

The Pennsylvania State University

The Graduate School

Department of Plant Pathology and Environmental Microbiology

**EFFECT OF HETEROTROPHIC BACTERIAL COMMUNITIES ON
PYTHIUM SPP. IN RECYCLED IRRIGATION WATER**

A Dissertation in

Plant Pathology

by

María L. Burgos-Garay

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The dissertation of María L. Burgos-Garay was reviewed and approved* by the following:

Gary W. Moorman
Professor of Plant Pathology
Dissertation Advisor
Chair of Committee

Edward G. Dudley
Associate Professor of Food Science

Beth Gugino
Assistant Professor of Plant Pathology

María del Mar Jiménez-Gasco
Assistant Professor of Plant Pathology

Timothy W. McNellis
Associate Professor of Plant Pathology

Frederick E. Gildow
Professor of Plant Pathology
Head of the Department of Plant Pathology

*Signatures are on file in the Graduate School

Abstract

A main concern in commercial greenhouses is the harboring of *Pythium* species in recycled irrigation water and their spread into susceptible crops. *Pythium* species are among the most damaging pathogens of horticultural crops causing damping-off of seedlings as well as root and stem rots in ornamental plants. Despite frequent attempts to recover *Pythium* from recycled water, success in isolation is sporadic. This raises the question of possible microbial suppression of *Pythium* present in recycled irrigation water.

The present study focused on the deleterious effect of microbial communities in recycled irrigation water on the development of *Pythium aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare*. Microscopic observations of the interaction between microorganisms in recycled irrigation water and *Pythium* species indicated a deleterious effect on *Pythium* development. The amount of sporangia produced in the presence of the microbial community was negatively affected when compared to water samples with no microorganisms. In addition, research was conducted to evaluate heterotrophic bacteria isolated from recycled irrigation water that reduced the growth *in vitro* of the three highly pathogenic *Pythium* species. We isolated a variety of bacteria some of which inhibited the three *Pythium* spp. growth, others bound to their hyphae, and still others seemed to enhance the growth of the *Pythium* species. Known biocontrol agents against *Pythium* (*Pseudomonas* and *Bacillus*) and some potentially new biocontrol agents (*Acidovorax*, *Microbacterium*, and *Chryseobacterium*) were identified as isolates inhibiting the growth of *P. aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare in vitro*. The use of biological control agents to control *Pythium* spp. is not new. However, in most cases, commercially available beneficial organisms are from other environments. Utilization of naturally occurring suppressive bacterial communities in recycled irrigation water may provide an environmentally friendly ecologically sound biological control strategy. We observed that *Pythium* grew slower when bacterial isolates bound to their hyphae. This phenomenon was previously observed but is not fully understood. These isolates were identified as *Acinetobacter*, *Enterobacter*, *Pantoea*, and *Pseudomonas* species. Surprisingly, we also isolated

bacteria that enhanced the growth of *Pythium in vitro*. More research is needed to understand this phenomenon.

Further studies were conducted to determine if changes in bacterial community composition were associated with the presence of *Pythium* species in the water. We used automated ribosomal intergenic spacer analysis (ARISA) to examine the impact the presence of *P. aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* had on bacteria diversity in recycled irrigated water. ARISA profiles indicated that all three *Pythium* species had an impact on the bacterial community. To confirm ARISA results, we used quantitative PCR (q-PCR) to quantify the change in γ -Proteobacteria populations when *P. aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* were added to recycled irrigation water samples. All samples showed a significant increase in γ -Proteobacteria population when one of the three *Pythium* was present in the water sample. The results suggest that *Pythium* presence plays a role in structuring bacterial community composition.

In an effort to gain knowledge of the aquatic ecology of *Pythium* spp., we isolated and identified *Pythium* species from greenhouse water reservoirs to elucidate temporal patterns of *Pythium* in that aquatic environment. This is an important aspect to increase farmer awareness of this genus and the species that are more prevalent in this ecosystem. The results suggest that some *Pythium* species survived for long periods (e.g. *Pythium* group F isolates) while other species are transient (e.g. *P. catenulatum*) in this environment. *P. aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare* were rarely isolated (one time only) from greenhouse water reservoirs suggesting that microbial populations in recycled irrigation water suppress these three pathogenic *Pythium* species.

TABLE OF CONTENTS

LIST OF FIGURES	viii
LIST OF TABLES.....	xi
ACKNOWLEDGEMENTS	xii
Chapter 1 Literature Review	1
Pythium species.....	1
Plant Pathogenic Species	3
<i>Pythium aphanidermatum</i> (Edson) Fitzp.	6
<i>Pythium irregulare</i> Buisman	6
<i>Pythium cryptoirregulare</i> Garzón, Yáñez & Moorman	7
Diseases Caused by <i>Pythium</i>	8
Sources of <i>Pythium</i> Inoculum in the Greenhouse	9
Survival of <i>Pythium</i> species in Recycled Irrigation Water.....	11
Recycled irrigation water.....	12
Water Environment.....	13
Microbial communities in Recycled Irrigation Water System	15
Biological control.....	16
Mode of Action	16
Biological control of <i>Pythium</i> diseases	17
Microbial Interaction in Recycled Irrigation Water	19
Research Objectives.....	19
Literature Cited	22
Chapter 2 Effect of microbial communities present in recycled irrigation water on the development of <i>P. aphanidermatum</i> , <i>P. irregulare</i> , and <i>P. cryptoirregulare</i> <i>in vitro</i>	32
Abstract.....	32
Introduction	32
Materials and Methods.....	34
<i>Pythium</i> species	34
Recycled irrigation water sampling.....	35
Microbial community fractionation	35
Testing the effect of microbial communities on <i>Pythium</i>	37
Microscopic observations.....	38
Data analysis.....	38
Results.....	39
Influence of microbial community on sporangia	39
A. <i>Pythium aphanidermatum</i>	39

B. <i>Pythium cryptoirregulare</i>	40
C. <i>Pythium irregulare</i>	42
Influence of microbial community in the production of zoospores	43
Discussion.....	45
Acknowledgments.....	47
Literature cited	48
 Chapter 3 Heterotrophic bacteria in recycled irrigation water and their <i>in vitro</i> interaction with <i>Pythium aphanidermatum</i> , <i>P. irregulare</i> , and <i>P. cryptoirregulare</i>	52
Abstract.....	52
Introduction	52
Materials and Methods.....	54
<i>Pythium</i> isolates	54
Isolation of heterotrophic bacteria	54
<i>In vitro</i> effect on three <i>Pythium</i> species.....	55
DNA extraction	56
PCR amplification of 16S rDNA from bacteria	56
DNA sequencing	57
Greenhouse study	57
Statistical analysis	59
Results.....	59
Heterotrophic bacteria isolates in recycled irrigation water	59
<i>In vitro</i> inhibitory effect on <i>Pythium</i>	62
<i>In vitro</i> attaching effect on <i>Pythium</i>	64
<i>In vitro</i> growth enhancing effect on <i>Pythium</i>	66
Greenhouse experiment.....	68
Discussion.....	70
Acknowledgments.....	74
Literature cited	74
 Chapter 4 Shift of Bacterial Community in Recycled Irrigation Water	80
Abstract.....	80
Introduction	80
Materials and Methods.....	82
<i>Pythium</i> inoculum	82
Water samples	83
Isolation of bacterial DNA and quantification.....	83
PCR amplification of the ITS region	83
Automated ribosomal intergenic spacer analysis (ARISA)	84
Genus-specific quantitative PCR (q-PCR).....	85
Data analysis.....	86
Results.....	86

ARISA fingerprinting.....	86
Community similarity	88
Nonmetric multidimensional plots.....	88
Quantitative PCR (q-PCR)	92
Discussion.....	94
Acknowledgments.....	95
Literature cited	96
 Chapter 5 <i>Pythium</i> species in recycled irrigation water reservoirs	101
Abstract.....	101
Introduction	101
Materials and Methods.....	103
<i>Pythium</i> isolation	103
Morphological identification	104
DNA isolation and PCR for amplification of the ITS1, 5.8, and ITS2 rDNA.....	104
DNA sequencing	104
Results.....	105
<i>Pythium</i> species identification	105
A. Greenhouse S	105
<i>Pythium</i> species identification	107
B. Greenhouse E	107
<i>Pythium</i> species frequency	110
Discussion.....	112
Acknowledgments.....	115
Literature cited	115
 Conclusions	118

LIST OF FIGURES

Figure 1-1: Structures produced by <i>Pythium</i> species.....	3
Figure 1-2: Disease cycle of <i>Pythium</i>	5
Figure 1-3: Symptoms caused by <i>Pythium</i> specie	9
Figure 1-4: Recycled irrigation water reservoir.....	13
Figure 2-1: Recycled irrigation water fraction samples filtrates	36
Figure 2-2: Schematic of water fraction samples	38
Figure 2-3: <i>P. aphanidermatum</i> sporangia formed in water fraction samples in greenhouse S.....	39
Figure 2-4: <i>P. aphanidermatum</i> sporangia formed in water fraction samples in greenhouse E.....	40
Figure 2-5: <i>P. cryptoirregulare</i> sporangia formed in water fraction samples in greenhouse S.....	41
Figure 2-6: <i>P. cryptoirregulare</i> sporangia formed in water fraction samples in greenhouse E.....	41
Figure 2-7: <i>P. irregulare</i> sporangia formed in water fraction samples in greenhouse S.....	42
Figure 2-8: <i>P. irregulare</i> sporangia formed in water fraction samples in greenhouse E.....	43
Figure 2-9: Zoospores produced by all three <i>Pythium</i> species in greenhouse S.....	44
Figure 2-10: Zoospores produced by all three <i>Pythium</i> species in greenhouse E.....	44
Figure 3-1: Number of copiotrophic and oligotrophic bacteria in greenhouse S.....	60
Figure 3-2: Number of copiotrophic and oligotrophic bacteria in greenhouse E.....	60

Figure 3-3: Photo of bacterial <i>in vitro</i> effects.....	62
Figure 3-4: Phylogenetic tree of inhibitory bacteria	64
Figure 3-5: Phylogenetic tree of attaching bacteria.....	66
Figure 3-6: Phylogenetic tree of enhancing bacteria.....	68
Figure 3-7: Disease progress curve of geranium under greenhouse conditions.....	69
Figure 3-8: Area under the progress curve of geranium disease under greenhouse conditions S	70
Figure 4-1: Operational taxonomic unit (OTU) number per greenhouse	87
Figure 4-2: Analysis of bacterial communities in recycled irrigation water from greenhouse S.....	89
Figure 4-3: Analysis of bacterial communities in recycled irrigation water from greenhouse E.....	90
Figure 4-4: Nonmetric multidimensional scaling (NMDS) plot was constructed using all ARISA profiles from greenhouses S and E and analyzed in XLStat (AddinSoft SARL) using total area of peaks	91
Figure 4-5: Nonmetric multidimensional scaling (NMDS) plot was constructed using all ARISA profiles from greenhouse S for time samples day 0, 2, 7 and analyzed in XLStat (AddinSoft SARL) using total area of peaks	91
Figure 4-6: Nonmetric multidimensional scaling (NMDS) plot was constructed using all ARISA profiles from greenhouse E for time samples day 0, 2, 7 and analyzed in XLStat (AddinSoft SARL) using total area of peaks	92
Figure 4-7: q-PCR results for the amount of 16S rRNA γ -Proteobacteria genes in water samples detected following 0 (d0), 2 (d2) and 7 (d7) days of incubation	93
Figure 5-1: Phylogenetic tree of <i>Pythium</i> species isolated from water tank in greenhouse S	106

Figure 5-2: Phylogenetic tree of <i>Pythium</i> species isolated from water tanks L in greenhouse E	108
Figure 5-3: Phylogenetic tree of <i>Pythium</i> species isolated from water tanks R in greenhouse E	108
Figure 5-4: Phylogenetic tree of <i>Pythium</i> species isolated from water tanks G in greenhouse E.....	109
Figure 5-5: Phylogenetic tree of <i>Pythium</i> species isolated from water tanks SB in greenhouse E.....	109
Figure 5-6: Distribution of <i>Pythium</i> species isolated according to the month	112

LIST OF TABLES

Table 1-1: Sources of <i>Pythium</i> inoculum in the greenhouse	11
Table 1-2: Bacteria-fungal interaction leading to biological control by bacteria.....	17
Table 2-1: Identification of <i>Pythium</i> species used in the study.....	35
Table 2-2: Average measurements of recycled irrigation water sample.....	37
Table 3-1: Bacterial isolates identification list.....	61
Table 3-2: Bacterial isolates inhibiting <i>Pythium in vitro</i> list.....	63
Table 3-3: Bacterial isolates attaching to <i>Pythium in vitro</i> list.....	65
Table 3-4: Bacterial isolates enhancing <i>Pythium in vitro</i> list	67
Table4-1: Diversity index for greenhouse S and E	88
Table 4-2: Analysis of similarity (ANOSIM) between communities in recycled irrigation water for ARISA profiles based on Bray-Curtis similarity matrix	92
Table 5-1: Amount of each <i>Pythium</i> species isolated from the two greenhouses	111

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CHAPTER 1

Literature Review

Pythium species

Pythium is an ubiquitous genus that includes a variety of species found in terrestrial and aquatic habitats where they can live as saprophytes, plant or animal parasites, or mycoparasites. The genus was established by Pringsheim in 1858 [2] and in the current taxonomic scheme it is placed in the Kingdom Stramenopila, Phylum Oomycota, Class Peronosporomycetes, Order Pythiales, Family Pythiaceae, Genus *Pythium* [25]. The majority of plant pathogenic oomycetes belong to two orders within the Peronosporomycetidae, the Peronosporales and Pythiales [103]. The order Pythiales includes *Pythium*, *Phytophthora*, and *Albugo*. *Pythium* species are eukaryotes, fungi-like organisms that were long considered to belong within the kingdom 'Fungi' [30]. Both oomycetes and fungi share distinct similarities. Both have filamentous growth in their vegetative stage, produce spores, and have a heterotrophic life style. Although they share these similarities, important differences are known that separate them into different Kingdoms [16, 21, 82] including: vegetative nuclear state of oomycetes is diploid (2n), while fungi are haploid (n) or dikaryon (n+n); and oomycetes hyphae are coenocytic or non-septate, while fungal hyphae are septate. In addition, the cell wall of many oomycetes is composed of cellulose and β -1, 3 glucan with minimal amounts of chitin, which is a primary component of the fungal cell wall. The sequence of conserved genes positions the oomycetes as heterokonts [17]. Morphological structures have traditionally been used to classify isolates of the genus into species (Fig. 1-1). Standard keys [48] are employed to identify isolates based on morphological diversity in the genus. The keys include the shapes and sizes of the sporangium, antheridium, oogonium, and oospore. Some *Pythium* species are heterothallic and require opposite mating types to reproduce sexually but most of them are homothallic. In the homothallic species, the sexual process occurs in a hypha or between two different hyphae close to each other where the oogonium and antheridia come in contact.

