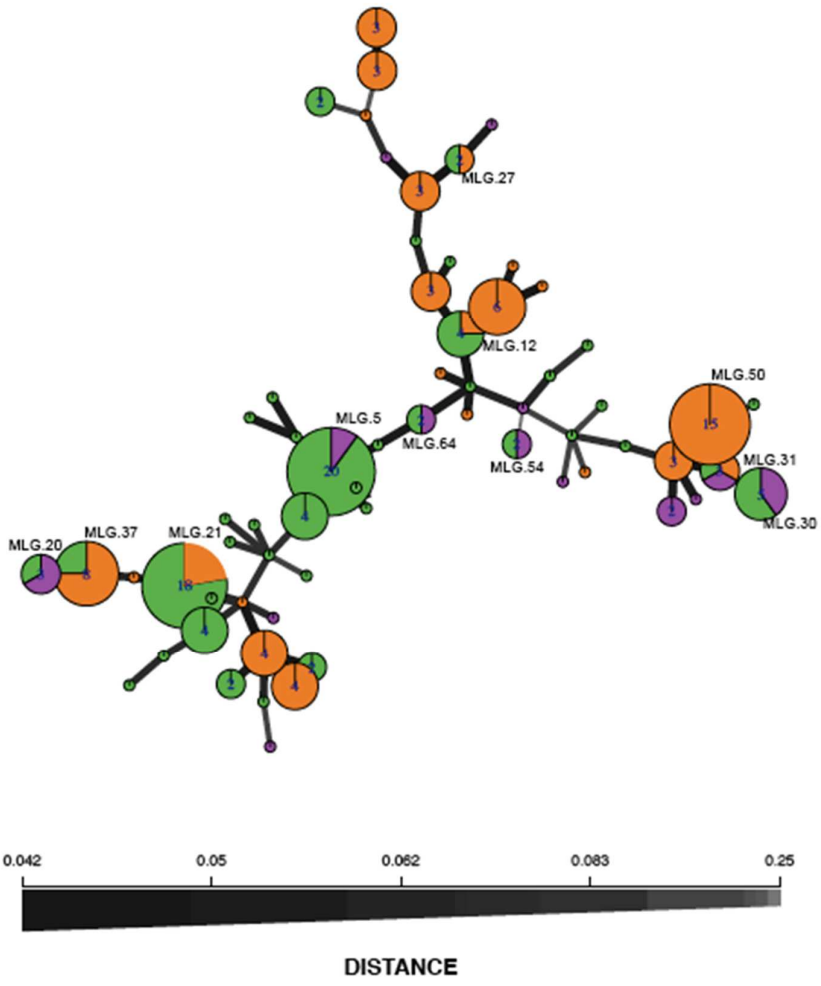


**Fig 4.2**

**Figure 4.2.** Minimum spanning network for *P. ultimum* multilocus genotypes from Michigan. Each node (circle) represents a multilocus genotype. Distances between nodes are proportional to Bruvo's distance (Bruvo et al., 2004). Node sizes are indicators of frequency and are colored in proportion of their frequencies in Fall 2011 (green), Spring (purple), and Fall 2012 (orange).