The genus contains economically important plant pathogens that cause diseases in many crops. *Pythium* produces some enzymes that aid in the degradation of cell walls of susceptible hosts. Extracellular hydrolytic enzymes such as pectinase, cellulase, and hemicellulase cause plant tissue to lose their structural integrity [98]. Oomycetes evolved the ability to infect plants and animals independently of other eukaryotic microbes, and probably developed unique mechanisms of pathogenicity [49]. *Pythium* has several of the most important casual agents of seed rot, seedling damping-off, root rot of all types of plants, and soft rot of fleshy fruits in contact with the soil [2]. They can be especially severe in soilless culture where they can spread easily in the nutrient solution [48, 71] with the production of zoospores.

Many horticultural operations reuse effluent water to reduce the release of fertilizers and pesticides into the environment and, in some cases, the recycled effluent is pumped to an irrigation reservoir where it is mixed with fresh water [15, 48]. These practices have increased plant disease risks and the need to implement pathogen management protocols to minimize the dispersal to irrigated plants. Host resistance (e.g. plant breeding) to *Pythium* is generally not an option for greenhouse and field crops because of the very large host range of most species. *Pythium* diseases are usually controlled through cultural practices, fungicides, and biocontrol methods. Some irrigation methods and timing seem to influence disease [94]. Good soil drainage, soil moisture management, nutrition of the crop (especially control of nitrogen fertilization), crop rotation with non-host plants, and treatment of seeds with fungicides are some the effective management tactics used to control *Pythium* diseases [98].

It is an exciting time to study this genus. New discoveries are modifying what is known about their ecology. A new report supports the idea that *P. ultimum* may play a role in bacterial movement and growth by providing both migration pathways and growth-promoting exudates [109]. This report provides evidence of how complex and dynamic the epidemiology of *Pythium* species are in the different ecological niches they occupy. It is believed that many *Pythium* species are yet to be discovered, especially in forest and natural water habitats. Due to their association with many important crops, there is a long history of research pertaining to their life and disease cycles, control

managements, and agroecosystem niches. The genus *Pythium* has about 120 species according to Hawksworth et al. 1995 [40]. This number keeps growing with the description of new species isolated and described in different parts of the world. Furthermore with each new species described, more questions will need to be answered to add to the current understanding of the genus.

Of the fundamental questions of their biology we should focus our attention on the different ecological niches where *Pythium* can be found. This can help us learn about the differences in their survival strategies and their interaction and possible communication strategies with other microorganisms in the ecosystem. Where *Pythium* species are known to survive and thrive included soil, fresh and salt water bodies, and in plants. If we can elucidate differences among the species this may help us manage the various species in their different ecosystems.

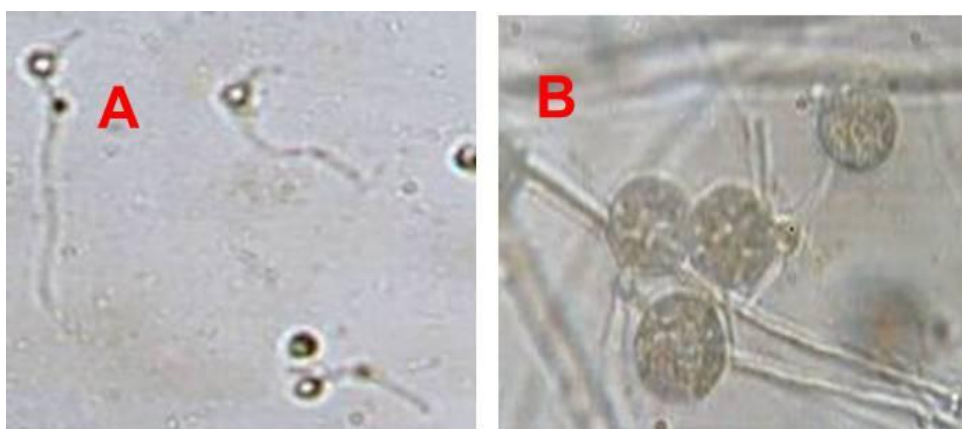


Figure 1-1: Some structures produced by *Pythium* species. A) *P. aphanidermatum* zoospores
B) *P. irregulare* sporangia.

Plant Pathogenic Species

Many of the plant pathogenic oomycetes cause devastating diseases on several important crops including a wide variety of ornamental plants. Soilborne root diseases caused by plant pathogenic *Pythium* species cause serious losses in a number of agricultural production systems, which has led to a considerable effort to develop biological agents for disease control [66]. They have a broad host range which makes

their management difficult. Disease development depends on environmental conditions, availability of susceptible hosts, and the species of *Pythium* involved. In most cases, moist and cool conditions are conducive to disease development but in some cases, high temperatures favor it. Diseases caused by *P. ultimum* and *P. irregulare* tend to be more severe at cooler temperatures while diseases caused by *P. aphanidermatum*, *P. myriotylum*, and *P. helicoides* are more severe at higher temperatures [66].

Plant diseases caused by *Pythium* are divided in two types: disease that affect plant parts in contact with the soil (roots, lower stem, seeds, tubers, and fleshy fruits) and diseases that affect above ground parts including leaves, young stems, and fruits [2]. Some *Pythium* species also infect roots of mature plants, typically causing necrotic lesions on root tips or fine feeder roots and, less commonly, on tap roots [66]. The lifecycle of the genus includes the production of several spore types and somatic hyphae. Most *Pythium* species produce an asexual spore called the sporangium. This propagule may germinate directly and produce a germ tube or may produce zoospores. Germination is dependent on temperature and varies with species. Zoospores are asexual, bi-flagellated spores that use water to move toward host roots where they encyst. The cysts then produce a germ tube that aids in the penetration of the host's roots. However, not all species of *Pythium* or all isolates within a given species form zoospores. The differences between species that produce abundant zoospores and those that do not may be the reason why some species are more prevalent in aquatic ecosystems and others in soil. *Pythium* species produce sexual spores called oospores resulting from the fertilization of the oogonium by the antheridium. The thick walled oospore is suited to survive desiccation, microbial infection, and adverse temperatures. Together with sporangia, they are considered the primary survival structures or sources of inoculum of several *Pythium* species [59]. The dissimilarities in the life cycle and spore production among species are an aspect of their disease cycle that deserves closer attention. Usually, the management is the same regardless of which species are present in the greenhouse. External factors are involved in oosporogenesis reflecting the diversity of oomycetes [47]. These factors are directly related to the ecology of the species. For example, oosporogenesis is favored by salinity levels consistent with their

normal habitats (eg. water vs. soil) [46] as well as low ratios of carbon/nitrogen typical in infected host tissue [34, 55]. A distinction should be made among species which are causing disease and those species which are present in the ecosystem without causing disease. In addition, it is key to identify the environmental effects that may influence pathogenicity according to the species occupying the niche. The identification and classification of the different species may aid in assessing different management strategies needed to control each species.

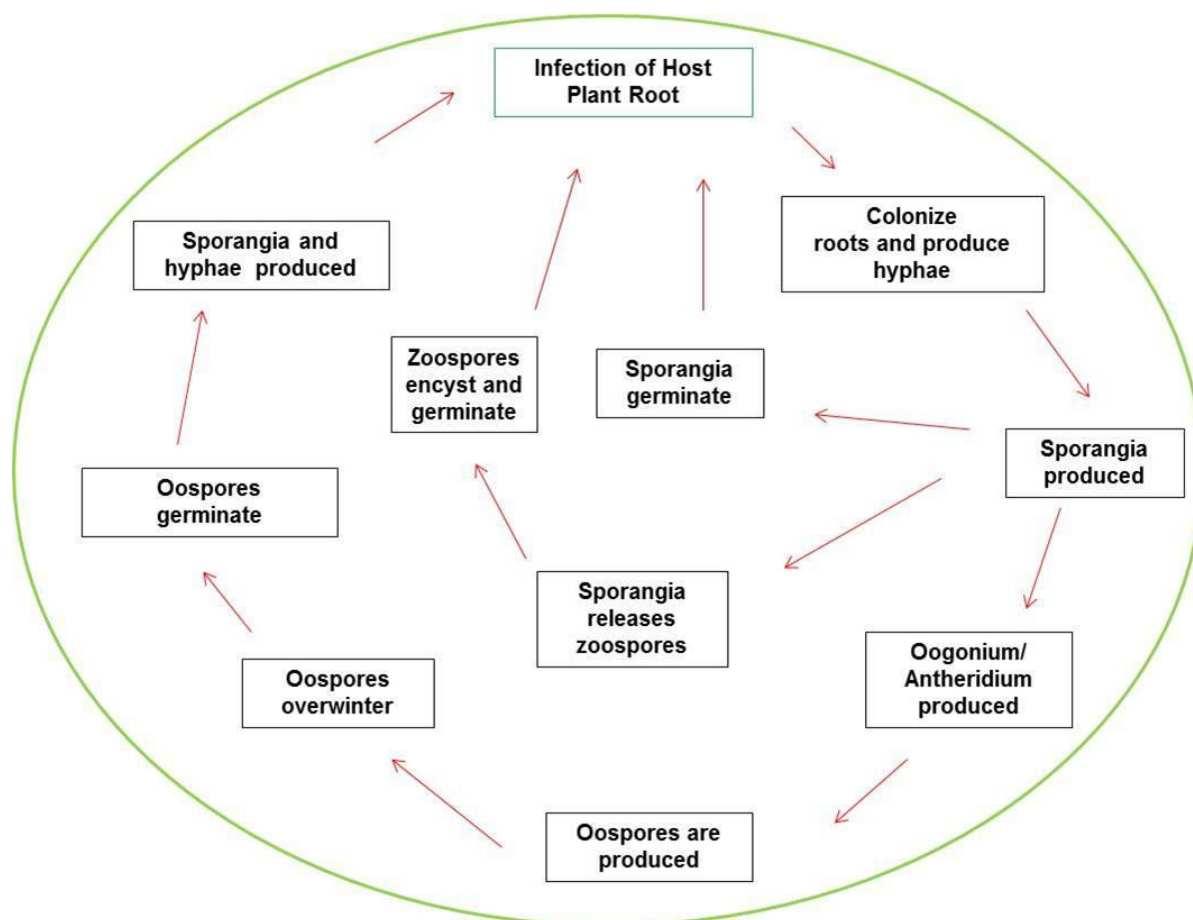


Figure 1-2: Disease cycle of typical *Pythium* diseases: The disease cycle starts with the introduction, colonization, and proliferation of *Pythium* in host plant tissue.

Two *Pythium* species, *P. irregulare* and *P. aphanidermatum*, are found as major pathogens associated with greenhouse crops in Pennsylvania [69]. These two species and *P. cryptoirregulare* are the three pathogenic species relevant to this research. These species are consistently isolated from infected plants submitted from commercial greenhouses in Pennsylvania. In this study, we selected three isolates, one of each species, which were highly pathogenic on geranium seedlings. In addition,

these three species were rarely isolated from recycled irrigation water from the two commercial greenhouses used in this study. We are using them to learn about their survival in recycled irrigation water throughout the growing season and their interaction with local bacterial communities.

***Pythium aphanidermatum* (Edson) Fitzp.**

P. aphanidermatum occurs worldwide and produces filamentous inflated sporangia, mycelium with intercalary antheridia, aplerotic oospores, and oogonia [48, 60]. It can grow at temperatures higher than 40°C, making it possible to survive and prevail under greenhouse conditions. This producer of abundant zoospores is a serious pathogen for many important crops worldwide. Of special interest is the devastation that it can cause in soilless systems [80] due to the formation of zoospores. This swimming spore can be easily dispersed in water, which aids the pathogen reaching susceptible hosts. Multiple biological control agents (BCA) with antagonisms to different stages of their development, particularly zoospores, may be used to decrease *P. aphanidermatum* numbers and survival in water. *P. aphanidermatum* has been isolated from soil and aquatic habitats. Large populations of dormant oospores occur in soil [96]. It is a root pathogen on most susceptible plants and has a wide host range. A close association of this species with poinsettia (*Euphorbia pulcherrima*) has been observed [58]. Some populations of *P. aphanidermatum* contain resistance to fungicides such as metalaxyl, mefenoxam, and propamocarb [70]. Although little is known about the genetic diversity of populations of *P. aphanidermatum* [57] and the changes overtime in metalaxyl sensitivity from a single greenhouse [5], it is of great urgency to find alternative controls to minimize their spread in water, decrease their movement, and inhibit their dispersion in the greenhouse production. This species is one of the most studied species from this genus.

***Pythium irregulare* Buisman**

Members of the *P. irregulare* complex are soilborne organisms found in association with many different plant hosts and are of worldwide distribution. The species exhibits high genetic and morphological diversity [10] which is why it is

considered to be a complex of species [67]. The species produces intercalary or terminal oogonia; 1-3 monoclinal, hypogynous or diclinous antheridia per oogonium; aplerotic or plerotic oospores [100]; spherical sporangia and ornamentation on the oogonial wall. Members of the complex cannot be differentiated solely based on morphology because of this very diverse morphology. DNA sequences of internal transcribed spacer regions (ITS) of the ribosomal DNA and the cytochrome oxidase II (cox II) genes are being used to separate them [35]. Members of the complex have been isolated from water and soil habitats with variation in the degree of disease caused on susceptible crops. This species is generally isolated more frequently than *P. aphanidermatum* from soil, plants, and water sampled from greenhouses [58]. Resistance to mefenoxam, a systemic fungicide widely used for the control of root rot and damping off of seedlings, has been reported in *P. irregulare* isolates [1]. It is considered a re-emerging disease due to the resistance to mefenoxam. New methods are being explored to substitute for the use of fungicides to control the pathogen. Distinct groups, probably different species, within *P. irregulare* have been revealed by ribosomal DNA internal transcribed spacers restriction fragment length polymorphism (ITS-RFLP) and random amplification of polymorphic DNA (RAPD) analyses [10], isozyme-based genetic diversity assessments [67], and DNA sequencing and amplified fragment length polymorphism (AFLP) analysis [35]. Members of this complex can cause pre- and post- emergence damping-off of seed and seedlings and root rot of older plants [42]. The species is being studied for their production of eicosapentaenoic acid (EPA), a major byproduct with usage in ethanol plants [62]. It is known to survive well in soil. Although it has been recovered in water there is a lack of information on how long they can survive in this environment. This is a key aspect of their life cycle that deserves consideration. The information would help to understand their survival and dispersal through the water reservoir. It is beneficial to know if they can survive in water and how long they are viable.

***Pythium cryptoirregulare* Garzón, Yáñez & Moorman**

This recently described species is a member of the *P. irregulare* complex. Molecular analyses conducted by Garzón et al. (2007) [35], differentiated *P.*

cryptoirregulare isolates from *P. irregulare* based on genetic analysis of multilocus DNA fingerprints. Both are morphologically similar and cannot be separated based on growth rate or morphological measures alone. Little is known about its habitat, pathogenicity, or distribution. Until recently, it was not differentiated from *P. irregulare*. Therefore due to its close association with *P. irregulare*, it is considered to have similar habitat and pathogenesis toward susceptible crops as *P. irregulare*. It is important to distinguish the species from *P. irregulare* isolates using PCR tools.

Diseases Caused by *Pythium*

Pythium species are among the most damaging pathogens in herbaceous ornamental plants causing damping-off, root rots, and stem rots. *Pythium* infections are usually limited to the meristematic root tips, root epidermis, cortex of roots, and fruits; but occasionally, severe infections occur when the pathogen moves deeper into the plant tissue and reaches the vascular system [49]. They can be found associated with seeds, seedlings, and young plants. They are important pathogens that cause root rot of several crops in hydroponic culture and in ebb-and-flow irrigation systems [107]. If soil is infested with *Pythium*, pre-emergence damping-off occurs when the seeds are colonized during germination and become soft, turn brown, and disintegrate [2]. In post-emergence damping-off the seedlings are infected right after germination. Seedlings that emerge are usually infected at the roots or stem below the soil line. The area becomes water soaked and collapses causing the seedling to collapse at the soil line. When older plants are infected with *Pythium* they often are not killed even though they develop root rot. After *Pythium* colonizes the roots it will cause extensive damage to the root system. The symptoms associated with root rot include lack of small feeder roots, brown lesions in the roots, and loss of cortex. The pathogen may proliferate and move from the root to other parts of the host plant. If the plant survives, *Pythium* may colonize the stem and cause stem rot (Fig. 1-3). The stem will turn brown with extensive damage to the vascular system leading to the lack of water transportation to the leaves and the death of the plant. Sometimes the plants will be infected with *Pythium* and the growth may be retarded as compared to a healthy plant. This is known as stunting.

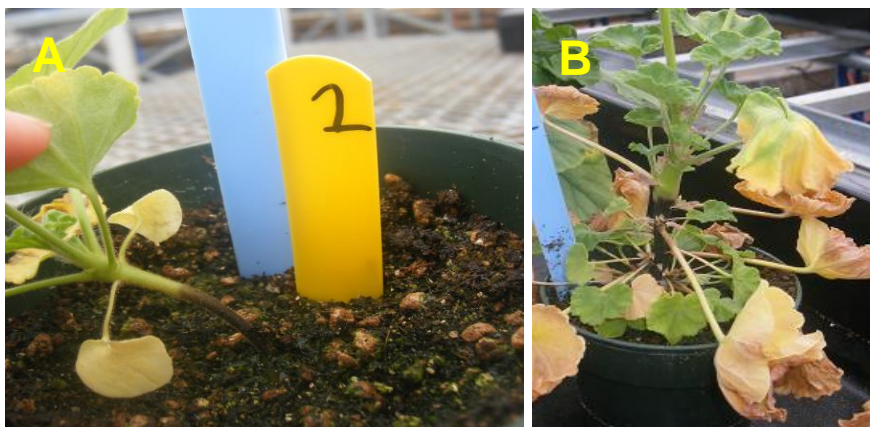


Figure 1-3: Stem rot symptoms caused by *P. aphanidermatum* infection of geranium (*Pelargonium*). Symptoms are due to colonization of the stem by *Pythium*, damage to the vascular system, causing the stem to turn brown and usually the death of the plant.

Sources of *Pythium* Inoculum in the Greenhouse

Pythium in the greenhouse may come from several sources at different times during crop production. The survival and continuous movement of *Pythium* inoculum in the greenhouse makes it difficult to trace the sources of inoculum. It is extremely difficult to determine how many different species are present, the identity of all species associated with the disease, and which species are the most abundant and most virulent [13]. Although it's not a trivial task, effort should be made to elucidate some of these questions and add to the current understanding of this genus. This is a key aspect of the management of this genus that deserves close attention. There is a need to trace the inoculum of particular species to its harbor and to determine how long it is present in the greenhouse. Recently, a study found that insects common to greenhouses, fungus gnats (*Bradysia impatiens*) and shoreflies (*Scatella stagnalis*), excreted viable *P. aphanidermatum* oospores after ingestion [44]. Some *Pythium* species are adapted to live in water producing swimming zoospores that can easily travel to other parts of the greenhouse. Others are adapted to live in the soil producing oospores which serve as resting structures that survive desiccation and microbial infection. Previous studies suggested that populations of *Pythium* are relatively stable in soil when optimal conditions are present. Al-Sadi et al. 2008 [6], investigated the potential source of inoculum in greenhouse soils. They found that 7% of fallow soil was infested before introducing it to the greenhouse [6]. They also found that other sources

of *Pythium* included potting mixes; soil adhered to cultivation equipment and grower's shoes and reused irrigation pipes. The consensus is that the main source of *Pythium* inoculum comes from soil and infected plants introduced to the greenhouse (table 1-1). In addition, *Pythium* species have been found to reside in soil, in aisles, on tools, and in irrigation water (Moorman and Daughtrey, unpublished). For this study, three isolates representing each of the species were selected that were highly pathogenic on geranium seedlings.

Contaminated soil mix, and infected plants are just some of the potential sources of *Pythium* in the greenhouse. Untreated irrigation water from streams, rivers, lakes, and ponds may pose a threat to crop production. Dispersal of root pathogens is a major concern especially in hydroponic culture [110]. MacDonald et al. (1994) [65], showed that recycled irrigated water could harbor significant levels of fungal propagules that can result in contamination of container crops and colonization by members of the *Pythiaceae*. Although *Pythium* was isolated from irrigation water, they were not identified to species. It is not known whether they were consistently the same species in the water or if those in the water were causing crop losses in the greenhouse. Research should target this aspect of the production in the greenhouse in order to have a better understanding of the different species residing in recycled irrigation water reservoirs and the risk they pose to crops. Although water is suspected to be a point of distribution for some *Pythium* species, it is unknown if those species are viable for long periods of time. The irrigation water environment may be a potential point to inhibit *Pythium* distribution through the addition of biological control agents (BCAs) to recycled irrigation water.

Two commercial greenhouses in Pennsylvania were selected for this study. Greenhouses S and E are similar in production practices. Depending on the demand and savings, they may produce their own cuttings or start seedlings; buy rooted and unrooted cuttings or seedlings to grow crops. Both greenhouses use cement flood floors and ebb and flood benches to grow a variety of plants. They produce bedding plants, potted, and hanging basket plants in the summer. In fall, they produce chrysanthemums and poinsettia in the winter. Watering of the plants it's done using

drip irrigation, ebb and flow, and overhead misting systems. They used a variety of soluble fertilization, depending on the crop, through their irrigation system. The initial water for the recycling irrigation systems in both greenhouses originated from onsite wells. Both greenhouses operate year round.

Table 1-1: Sources of *Pythium* spp. inoculum in the greenhouse

Sources of Inoculum	Description
Soil	From outdoors Contaminated potting mix Debris from previous production Contaminated machinery Contaminated pots or flats Contaminated shoes or other articles of clothing
Water	Contaminated water source Contaminated water containers Contaminated plumbing
Plants	Infested seeds Infested seedlings Infested plants or cuttings Debris from previous production

Survival of *Pythium* species in Recycled Irrigation Water

The contribution of recycled irrigation water to the survival and dispersal of *Pythium* in the greenhouse is largely unknown. Soilless culture was developed to control soilborne diseases [24]. Recycled water may introduce or re-introduce *Pythium* inoculum into new areas of the greenhouse. Harboring of pathogenic *Pythium* in water deserves close attention. Several *Pythium* species are important pathogens that cause disease in several crops in hydroponic culture and ebb and flow irrigation systems [107]. Zoosporic oomycetes and other organisms are commonly recovered from water and it is known to help in the dispersal of the asexually produced zoospores [43]. Nevertheless, as mentioned earlier, is unknown how long they survive in this environment. According to a new study, unidentified species exist in soil of uncultivated fields and in relatively unexplored habitats increasing the importance of understanding their ecological roles, physiological features, and the taxonomy and phylogeny of the genus [99]. Knowledge of *Pythium* ecology is changing with new discoveries, especially

the discovery of new aquatic *Pythium* species. New discoveries give rise to new questions. Aspects that deserve close attention are the contribution of water to the survival, establishment, and distribution of saprophytic and pathogenic *Pythium* species residing in recycled irrigated water in the greenhouse. *Pythium* is frequently isolated from irrigation water samples in nurseries [15, 77]. However, most researchers do not identify *Pythium* isolates to species. During 1996-2001, *P. aphanidermatum* and *P. irregulare* were rarely isolated from water even if they were present on plant tissue or in soil in the same facility [69]. It is unclear if they were present in the water at low quantities and were not detected or if they were not able to survive in this ecosystem. New studies of the ecology of *Pythium* should include the examination of aquatic oomycetes and their role in pathogenesis in commercial greenhouses, their saprophytic life stages, and their influence on aquatic microbial communities. A better understanding of the survival, distribution, and epidemiology of *Pythium* in water is very important to help control this pathogen. Not all *Pythium* seemed to survive in all environments (soil, plant material, and water reservoir). It is not clearly understood which species are endemic and which are transient in different ecosystems. The identification of transient species vs. endemic species is another important aspect to the overall understanding of the life cycle of individual *Pythium* species.

It is important to identify which species are surviving from season to season and what may be the source of the inoculum. Logic suggests that they may be introduced to the production by external means (infested soil, seeds, transplants, water), and which are only present in one season. The characterization of the temporal distribution of *Pythium* may give us a better idea of which species are not causing any problem and which should be closely monitored and controlled.

Recycled irrigation water

The capture and recycling of irrigation water in greenhouses and nurseries was implemented to decrease water use and labor costs and to conserve fertilizer and lessen groundwater contamination with fertilizer and pesticides. This strategy

decreases nutrient run-off into the surrounding environment and lessens the negative environmental issues in greenhouse production. Collecting and recycling effluent however, may also collect and disperse plant pathogens [15] and adds a new consideration to the management of pathogenic species surviving in water. The often marginal quality of the water can present agricultural challenges [50]. In addition, the transfer of water to cities and for environmental protection of rivers, wetlands and deltas will decrease agricultural production [106]. Irrigation water recycling is an increasingly important practice in agriculture in the context of diminishing water supply and the regulation requirements in some parts of the world [36]. This practice has created the need for more studies on the ecology of microbial communities in this highly managed habitat. Chemical analyses of water samples are needed to study water quality for commercial greenhouse usage. To maintain production quality, constant monitoring of water parameters is needed. Other water users are increasing their attention to recycling water including food production and processing [76]. New laws regulating water saving and polluting compel users to reduce effluents especially in soilless culture [79]. The soilless production system merits our attention since it may be used to distribute BCA's that help control *Pythium*.



Figure 1-4: Recycled irrigation water reservoir in a commercial greenhouse in Pennsylvania

Water Environment

Water is an environment known to sustain the survival and development of different groups of microorganisms. Viruses, bacteria, archaea, algae, oomycetes, and

fungi are some of the groups that can live in this ecosystem. In most aquatic systems the microbes, primarily heterotrophic bacteria, are responsible for most of the respiratory losses; with a range of 25% to 90% of community respiration attributed to bacterial respiration [52, 73, 74, 85, 86, 87]. Water quality is a term that encompasses the chemical, physical, and biological characteristics of water [78]. As water users increase in numbers and demand, the concern for water quality has increased. Few states or regions list specific water quality criteria for industrial and agricultural water [78]. According to Li (2001), the explanation for this lack of specifics is likely due to the designated uses and more sensitive water quality criteria such as aquatic life use. States assess variables that will indicate potential quality degradation due to nutrient enrichment [78]. Some of these variables are water turbidity, chlorophyll concentration, dissolved oxygen (DO), pH fluctuations, electrical conductivity (EC), and aquatic life communities. Dissolved oxygen, turbidity, electrical conductivity, and pH are used as indirect assessments related to nutrients because these change in response to changing nutrients content and have numeric criteria that influence aquatic life [78]. A study noted that rates of bacterial respiration were observed to be higher during spring and summer as compared to winter and early spring [74] which can be the result of the increased population in these seasons.

Nutrient supply, pH, and electrical conductivity are regularly monitored by the growers in commercial greenhouses in Pennsylvania. Many physical and biological factors have been found that affect the development of *Pythium*. Competition for plant derived chemicals and antagonistic activity against *Pythium* inoculum (zoospores, mycelium, and oospores) has been reported [72, 101]. There are some reports about the adverse effect of different chemical parameters that control the survival of *Pythium*. It is important to establish not only what parameters adversely affect *Pythium*, but what parameters are needed to maintain and increase the microbial community known to suppress this genus. Both aspects are linked and should be taken in consideration when establishing a management strategy.

Microbial Communities in Recycled Irrigation Water Systems

The characterization of microbes and microbial communities capable of suppressing pathogenic *Pythium* could be exploited in order to manage disease outbreaks in commercial greenhouses. The use of beneficial microorganisms via seed treatment, soil amendments, and enhancement of plant defenses are some of the practices already implemented to suppress pathogens [3, 8, 12, 80]. However in most cases, commercially available beneficial organisms are from other environments or from other parts of the world rather than from the local environment where crop production is occurring. Microorganisms present in the same habitat as *Pythium* species should be explored as they may survive, reside, and proliferate in the habitat. Local microbial communities in water may play a role in suppressing the incidence of disease caused by *Pythium* species in the greenhouse and should be examined. Several methods, such as heat treatment, ozonization, ultraviolet radiation and chlorination, are used to disinfect nutrient solutions [23]. Naturally occurring microorganisms are an element that may be used to complement these management practices. Unfortunately, the methods mentioned above do not discriminate between target pathogens and non-target, beneficial microbes.