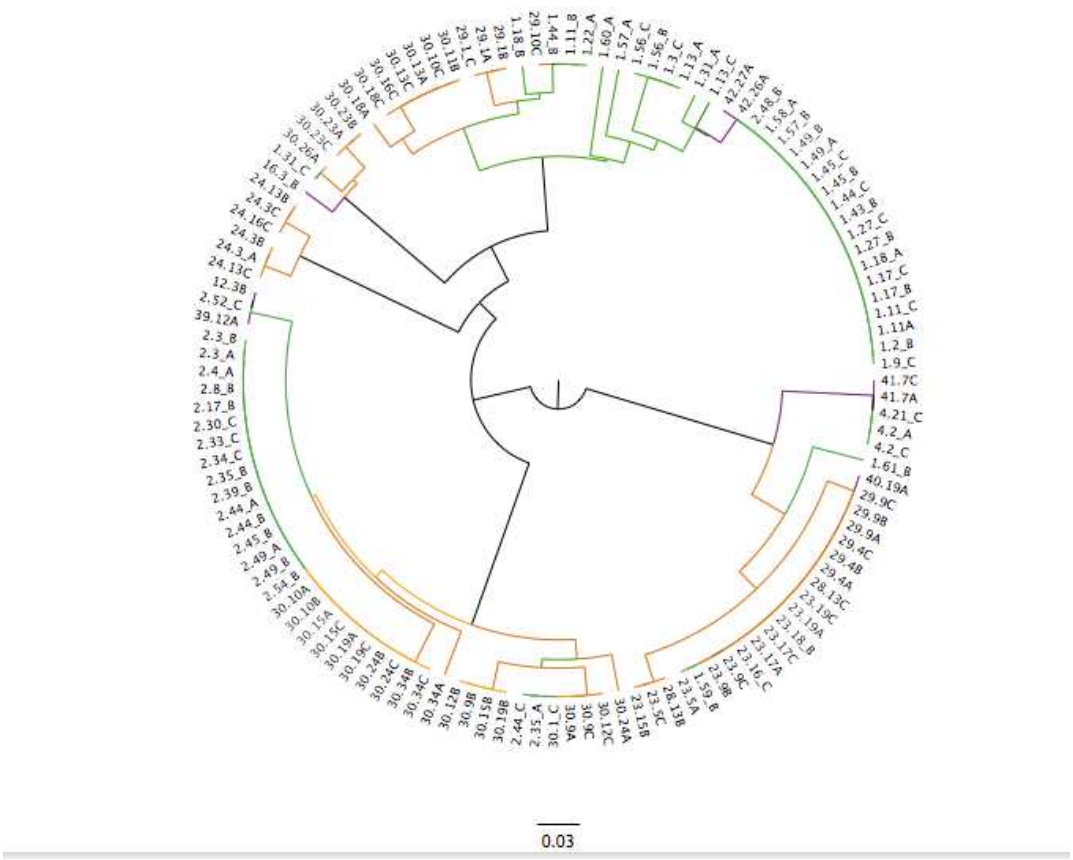


**Fig. 4.3**

**Figure 4.3.** UPGMA tree of *P. ultimum* based on Bruvo's genetic distance obtained with SSRs loci. Colors branches indicate the population established: Fall 2011 (green), Spring (purple), Fall 2012 (orange).

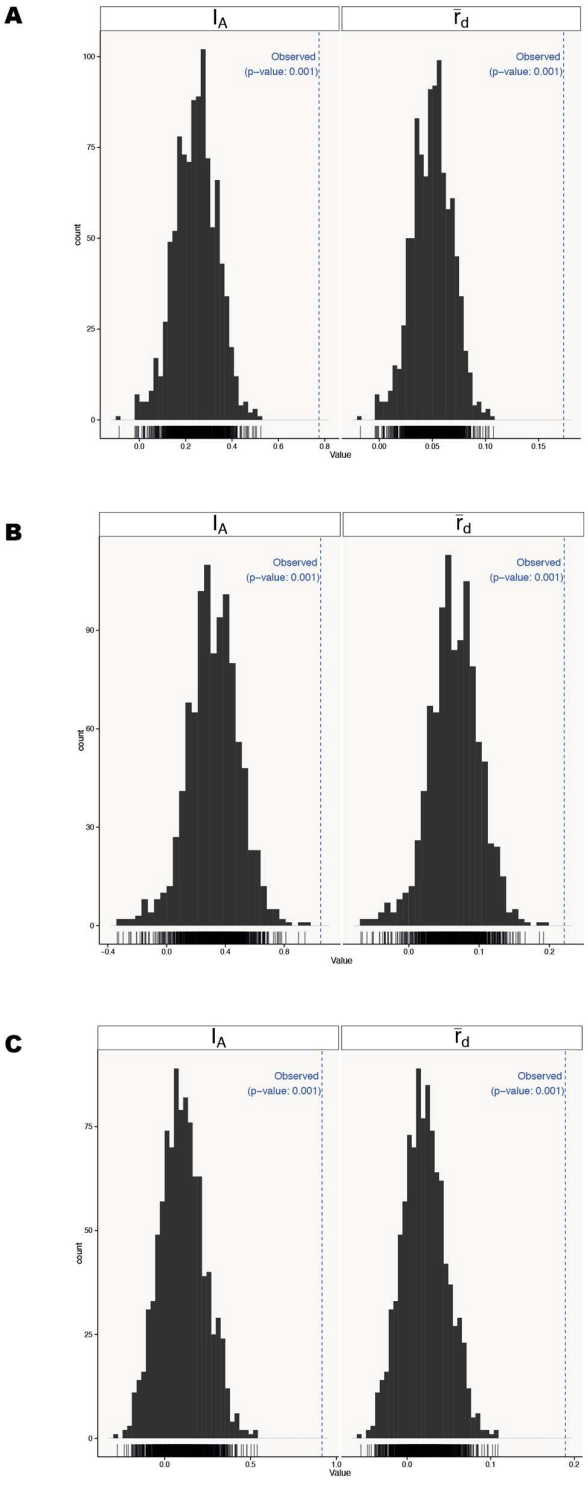


**Fig.4.4**

**Figure 4.4.** The null distribution of the Index of association $I_A$ and the standardized index of association ($\check{r}_d$), compared to the observed value (blue line) on clone-

**Figure 4.4. (cont'd)**

corrected data from *P. ultimum* in **A.** Fall 2011, **B.** Spring 2012 and 2013 and **C.** Fall 2012, rejecting the null hypothesis of random mating.

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## LITERATURE CITED


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