The interaction among organisms in a given ecosystem is complex and usually not well understood. This is particularly true for the aquatic environment in greenhouse crop production. Postma et al. 2000, studied the role that natural microflora in rockwool had in suppressing disease with and without the original microflora [81]. Naturally occurring microorganisms have the ability to suppress disease development [19, 102]. The microbial diversity in recycled irrigation water is poorly understood. If bacterial communities present in recycled irrigation water significantly suppress phytopathogenic *Pythium* species, efforts should be taken to minimize the elimination of the community when sanitation methods are used in the greenhouse. In addition to the need for experimental designs that identify naturally occurring BCAs effective against *Pythium in vitro* it is important to explore the outcome of the interactions between *Pythium* and these BCAs in recycled irrigation water under greenhouse conditions.

Biological control

Mode of Action

The concern about environmental contamination and the increase of fungicide-resistant pathogen populations resulting from the use of chemicals are the main forces for the development of alternative approaches to controlling diseases caused by *Pythium*. There are many research programs worldwide studying modes of action, pathogens, antagonist, and host interaction to increase the potential of biocontrol strategies [75]. Researchers have concluded that the best strategy is to use more than one approach to control plant pathogens. The combination of two or more BCAs should only be used with a clear understanding of the main biocontrol mechanisms and experimental evaluation [92]. Not all of the suppressive mechanisms are understood completely.

One example of a successful suppression of soilborne pathogens is suppressive soil. It is known that microbial communities are involved in the suppression, but the mechanisms are not completely understood. Suppressive soils hold considerable potential to manage soilborne pathogens [11]. One aspect of this method is to identify the microorganisms present in the soil that may be involved in the suppression. Some bacterial strains from soil that suppressed *Pythium* damping-off of cucumber, including *Pseudomonas aeruginosa*, [4] have been characterized as well as bacteria from municipal biosolids compost that colonize and protect the seeds [20]. Arndt et al. 1998 [7] found a marked stimulation of callose deposition in roots of the susceptible host. Even mangrove microorganisms have been evaluated for their potential to control root rot caused by *P. aphanidermatum* in hydroponic cucumbers [22]. These studies have a common theme: bacteria vs. fungal-like pathogen interactions. The modes of action associated with this interaction include antibiosis, competition for iron and other nutrients; competitive exclusion; parasitism; induced resistance; and plant-growth-promotion [27, 39, 108].

The genus most extensively studied as potential BCAs is *Pseudomonas* [9, 26, 31, 38, 45, 56, 68, 83, 91, 93]. They are fast growing bacteria that utilize a vast number of organic compounds.

Table 1-2: Bacteria-fungal interactions leading to inhibition of plant pathogens. (Adapted from Whipps 2001).

Type	Mode of Action	Examples in Literature
Antibiosis	Antimicrobial compounds	<i>Pseudomonas</i> [97]
Competition for Iron	Production of Siderophores	<i>Pseudomonas</i> [28, 64]
Parasitism	Production of Enzymes and lyse of pathogen cell wall	<i>Actinomycetes</i> [29] <i>Enterobacter cloacae</i> [73]
Induced Resistance	Induction of Plant Resistance	<i>Bacillus</i> [95] <i>Pseudomonas</i> [18]
Plant-Growth-Promoting-Rhizobacteria (PGPR)	Increase plant growth by different methods	<i>Pseudomonas fluorescens</i> [105] <i>Bacillus polymyxa</i> [88]

Biological control of *Pythium* diseases

Integrated pest management in the production of greenhouse crops is the most used and reliable strategy to control the spread of *Pythium*. Non-chemical control measures to reduce damage caused by *Pythium* spp. usually consist of modification of cultural practices [66]. Biological control strategies with bacterial strains, have given mixed results and it is not always successful, possibly because they are introduced rather than endemic strains. Biological suppression by bacteria is one aspect that holds promise in the management of *Pythium* in aquatic systems. *Pseudomonas chlororaphis* 63-28 was used to suppress *Pythium* root rot effectively in vegetative-stage cucumbers in greenhouse hydroponic systems [63]. Other bacteria and fungi-like organisms have been used to suppress *Pythium* including other *Pseudomonas* species [7], *Bacillus* [88], *Paenibacillus* [14], *Actinoplanes* [32], *Lysobacter enzymogenes* [53], *Enterobacter cloacae* [73], *Streptosporangium* [104], *Trichoderma* [89], and *Pythium oligandrum* [84]. It is thought that naturally occurring microbial communities play a role in suppressing *Pythium* spp. in soil. Following this concept, microbial communities similarly may aid in

the suppression of *Pythium* spp. residing in water. The interaction may not be direct. Nevertheless, major efforts are underway to find new candidates that suppress pathogenic *Pythium* in several important crops [11].

Innovative research is offering insights into diverse factors that may influence the outcome of specific interactions in a given ecosystem. The Australian macro-alga, *Delisea pulchra*, produces secondary metabolites similar to acyl-homoserine lactone (AHL) molecules [37], important in chemical communication signaling. Kong et al. (2010) found that oomycetes produced and used molecules to regulate zoospore aggregation and plant infection. This cooperation may be advantageous and allow one species to out compete others in a particular environment. The signal molecules for *Phytophthora* zoospore aggregation and plant infection are distinct from the known AHLs produced by bacteria [54] but raises the possibility of communication between these two diverse groups of microorganisms. There is a possibility that species of *Pythium* may communicate with bacteria. Frequent co-isolation of bacteria with *Phytophthora* and *Pythium* suggests interspecies communication [54].

Although some bacteria may produce chemicals that are harmful to the development of *Pythium in vitro*, the interaction probably cannot be sustained for long periods in the field because of the diversity and intense activity of the total microbial community in water. Characterization of bacteria coexisting in the same niche as aquatic *Pythium* is of great interest to this research. More importantly, are the close interactions, outcome of the interactions, and adaptation to the niche they share.

Biological control agents against *Pythium* are available [51, 61, 80]. Although many biological control studies have been conducted, they usually target one particular stage of the *Pythium* life cycle even though more than one type of inoculum may be produced. To implement a durable biological management strategy, disruption at several different stages of the development of the *Pythium* life cycle, may be more effective. Integration of various organisms into a production system that disrupt *Pythium* activity at different points of its development could be of importance.

Microbial Interaction in Recycled Irrigation Water

Soil microbial communities associated with suppressing *Pythium* species usually include fungi and bacteria. Following this concept, microbial communities similarly may aid in the suppression of *Pythium* spp. in water. Although aquatic systems contain many other microbes, our effort will be concentrated in heterotrophic bacteria. Our interest is to determine if these organisms are capable of deleterious effects toward *Pythium*. Their ubiquitous presence makes heterotrophic bacteria good subjects to evaluate as potential *Pythium* suppression agents in recycled irrigation water. The interaction between heterotrophic bacteria and *Pythium* inoculum in the water can be studied to learn their effect on *Pythium*.

The composition of any community is determined in part by the species that happen to be distributed in the area and can survive its environmental conditions [90]. The influence of aquatic heterotrophic bacteria on the *Pythium* in water is not known. Research that provides insights into the relationship and outcome among microorganisms in the same niche may help explain the transient nature of some *Pythium* species in water. The form and structure of terrestrial communities can be characterized by the nature of the vegetation [90]. Perhaps a parallel phenomenon can be found in recycled irrigation water. It is possible that the bacterial community can be structured and re-defined when a particular *Pythium* species is present in the system. Although dominance of a species does not necessarily mean more influence in the environment; it's usually implied that dominance is achieved due to their ability to exploit the range of environmental requirements more efficiently than others [90]. Microbial influence on the behavior and survival of *Pythium* in recycled irrigation water has not been addressed.

Research Objectives

One common factor to all crop production is the need for water. Commercial greenhouses and nurseries, particularly in the U.S. and Europe are under major

pressure to decrease the usage of water and prevent the run-off of fertilizers and pesticides into surface and ground water. The recycling of irrigation water is a significant innovation to address those concerns but raises the issue of the possible accumulation and dispersal of plant pathogens to the crops that are irrigated with that water. Many studies on the survival of oomycetes in water note the species that appear to be adapted to the aquatic environment [43]. Not all those oomycetes formed zoospores or survived in water for long periods [65]. Few *Pythium* species have been studied extensively other than *P. aphanidermatum*, *P. irregulare*, and *P. ultimum*. Some of the more than 120 known *Pythium* species identified to date seem to survive in water while others survive in soil. The development of effective disease management strategies should be based on an understanding of basic differences between soilborne and waterborne *Pythium* species. In addition, the bacterial community in the aquatic ecosystem may play a role in their survival. A high bacterial population diversity can play a role in plant pathogen suppression and nutrient cycling [41]. For this reason the research here characterizes some of the bacterial isolates that interact *in vitro* with *Pythium* species known to be responsible for crop losses in commercial greenhouses.

Objectives of this research

1. To determine the effect that microbial communities present in recycled irrigation water have on the development and survival of *Pythium aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare*

Each *Pythium* species was cultured in water collected from a recycling irrigation water system and the amount of sporangia formed after five days of incubation was quantified. The water samples were fractionated using membrane filters of various pore sizes, thus avoiding the use of antibiotics in the experiment that could also inhibit *Pythium*.

2. To test *in vitro* inhibition of *Pythium* species by heterotrophic bacterial isolates found in recycled irrigation water

Individual heterotrophic bacteria from the recycled irrigation water reservoirs were tested for their effects on the development of *Pythium aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare*. Our hypothesis is that naturally occurring heterotrophic bacteria survive in the reservoir of recycled water and that these bacteria interact with *Pythium*. Those that have deleterious effects on *Pythium* may be exploited as an alternative to adding chemicals or even non-native bacteria to the system. Furthermore, these microorganisms may explain why *P. aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* were not isolated from the water system during previous research. This approach is being taken keeping in mind the possibility that such organisms could be grown and added to irrigation systems.

3. To identify changes in the bacterial community in recycled irrigation water

This objective aims to identify changes in the bacteria community in recycled irrigation water when *P. aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* is present in the water. We hypothesize that the presence of *Pythium* in irrigation water influences the composition of the bacterial community in that water. We used Autosomal Ribose Intergenic Spacer Analysis (ARISA) [33] to determine if bacterial communities change in the presence of *P. aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare*, three species often associated with diseased plants in commercial greenhouses in PA [69]. In addition, we used another culture-independent technique, quantitative polymerase chain reaction (q-PCR), to determine which taxa of bacteria increased after adding mycelium of one of the three *Pythium* species to recycled irrigation water.

4. To identify *Pythium* species in recycled irrigation water

Determine which *Pythium* species may be permanent residents or endemic to recycled irrigation water and which species are transient in water used in commercial greenhouses. Our hypothesis is that pathogenic and non-pathogenic species of *Pythium* are residents of recycled irrigation water systems in the greenhouses regardless of whether crop losses due to *Pythium* are occurring.

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CHAPTER 2

Effect of microbial communities present in recycled irrigation water on the development of *P. aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare* *in vitro*

Abstract

Microbial communities in recycled greenhouse irrigation water were analyzed for their effect on sporangia and zoospore production by *P. aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare*. Recycled irrigation water samples were fractionated by filtration to separate microorganisms by their size. Filtrates with microorganisms of different sizes were inoculated with *Pythium*. All water fractions containing microbes had a negative effect on the number of sporangia formed by all three *Pythium* species used in this study. It was not possible to identify one group of microorganisms solely responsible for the reducing sporangia numbers using this technique. Therefore, we hypothesize that multiple microorganisms are responsible for the deleterious effect on the development of all three *Pythium* species.

Introduction

One of the challenges in greenhouses where irrigation water is recycled is the potential for the accumulation and dispersal of zoosporic plant pathogens throughout the crop when watering. Plant pathogenic species of *Pythium* and *Phytophthora* can spread and infect roots, kill plants, and cause substantial losses in some production systems [13, 30] if the water is not treated to remove them. *Pythium* is a ubiquitous genus, some species of which may survive in terrestrial and aquatic ecosystems as saprophytes or as plant or animal parasites. Several *Pythium* species are plant pathogens with host ranges much larger than species of *Phytophthora* [34]. Some species seem to be more host specific (*P. graminicola*), while others have a broad host range (*P. aphanidermatum*, *P. irregulare*, *P. ultimum*). Monitoring studies have shown that some *Pythium* species are frequently recovered from irrigation water, ponds, and nutrient solutions. While recycled irrigation water (RIW) has been shown to harbor both *Phytophthora* and *Pythium* species [1, 5, 13, 24, 26], *Pythium* species were recovered more frequently and in greater numbers than *Phytophthora* species. Water reservoirs

are an ideal vehicle for zoosporic fungi [29]. The main concern for commercial greenhouses is that plant pathogens may be spread through recycled irrigation water to susceptible crops. However when a *Pythium* disease epidemic occurs in a commercial greenhouse where potted plants are grown and irrigation water is recycled, such as poinsettias, the distribution of infected plants usually appears to be random (G. W. Moorman, personal communication). Seldom are very large numbers of potted plants sharing the same irrigation water found infected, as would be expected if the water harbored large numbers of zoospores from the phytopathogenic species. Preliminary research has indicated that *Pythium* species, although often recovered from water reservoirs in greenhouses, vary from sampling to sampling with no one species dominating the habitat. The species isolated are usually weakly pathogenic ones while those causing crop losses are seldom recovered from the water. During these isolations, bacteria are usually co-isolated with *Pythium*.

It is known that naturally occurring microbial communities can play a role in suppressing *Pythium* in soil. Competition for plant derived, antagonistic activity against *Pythium* inoculum (zoospores, mycelium, and oospores) has been shown to suppress *Pythium* in soil [35]. Biological control agents against *Pythium* diseases have been identified. *Pseudomonas* spp. [19, 32], *Actinomycetes* [8], *Trichoderma* spp. [18], *Bacillus* spp. [28, 31], *Enterobacter cloacae* [23], and *Pythium oligandrum* [3] effectively control or compete with *Pythium* in various environments. There are several studies on the suppression of *Pythium* by beneficial microorganisms in compost [2, 11].

Natural suppression phenomena in different environments are widely recognized. Mechanisms thought to be involved include (1) competition for nutrients, (2) predation of pathogens, (3) antibiosis, and (4) activation of resistance mechanisms in plants by composts [12]. Microbiostasis is known to be involved in the suppression of *Pythium* [39]. Similarly, naturally occurring microbial communities may aid in the suppression of *Pythium* spp. residing in water. A great diversity of microbes may inhabit RIW reservoirs. The isolation of microorganisms can be used to identify microbial diversity in soil, compost, and water, but this does not identify the group of microbial species involved in pathogen suppression in a given ecosystem,

especially if those species are unculturable. The identification of the microorganisms responsible for the suppression of *Pythium* in recycled irrigation water may help to improve biological strategies in that production system.

The objective of this study was to investigate the effect that subgroups of the microbial community present in recycle irrigation water have on the development of three *Pythium* species commonly found to cause substantial crop losses in commercial greenhouses in the northeastern U. S., *Pythium aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare*. We used three isolates, representing each of the species, which were pathogenic on geranium seedlings. Based on the hypothesis that suppression of *Pythium* is induced by microbiostasis, the microbial community was fractionated by size using filtration. Each sample contains different quantities of bacteria, fungi, and protists which then were tested for their effects on *Pythium* sporangia and zoospore production in culture. This allowed the study of different subgroups of microorganisms that may be involved in the suppression of *Pythium*. By separating the microorganisms into groups by physical fractionation, we wanted to determine if general suppression or specific suppression mechanisms against one or all of the *Pythium* spp. could be attributed to a particular subgroup of microorganisms and do this without the complicating factor of using antibiotics in the tests that may also inhibit *Pythium*.

Materials and Methods

***Pythium* species**

Three *Pythium* species used in this study (*Pythium aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare*) were obtained from commercial greenhouses in Pennsylvania (Table 2-1). Usually, these are the species found to cause substantial crop losses in PA. They were identified to species using morphology and sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA [22]. Cultures were stored on colonized water agar blocks in tubes with sterile tap water at room temperature and transferred to petri plates containing potato dextrose agar (PDA)

when active cultures were needed. Subcultures were transferred to water agar (WA) plates and incubated at room temperature for 2 days to provide inoculum for testing.

Table 2-1: Identification of *Pythium* species from commercial greenhouses in Pennsylvania used to determine the effects of microbial communities on their sporangia and zoospore production.

<i>Pythium</i> isolate	Identification (ITS)	Origin
P128	<i>P. aphanidermatum</i>	Chrysanthemum
P123	<i>P. cryptoirregulare</i>	Impatiens
P84	<i>P. irregulare</i>	Water

Recycled irrigation water sampling

Recycled irrigation water (RIW) samples were collected every 60 days from reservoirs constructed of cement (23,000 L tank in greenhouse S, 40°49'31.30"N, 76°48'18.17"W; and 76,000 L tank in greenhouse E, 40°13'21.56"N, 76°16'13.60"W) located inside the greenhouses. Water was collected in sterile, 1 L glass jars near the surface (< 1 meter depth) and from the bottom (> 2 meters depth) using a device that kept the jars sealed until they were at the desired depth. The jars were placed in a cooler containing ice packs that were not in direct contact with the jars. Dissolved oxygen levels were immediately measured (Traceable® Digital Oxygen by Control Company, Friendswood, Texas) at the time of collection. Electrical conductivity (Model SD-B15, Beckman Industrial) and pH (Mettler-Toledo SevenEasy model, Columbus, OH) were measured after transport to the laboratory.

Microbial community fractionation

RIW samples were passed through membrane filters of different pores sizes to separate the microorganisms as follows. Two water samples were filtered through a 5.0- μ m pore size membrane filter, to exclude protozoa, fungi, small animals, and algae larger than 5.0 μ m. The filtrates were kept and used as separate samples. One filtrate represents water fraction sample 5.0 μ m. The other filtrate was re-filtered through a 0.8 μ m pore size membrane filter. The 0.8 μ m membrane filter was transferred to a sterile

tube with 10 ml of autoclaved recycled irrigation water to resuspend the organisms, 0.8-5.0 μm in size, retained on the filter. This is water fraction sample 0.8-5.0 μm . A third sample was filtered through a 1.0 μm pore size membrane filter to exclude microorganisms larger than 1.0 μm in size (water fraction sample 1.0 μm). Planktonic bacteria of natural waters tend to be smaller than 0.5 μm in diameter [7]. This water fraction sample contained mostly bacteria present in the RIW sample. A fourth sample was filtered through a 0.2 μm membrane filter in order to remove most microorganisms (water fraction sample 0.2 μm). Extremely small free living ultramicrobacteria are known to live in a wide range of natural environments [16] including waterborne 0.2 μm filterable bacteria [14]. A fifth sample used in the study was RIW with no filtration (Unf-RIW). As controls autoclaved recycled irrigation water (A-RIW) and sterile distilled water (SDW) were used.

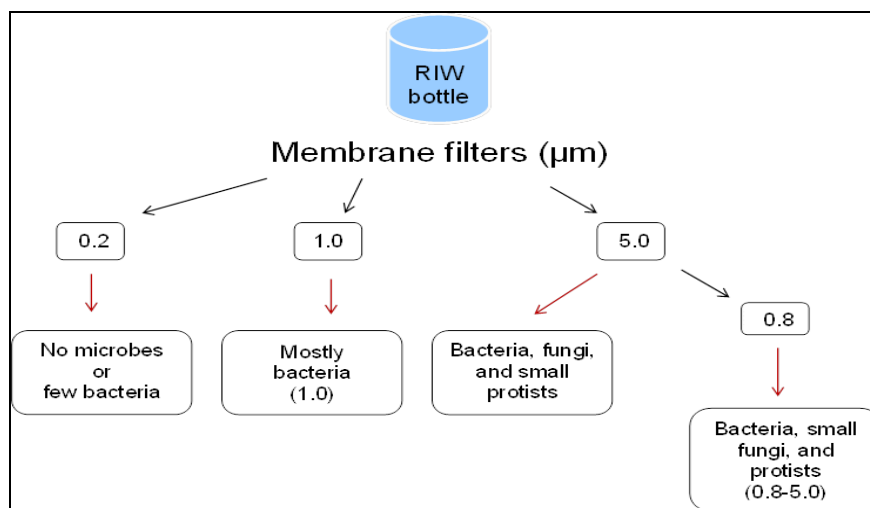


Figure 2-1: Recycled irrigation water (RIW) fractions obtained by filtration in order to separate microorganisms by sizes. In addition, three control water fraction samples were used in this study (unfiltered recycled irrigation water (Unf-RIW); autoclaved recycled irrigation water (A-RIW); and sterile distilled water (SDW)).

Table 2-2: Average measurements (n = 40) of recycled irrigation water (RIW) samples used in this study

Description ^a	Chemical properties ^b		
	pH	Dissolved oxygen (mg.O ₂ /L)	Electric conductivity (mS/cm)
Mean	6.16	4.13	1.49
Maximum	7.8	6.5	2.9
Minimum	5.3	2.0	0.3
Standard deviation	± 0.447	± 1.05	± 0.40

^a Results are for all samples from both greenhouses sampled.

^b Dissolved oxygen measurements were taken at the time of sampling, on site. Electric conductivity and pH measurements were taken in the laboratory.

Testing the effect of microbial communities on *Pythium*

To determine the influence of subgroups of microorganisms on the development of each *Pythium* species, sterile individual glass petri plates (60mm x 15mm) were prepared as follows. A 5.0 mm diameter WA mycelia plug of *P. aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* was taken from the edge of a 3 day old colony and placed upside down centrally in the glass petri plate. Four ml of a water fraction sample was added to the petri plate containing mycelia plug. In addition, two pieces of creeping bentgrass (*Agrostis stolonifera* L. 'Penn Eagle') or rye (*Lolium perenne* L.) leaf blades that had been boiled for 10 min. in distilled water were placed on top of the WA mycelia plug (Figure 2-2). All water fraction samples were done in duplicates. The glass petri plates were transferred to a plastic tray. Small plastic containers of water were added to the trays to minimize water evaporation. The trays were covered with a clear plastic dome and incubated at 21°C under fluorescent lights for 5 days.

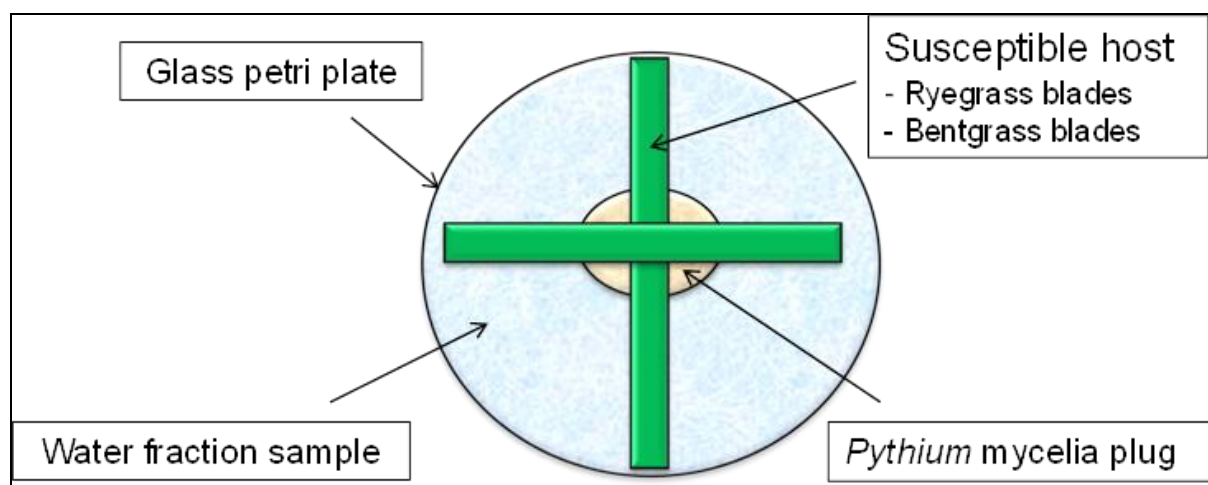


Figure 2-2: Schematic of glass plate containing a water fraction sample and *Pythium* spp.

Microscopic observations

Microscopic observations were conducted after 2, 4, and 5 days of incubation. The general health of the mycelium was noted (data not shown), whether zoospores formed, and number of sporangia per five random microscope fields on the mycelia attached to the grass blades was recorded for *P. aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare*. The number of swimming zoospores was counted on the same five microscopic fields as the number of sporangia. Not all water fraction samples were used for zoospores assessment due to their similarity in size to protists in the water samples.

Data analysis

Water sample chemical properties were documented. The mean, standard deviation, minimum and maximum values for pH, dissolved oxygen, and electric conductivity were calculated. The number of sporangia was expressed as a mean. The number of zoospores was transformed to logarithmic natural number. Each water fraction sample treatment was done in duplicate. The sporangia data was subjected to one-way analysis of variance (ANOVA) using Minitab 16 statistical software (Minitab Inc., State College, PA). Mean separation was calculated using Fisher test ($P < 0.1$).

Results

Influence of microbial community on sporangia

A. *Pythium aphanidermatum*

The results for the mean number of sporangia recorded by water fraction sample, day, and greenhouse are summarized in Fig. 2-3 for greenhouse S and 2-4 for greenhouse E. The number of sporangia formed was reduced in microbe-containing water fractions samples when compared to A-RIW, SDW, and 0.2 filtrate samples. Results showed that the mean of A-RIW, SDW and 0.2 filtrates were significantly more than microbe-containing water fractions samples. Most water fraction samples had similar effects regardless of where the water sample originated (greenhouse S or E). The highest number of sporangia were produced in A-RIW followed by SDW, and 0.2 filtrate in greenhouse S. All microbe-containing samples were statistically similar. In greenhouse E, the highest number of sporangia were produced in SDW followed by A-RIW and 0.2 filtrate samples. Most microbe-containing samples were statistically similar. Unf-RIW produced the fewest sporangia and was statistically different to the other microbial-containing samples in greenhouse E.

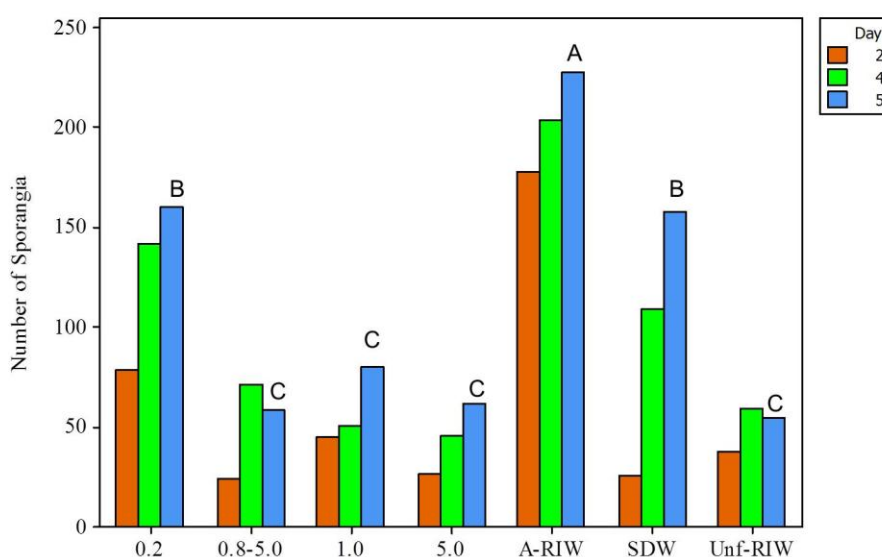


Figure 2.3: Mean number of sporangia formed by *Pythium aphanidermatum* in water fraction samples from greenhouse S. Results comparing all water fraction samples recorded on day 2, 4, 5. Mean results from day 5 were used to calculate statistical test using Fisher test. Means that do not share a letter are significantly different ($P < 0.1$).

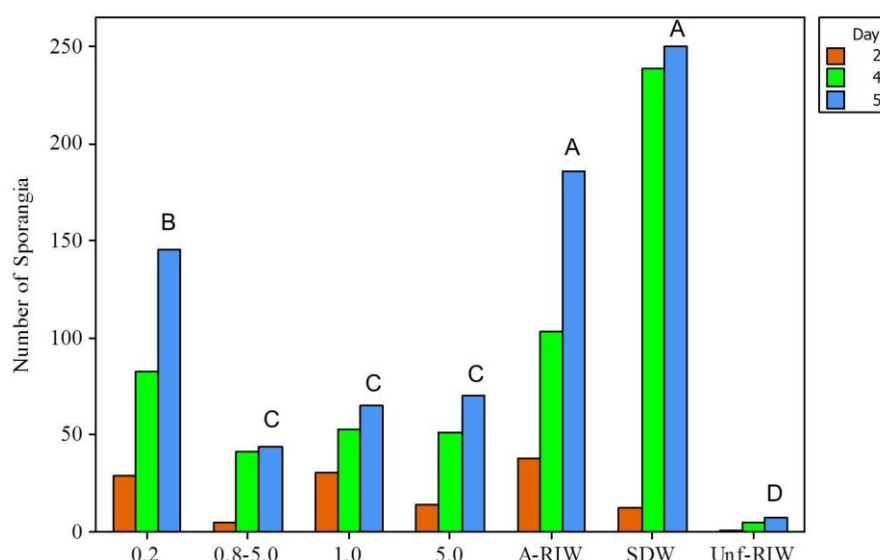


Figure 2.4: Mean number of sporangia formed by *Pythium aphanidermatum* in water fraction samples from greenhouse E. Results comparing all water fraction samples recorded on day 2, 4, 5. Mean results from day 5 were used to calculate statistical test using Fisher test. Means that do not share a letter are significantly different ($P < 0.1$).

B. *Pythium cryptoirregulare*

Similar to *P. aphanidermatum*, the number of sporangia was less in microbe-containing water fractions as compared to A-RIW, SDW, and 0.2 filtrate sample. For this species the mean number of sporangia produced in A-RIW and in SDW was significantly greater than all microbe-containing water fractions from greenhouse S. Most microbe-containing samples were statistically similar. The highest number of sporangia were produced in SDW and 0.2 filtrate samples followed by A-RIW in greenhouse E. The least amount of sporangia formed was in Unf-RIW in both greenhouses. Results of the mean of sporangia are summarized in Fig. 2-5 for greenhouse S and Fig. 2-6 for greenhouse E.

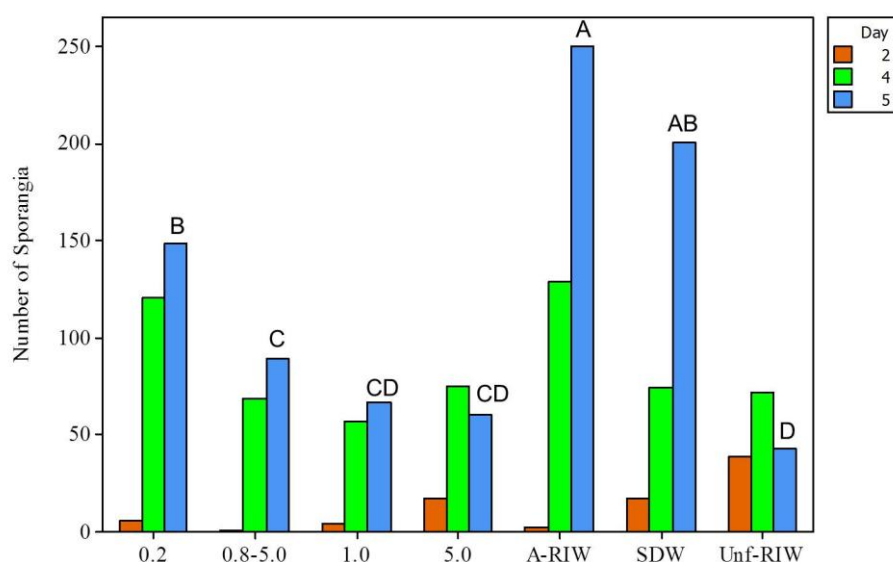


Figure 2.5: Mean number of sporangia formed by *Pythium cryptoirregulare* in water fraction samples from greenhouse S. Results comparing all water fraction samples recorded on day 2, 4, 5. Mean results from day 5 were used to calculate statistical test using Fisher test. Means that do not share a letter are significantly different ($P < 0.1$).

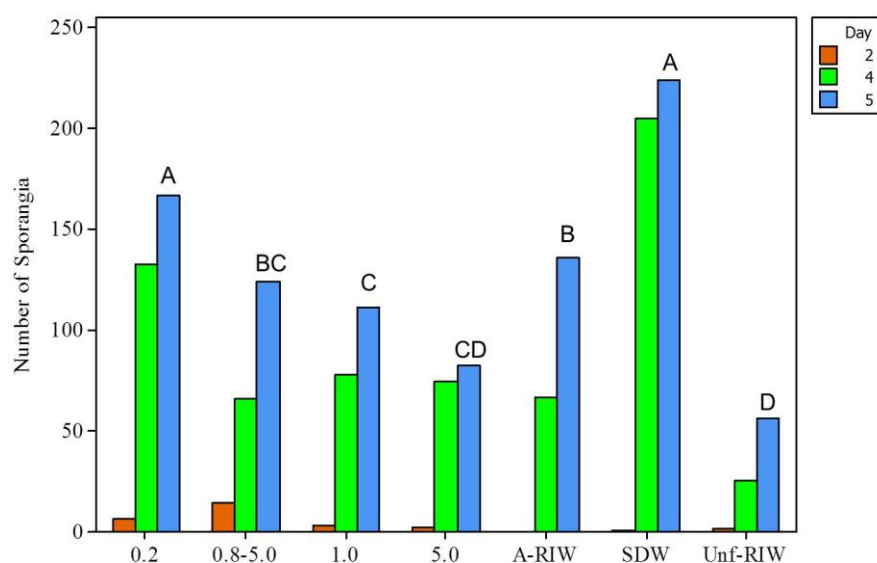


Figure 2.6: Mean number of sporangia formed by *Pythium cryptoirregulare* in water fraction samples from greenhouse E. Results comparing all water fraction samples recorded on day 2, 4, 5. Mean results from day 5 were used to calculate statistical test using Fisher test. Means that do not share a letter are significantly different ($P < 0.1$).

C. *P. irregulare*

The results for the mean number of sporangia formed were more variable among greenhouses for this species than for *P. aphanidermatum* or *P. cryptoirregulare*. The number of sporangia was less in microbe-containing water fractions when compared to the A-RIW and SDW controls and to the 0.2 filtrate from greenhouse S. The mean number of sporangia produced in A-RIW, SDW, and 0.2 filtrate were significantly greater than that produced in all microbe-containing water fraction samples for greenhouse S. In water samples from greenhouse E, the greatest mean number of sporangia were produced in A-RIW, SDW, and 0.2 filtrate but they were not statistically significantly different from some of the water fraction samples containing microbes. Unf-RIW and 5.0 filtrate were the samples with the fewest amount of sporangia in greenhouse E. Results of the mean number of sporangia are summarized in Fig. 2-7 for greenhouse S and Fig. 2-8 for greenhouse E.

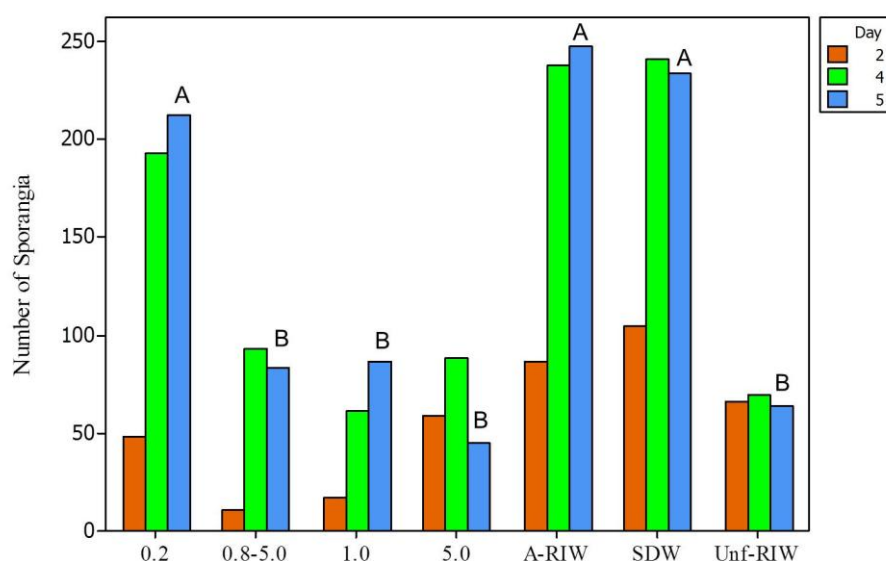


Figure 2.7: Mean number of sporangia formed by *Pythium irregulare* in water fraction samples from greenhouse S. Results comparing all water fraction samples recorded on day 2, 4, 5. Mean results from day 5 were used to calculate statistical test using Fisher test. Means that do not share a letter are significantly different ($P < 0.1$).

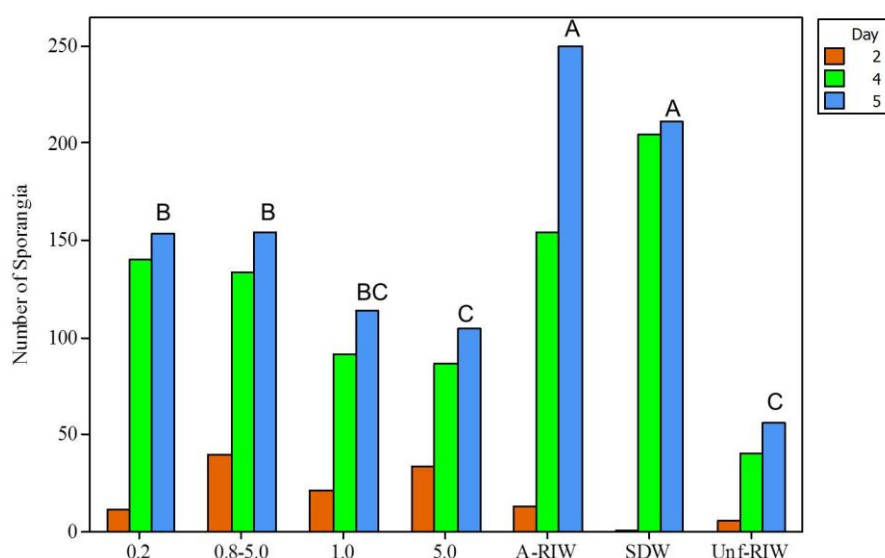


Figure 2.8: Mean number of sporangia formed by *Pythium irregulare* in water fraction samples from greenhouse E. Results comparing all water fraction samples recorded on day 2, 4, 5. Mean results from day 5 were used to calculate statistical test using Fisher test. Means that do not share a letter are significantly different ($P < 0.1$).

Influence of microbial community in the production of zoospores

Overall, zoospore formation by *P. aphanidermatum* in RIW samples containing microorganisms was not inhibited (Fig. 2-9 and 2-10). Zoospore formation by *P. aphanidermatum* was observed after 24 hours on all plates assessed. Results for water fractions from greenhouse S and E were similar.

Zoospore formation by *P. irregulare* and *P. cryptoirregulare* was observed occasionally after 48 hours. The results varied among water samples for greenhouse S and E. In SDW and 0.2 filtrate samples from both greenhouses, *P. irregulare* and *P. cryptoirregulare* produced zoospores. Nevertheless, results for A-RIW control sample and 1.0 filtrate were variable among greenhouses. In samples that contained microorganisms larger than 5.0 μm , *Pythium* zoospores could not be differentiated from other microorganisms and assessments were not attempted on those samples. Figure 2-9 shows the results for zoospore formation for greenhouse S water fraction samples and Fig. 2-10 shows the results for greenhouse E. *Pythium cryptoirregulare* failed to form zoospores in the 1.0 samples from both greenhouses while *P. irregulare* failed to form zoospores in the 1.0 samples only from greenhouse E.

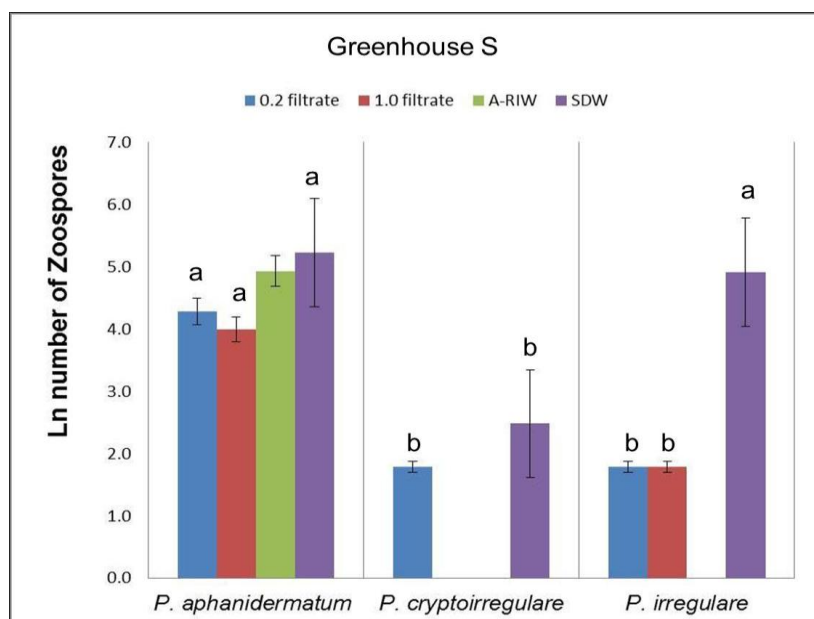


Figure 2-9: Log natural number of zoospores observed for all three *Pythium* species from greenhouse S. *P. aphanidermatum* produced zoospores in all samples. *P. cryptoirregulare* and *P. irregulare* did not produce zoospores in all water fraction samples. Zoospores production was assessed in SDW, A-RIW, 0.2 and 1.0 filtrates. Results that do not share a letter are significantly different ($P < 0.1$). Only water samples that gave results for all three *Pythium* species were compared. Comparisons were based on each water sample (e.g 1.0 filtrate) results for each *Pythium* species.

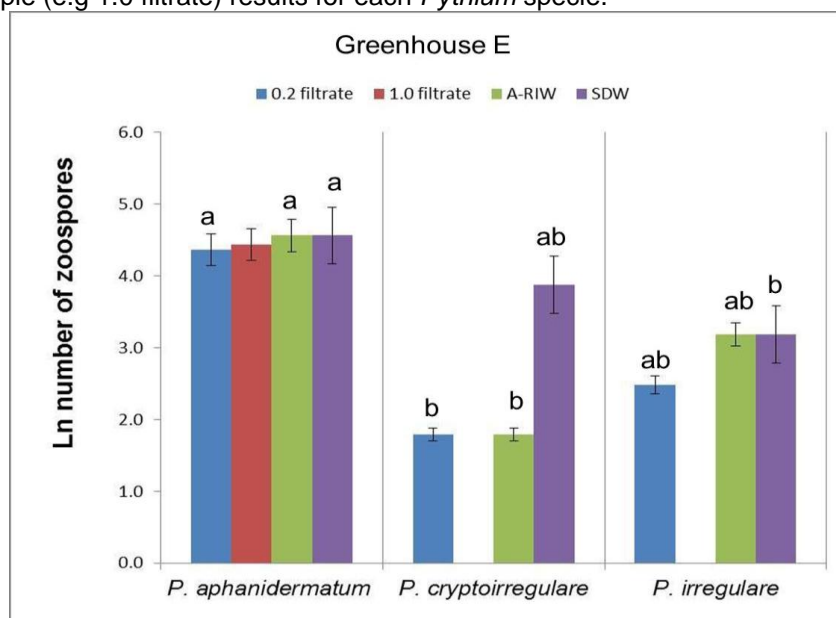


Figure 2-10: Log natural number of zoospores observed for all three *Pythium* species from greenhouse E. *P. aphanidermatum* produced zoospores in all samples. *P. cryptoirregulare* and *P. irregulare* did not produced zoospores in all water fraction samples. Zoospores production was assessed in SDW, A-RIW, 0.2 and 1.0 filtrates. Results that do not share a letter are significantly different ($P < 0.1$). Only water samples that gave results for all three *Pythium* species were compared. Comparisons were based on each water sample (e.g 1.0 filtrate) results for each *Pythium* species.

Discussion

Microbial ecology and its effect on plant ecosystem function, dynamics, and productivity currently is a vibrant area of research [21]. The assessment of microbial populations in recycled irrigation water reservoirs, a subset of microbial ecology, deserves attention because if naturally occurring microorganisms are responsible for suppressing the survival of *P. aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare* it may be possible to increase the phenomenon in greenhouse production systems.

In recent years the addition of antagonistic bacteria to slow filters has been recognized as a possible strategy to control pathogens in soilless cultures [25]. The present research indicates that a naturally occurring microbial community capable of suppressing the development of the three *Pythium* spp. is present throughout the year in the two greenhouses. Regardless of when the water samples were collected, suppression of sporangia formation by the three *Pythium* species was observed. Most water samples containing microorganisms had a deleterious effect on the development of sporangia by *P. aphanidermatum*, *P. irregulare* and *P. cryptoirregulare* when compared to their development in autoclaved irrigation water (A-RIW), sterile distilled water (SDW), and water passed through the 0.2 μm pore size filter to remove most microbes. The inhibitory effect occurred within 72 hours and the number of sporangia produced after 72 hours did not change significantly. The negative effect on sporangia produced by *Pythium* species varied by water source. It is likely that the two greenhouse reservoirs contained different microbial populations and this may explain the differences between the water sample fractions containing similar sized microbes that were from the different greenhouses. Bacteria termed ultramicrobacteria (UMB) [33] are generally smaller than 0.2 μm [14, 15, 17, 20] with some identified as *Pseudomonas* spp. [27]. It has been shown that different populations of microflora may stimulate or inhibit *Phytophthora* spp. sporangial formation [4, 9]. *Pseudomonas* spp. have been implicated in the stimulation of sporangia production in *Phytophthora* spp. [6, 38]. In the present research, *Pseudomonas* spp. were isolated and identified from water collected at both greenhouses (data not shown). If *Pythium* species respond similarly to *Phytophthora*, then small bacteria may be responsible for some of the effect

In this study, it was not possible to definitively identify specific microbial communities, fractionated by organism size, responsible for the suppression of *Pythium* sporulation in the water samples. General suppression against all three *Pythium* species was observed for all samples containing microbes. The results do not correlate the suppression of sporangia formation with a particular subgroup of microorganisms present in recycled irrigation water. It is not known if *Pythium* disease suppression is caused by a general nutrient limitation on *Pythium* as a result of high level microbial activity [10, 36], or if specific microbes antagonize the pathogen. However in the present research, the suppression was most likely due to the presence of microbes rather than a depletion of nutrients available to *Pythium*, based on the comparison to the water passed through 0.2 μm filters, SDW, and A-RIW. In artificial media of high nutritional content, *Pythium* forms abundant mycelium with few or no sporangia. Sporangia form readily in a low nutrition *in vitro* environment. For this reason in the present research, the relatively high number of sporangia formed in SDW containing only boiled grass blades as the main nutritive source was expected. Initially, each fraction of a given greenhouse sample probably contained the same amount of nutrients but differed in microbes except, for the 0.2 fraction which probably contained few, if any, microbes. The 0.2 filtrate probably contained bacteria termed ultramicrobacteria (UMB) [33], which are generally smaller than 0.2 μm [14, 15, 17, 20]. Some of these UMB may stimulate or inhibit *Pythium* spp. sporangial formation. This may explained the variable results for 0.2 filtrates. Usually this sample was similar to A-RIW and SDW in most results. The samples known to contained microbes had similar statistical results and were different from A-RIW, SDW, and, in most cases, 0.2 filtrate. Based on this, it could be concluded that in general, the development of sporangia was negatively affected by the microorganisms in RIW.

Since zoospores arise from sporangia, the formation of zoospores was significantly affected. In general, *P. aphanidermatum* zoospores formation was not suppressed in none of the water samples. *P. cryptoirregulare* and *P. irregulare* zoospores were, in some cases, inhibited by water samples with and without microbes depending on the greenhouse sample (S or E). *P. cryptoirregulare* and *P. irregulare* formed zoospores in 0.2 filtrate and SDW from both greenhouses samples. The

differences in zoospores formation were observed in 1.0 filtrate and A-RIW. *P. cryptoirregulare* did not form zoospores in 1.0 and A-RIW samples while *P. irregulare* did not form in A-RIW on water sample from greenhouse S. High nutrient content (A-RIW) may explain zoospore inhibition in *P. cryptoirregulare* and *P. irregulare*. As mentioned before, in artificial media of high nutritional content, *Pythium* forms abundant mycelium with few or no sporangia. Although we did not observed sporangia suppression in A-RIW sample, this may explain zoospore suppression. It is not known what the mechanisms are behind the suppression of sporangia or zoospores in media of high nutrient content. In addition, greenhouse S contained higher amounts of bacteria (see chapter 3) when compared to greenhouse E. This may explain the differences in 1.0 filtrate for each greenhouse. The primary phytopathogenic role of zoospores is transmission of the pathogen from host to host [37]. Although it is assumed that zoospores may be spread through water easily, this does not seem to be the case in RIW under commercial greenhouse conditions where potted plants are grown. *P. aphanidermatum* was isolated one time from RIW collected during three years of sampling at the two commercial greenhouses. *P. cryptoirregulare* and *P. irregulare* were never isolated from RIW samples. An experiment that minimizes or eliminates one group of microorganisms from the water (e.g. bacteria, protozoa, or fungi) may help to elucidate if a certain group is primarily responsible for the deleterious effect on sporangium formation by *P. aphanidermatum*, *P. cryptoirregulare* or *P. irregulare* revealed in the present research. It is hypothesized that the microbial community is inhibiting the spread of all three *Pythium* species via recycled irrigation as a result of the activity of the microbes in the water. Additional research is required to determine which organism or organisms are actually responsible for this suppression.

Acknowledgments

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CHAPTER 3

Heterotrophic bacteria in recycled irrigation water and their *in vitro* interaction with *Pythium aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare*

Abstract

Heterotrophic bacteria present in recycled irrigation water (RIW) were evaluated for their *in vitro* effect on *P. aphanidermatum*, *P. cryptoirregulare*, and *P. irregulare*. To detect and compare heterotrophic bacteria we used two media, nutrient agar (NA) and R2A, to isolate copiotrophic and oligotrophic bacteria from water samples. Bacterial isolates recovered from RIW were classified by their *in vitro* mycelia growth inhibiting ability, attaching to *Pythium* hyphae, and enhancing mycelia growth of the three *Pythium* species. Disease development by *Pythium aphanidermatum* in geranium (*Pelargonium X hortorum* 'White Orbit') grown in pasteurized potting mix was evaluated in ebb and flow irrigation systems with recirculating water inoculated with one of three bacteria. *Sphingobium* sp. (431 1.0 N.1) inhibited, *Pseudomonas* sp. (438⁻³ 1.0 N.1) attached to, and *Cupriavidus* sp.(756⁻³ 1.0 N.3) enhanced the mycelial growth of *P. aphanidermatum in vitro*. Disease progress curves differed for each experimental unit but the differences were not statistically significant ($p < 0.1$). None of the bacterial isolates suppressed or increased disease development when experimental units were inoculated with *P. aphanidermatum*.

Introduction

Recycling of irrigation water in commercial greenhouses is implemented as a strategy to minimize water and pesticide or fertilizer run-off from contaminating the environment. However, the use of water effluent may increase the risk of spreading *Pythium* especially those that produce zoospores. *Pythium* members are ubiquitous and occupy several ecological niches [48] including water. This genus contains some phytopathogenic root pathogens that can cause severe losses in several greenhouse crops with soilless cultivation systems which are particularly prone to losses [37]. The need for biological agents to control and even eliminate pathogens is seen as a

measure to minimize the usage of chemicals to control the spread of the disease caused by *Pythium*.

Biological control of *Pythium* has had some successes and some disappointing results. Research that provides insights into the relationship among organisms of a microbial community in relation to *Pythium* spp. may help explain the mixed results. A closer look into unexplored habitats are important to understand *Pythium* species ecological roles [46] and their interaction with bacterial populations in the same ecosystem. The co-existence of bacterial isolates that inhibit or enhance the growth of *Pythium* should be considered when investigating an ecosystem. Naturally occurring microorganisms have the ability to suppress diseases [2, 5, 49] even in soilless systems [36, 38]. In addition, they may have an ability to stimulate the development of specific fungi for their advantage. The stimulation of specific indigenous fungi may be a strategy to mobilize bacteria in soil [21]. Frequent co-isolation of bacteria and *Pythium* from the environment suggests a close relationship among the two groups of microbes. The ubiquitous coexistence of bacteria and fungi in a niche may indicate a synchronous relationship with beneficial outcomes for one or both populations. Synergistic interaction studies between *Pythium* and co-niche inhabiting microbes are few. It is unknown to what extent bacteria may play a role in the survival and spread of *Pythium* in different ecosystems. The frequent co-isolation of bacteria with *Pythium* and *Phytophthora* species suggests possible interspecies communication [22]. Closer attention should be given to the interaction and outcome of different microorganisms co-existing in the same environment.

The biocontrol mode of action against *Pythium* in soil was described as a general suppression phenomenon [14] due to a diverse group of microorganisms and weak competitiveness of *Pythium* [13]. Naturally occurring bacteria in recycled irrigation water may play a role in the survival, suppression, and spread of *Pythium*. Therefore, the assessment of the microbial diversity in recycled irrigation water also deserves attention. The stimulation of beneficial microorganisms known to survive in RIW and able to suppress phytopathogenic *Pythium* species survival could be exploited as an alternative to chemically treated water in the greenhouse.

The goal of this study was to identify *Pythium*–enhancing and *Pythium*-inhibiting bacteria residing in recycled irrigation water. Furthermore, we wanted to determine if the *in vitro* effect could be used to predict their effect in disease suppression under greenhouse conditions. Three bacteria isolates, *Sphingobium* sp., *Pseudomonas* sp., and *Cupriavidus* sp. were assessed for their effect in disease suppression in geranium inoculated with *P. aphanidermatum* in ebb and flow irrigation systems.

Materials and Methods

***Pythium* isolates**

Isolates of three phytopathogenic *Pythium* species frequently found in commercial greenhouse crops in Pennsylvania were used for this study (*Pythium aphanidermatum* (Edson) Fitzp., *P. irregulare* Buisman, and *P. cryptoirregulare* Garzón, Yáñez, and Moorman). Cultures were maintained on colonized water agar blocks in sterile tap water tubes stored at room temperature. The water agar blocks were transferred to potato dextrose agar (PDA) in petri plates to start active cultures at room temperature. Subcultures were transferred to water agar (WA) or PDA plates and incubated at room temperature for 3 days to use as inoculum for further testing.

Isolation of heterotrophic bacteria

Recycled irrigation water (RIW) samples collected approximately every 60 days from cement tanks located in two commercial greenhouses (greenhouse S, 40°49'31.30"N, 76°48'18.17"W and greenhouse E, 40°13'21.56"N, 76°16'13.60"W) were processed to isolate heterotrophic bacteria. Water was collected in sterile 1 L glass jars, near the surface (< 1 meter depth) and from the bottom (> 2 meters depth) using a device that kept the jars sealed until they were at the desired depth. The jars were placed in a cooler containing ice packs that were not in direct contact with the jars. Dissolved oxygen levels were immediately measured (Traceable® Digital Oxygen, Control Company, Friendswood, Texas) at the time of collection. Electrical conductivity

(Model SD-B15, Beckman Industrial) and pH (Mettler-Toledo SevenEasy model, Columbus, OH) were measured after transport to the laboratory.

Two media were used for isolation of bacteria, nutrient agar (NA; Difco™ Nutrient Agar, Beckton, Dixon, and Co. Sparks, MD) and R2A (Difco™ R2A Agar, Beckton, Dixon, and Co. Sparks, MD). NA was used to isolate non-fastidious copiotrophic microorganisms and R2A for slow growing oligotrophic microorganisms using the streak plate technique. One ml of RIW was added to a glass tube containing 9 ml of sterile M9 (1X salt solution; 12.8 g Na₂HPO₄; 3 g KH₂PO₄; 0.5 mg NaCl; 1.0 g NH₄Cl, adjust to 1000 ml of sterile water). Serial dilution up to 10⁻⁹ was used to obtain colony counts (colony forming units per ml; CFU/ml) and individual, morphologically different colonies on each plate. Five hundred microliters from each dilution tube was transferred to NA and R2A media in petri plates and streaked with a sterile glass rod. NA plates were incubated at 23°C for 3 days and R2A plates at 15°C for 7 days. Morphologically different colonies were transferred to new media plates to obtain pure cultures. Each pure isolate was transferred to full strength nutrient broth (NB; EMD Chemicals, Inc., Gibbstown, NJ) from NA plates and one quarter strength NB from R2A plates and incubated at 23°C and 15°C respectively. Fifty microliters from NB tubes were streaked on NA and R2A to confirm the purity of the culture. Pure cultures were transferred to NB tubes for future studies and for DNA extraction.

***In vitro* effect on three *Pythium* species**

Fifty microliters of 48 hr. old bacterium isolates from NB were placed on the margin of M9 minimum media agar (12.5 g Na₂HPO₄; 3 g KH₂PO₄; 0.5 g NaCl; 1.0 g NH₄Cl; 1M MgSO₄; 1M MgSO₄, 20 ml of 20% glucose) in petri plates and incubated at 21°C in the dark. After 24 hours, a *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* colonized WA plug (5-mm) was placed on the opposite side of the plate containing the bacterium isolate. Plates were incubated at 21°C for 2 days, observed daily and assessed visually for an interaction with *Pythium*. A clear zone of growth inhibition was used as an indication of a deleterious effect on the development of *Pythium*. After 48 hr., radii of the mycelium growth in the direction of the bacterium was measured and percentage of inhibition was calculated ($[(R_1 - R_2) / R_1] \times 100$),

where R_1 is the maximum radius of *P. aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* colonies in bacteria-free control plates and R_2 is the radius of the colonies on the plates containing bacteria [34]. Similarly, enhancement of *Pythium* growth by bacterial isolates was calculated using a modified version of the equation above ($[(R_2 - R_1)/R_2] \times 100$). In addition, bacterial isolates attaching to the hyphae of *Pythium in vitro* were noted, isolated, and kept for identification. This mechanism was associated to growth reduction of *P. ultimum* [32]. All bacterial isolates were tested at least three times and only isolates with consistent results were kept for identification.

DNA extraction

Bacteria isolates were grown in NB tubes and incubated at 21°C for 48 hr. A modified thermal lysis DNA extraction method [39, 43] was used. Briefly, 1 ml of bacterium culture in NB was transferred into a 2 ml heat resistant microcentrifuge tube and centrifuged (Eppendorf model 5415C) at 15,000g for 15 min [39]. The supernatant was discarded and the pellet was resuspended in 50 µl of sterile distilled water (SDW). Tubes were transferred to a heating block at 99°C for 10 minutes, cooled on ice for 2 minutes, and centrifuged at 15,000g x for 1 min. Supernatant was transferred into a new tube and labeled. The DNA concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) adjusted to 25 ng/µl with SDW, and stored at -20°C until further use.

PCR amplification of 16S rDNA from bacteria

Polymerase chain reaction (PCR) technique was used to amplify the coding region of the 16S rDNA gene of bacteria with semi-universal primers EUBf933 (5'-GCACAAGCGGTGGAGCATGTGG-3') and EUBr1387 (5'-GCCCGGGAACGTATTCACCG-3') [15]. PCR master mix (Promega, Madison, WI) was used to carry out all the reactions as follow: 5 µl of PCR (10X) standard Taq buffer, 1 µl of dNTP's (10mM), 5 µl of each (10 µM) EUB f933/EUBr1387 primer, 0.1 µl (1.25U) of Taq polymerase, 31.9 µl of SDW, and 2 µl of DNA template (25 ng/ µl) for a 50 µl PCR reactions. PCR cycles were carried out in PTC-100TM or PTC-200TM Programmable Thermal Controller (MJ Research) with the following program: 95°C, 3 min; 10 cycles

of 94°C for 30 s, 68°C for 45 s, decreasing 1.0°C per cycle, 72°C for 1 min; followed by 25 cycles of 95°C for 30 s, 58°C for 45 s, 72°C for 1 min; finishing with an extension step of 72°C for 5 min. Electrophoresis was done on 2 µl of (6X) EZ-Vision™ Three, DNA dye Loading buffer (AMRESCO, Solon, OH) mixed with 3 µl of PCR product and loaded onto 1% agarose. Bands in the agarose gel were visualized in a UV transilluminator (256-366 nm).

DNA sequencing

PCR products were cleaned using ExoSAP (New England Biolabs) following the manufacturer's instructions. The DNA template was adjusted to 20 ng/µl for sequencing. The same primers used for the initial PCR reaction were used for DNA sequencing. Briefly, 2 µl of EUB f933/EUBr1387 (1X) and 2 µl of DNA template were transferred to a 96 well ultraAmp™ PCR plate. DNA sequencing was conducted in an ABI Hitachi 3730XL DNA analyzer at The Pennsylvania State University Genomic Core Facility, University Park, PA. Bacterial sequences from the forward and reverse primer were aligned using Sequencher 5.0 (Gene Codes, Ann Harbor, MI) to construct a contig of the sequence for each bacteria isolate. DNA contigs were compared to 16S rDNA gene sequences available using nucleotide BLAST from the National Center for Biotechnology Information (NCBI) GenBank and Seqmatch from the Ribosomal Database Project (RDP Release 10, 29) to identify the bacteria to genus [7]. Phylogenetic trees to infer relatedness of isolates were constructed using the Maximum Likelihood method based on the Kimura's two-parameter model [19] and the rate variation among sites was modeled with a gamma distribution (shape parameter = 5). The bootstrap consensus trees were inferred from 500 replicates [11]. Evolutionary analyses were conducted with MEGA 5.0 [23, 44].

Greenhouse study

Ebb and flow experimental units (American Hydroponics, Arcata, CA) were set up in a greenhouse at University Park, PA to evaluate the effect of Bac. 1 (*Sphingobium* sp.; 431 1.0 N.1); Bac. 2 (*Pseudomonas* sp.; 438[^]-3 1.0 N.1); and Bac. 3 (*Cupriavidus* sp.; 756[^]-3 1.0 N.3) on disease development by *P. aphanidermatum*.

Seeds of geranium (*Pelargonium x hortorum* cv. 'White Orbit') were planted in pasteurized peat/perlite potting mix (Fafard #2; Conrad Fafard, Inc., Agawam, MA) in plastic seedling trays (200 cells/tray, 10 ml/cell) and grown in the lab for 5 days at 26 °C. The seedlings were then transferred to the greenhouse to acclimate. After 48 hr., seedlings were transplanted to individual round, plastic pots (10 cm diameter X 8.5 cm) containing approximately 400 ml of pasteurized Fafard #2 potting mix. Each experimental unit consisted of sixteen pots (4 rows of 4 pots) in a 1 m X 1 m plastic bench tray. Each bench tray had its own 30 L water reservoir and pump beneath it. Tap water containing soluble fertilizer (15% N-16% P₂O₅-17% K₂O; Peters Professional, The Scotts Company LLC, Marysville, OH) filled each reservoir. After 1 week, selected units were inoculated with a 48 hr. old bacterium isolate (10⁷ cfu/ml) by adding it to the water reservoir. The following week, units were inoculated with 5 or 7 day old *P. aphanidermatum* inoculum applied to the surface of the potting mix close to the stem or directly into the water reservoir. When *Pythium* was applied to plants in pots, 8 plants were inoculated. Electronic timers controlled the pumps, such that they ran continuously for approximately 10 minutes. An outflow pipe connected to the reservoir allowed the water to reach a depth of about 2 cm before it began to drain back into the reservoir. The reservoirs were adjusted to 30 L with fertilizer-containing water and kept at that volume for the duration of the experiment. Geranium growth, the height from the soil line to the top of the plant, was measure weekly on 2 plants that were initially randomly selected in each experimental unit. In order to monitor the presence of *Pythium*, all reservoirs were baited with blades of creeping bentgrass (*Agrostis stolonifera* L. 'Penn Eagle') that had been grown in the laboratory. Grass blades cut into sections were sandwiched between pieces of fiberglass window screen and suspended in the water. At the end of the baiting period, the blades were individually placed on NARF agar (25 mg nystatin, 150 mg ampicillin, 5 mg rifampicin, 5 µl fluazinam; 500 ml clarified V8 juice agar) [30] for detection of *Pythium*. *Pythium* growing from the blades was transferred to water agar and then identified. This was done weekly to assess the survival of *P. aphanidermatum*. Experimental units were arranged in a randomized complete block design. The number of geranium plants

infected per week was recorded for each experimental unit. The experiment was done two times, first from January to March, 2012 and from April to June 2012.

Statistical analysis

Heterotrophic bacteria counts were log transformed before analysis. Area under the disease progress curve (AUDPC) was calculated with the trapezoidal equation [28] using disease incidence over time. Data were analyzed with ANOVA and Tukey's ($P < 0.05$) using Minitab 16 statistical software (Minitab Inc., State College, PA).

Results

Heterotrophic bacteria isolates in recycled irrigation water

Two media were used to isolate heterotrophic fast growing, non-fastidious copiotrophic bacteria and slow growing oligotrophic bacteria from recycled irrigation water collected at two different commercial greenhouses. The quantities of heterotrophic bacteria (cfu/ml) were different between greenhouses. Overall, greenhouse S had more heterotrophic bacteria in 60% of the water samples (fig 3-1 and fig 3-2). The quantities of copiotrophic and oligotrophic bacteria were different too. Water samples from greenhouse S had 50% more colony forming units (cfu/ml) of copiotrophic bacteria while 21% of the samples had more oligotrophic bacteria.

Many morphologically different bacteria colonies were isolated from the RIW samples. Approximately 900 bacterial isolates were tested for their *in vitro* effect on the hyphae of the three *Pythium* species. Although different bacterial isolates were collected using both media, only the isolates (5%) that showed one of the three *in vitro* reactions of interest (table 3-1) were identified. These genera were isolated repeatedly from the greenhouses using the two media. Figure 3-3 lists the phenotypes used to screen and classify the bacterial isolates in this study. In addition, we identified bacterial isolates that attached to *Pythium* hyphae *in vitro*. It was observed that the interaction inhibited *Pythium* hyphae growth when compared to control (no bacteria). Nelson et al. [32] observed that *Pythium ultimum-Enterobacter* association inhibited the

growth of hyphae. Lastly, we isolated and identified few bacteria isolates that seemed to enhance the growth of *Pythium in vitro*. We did not find any information related to this phenomenon in the literature.

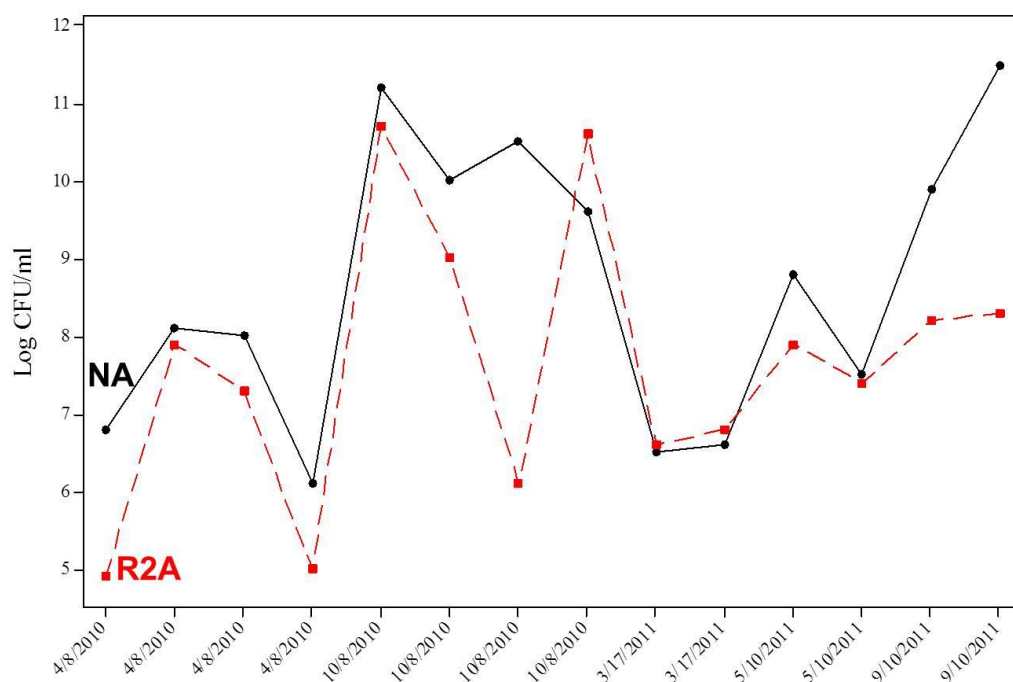


Figure 3-1: Heterotrophic bacteria (copiotrophic on nutrient agar, and oligotrophic on R2A) isolated from greenhouse S recycling irrigation water collected over three years.

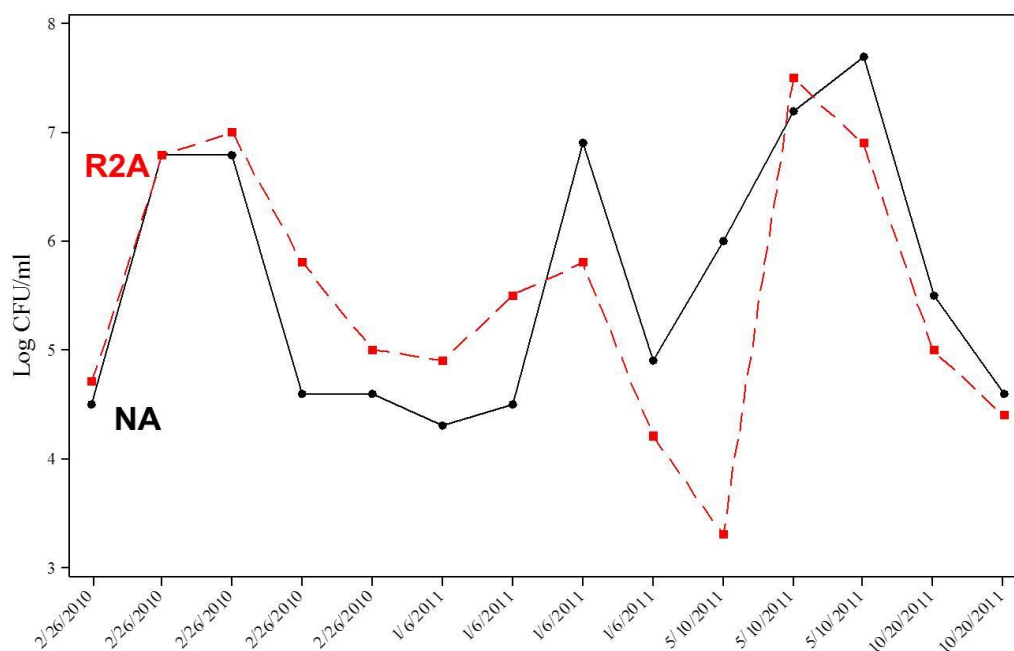


Figure3-2: Heterotrophic bacteria (copiotrophic on nutrient agar, and oligotrophic on R2A) isolated from greenhouse E recycling irrigation water collected over three years.

Table 3-1: Identification of bacterial isolates with *in vitro* effects on *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare*.

Greenhouse ^a	Medium ^b	Strain	Closest match ^c	Sequence identity (%)
S	NA	432 [^] -2 1.0 NA	<i>Acidovorax</i> sp.	99
	NA	439 [^] -2 N.6	<i>Bacillus</i> sp.	99
	NA	439 [^] -1 NA	<i>Bacillus</i> sp.	99
	R2A	440 [^] -4 R.3	<i>Bacillus</i> sp.	100
	NA	431 1.0 N.2	<i>Bosea</i> sp.	99
	NA	439 [^] -2 N.4	<i>Chryseobacterium</i> sp.	99
	NA	434 1.0 N.2	<i>Microbacterium</i> sp.	99
	R2A	437-5 1.0 R.1	<i>Mycobacterium</i> sp.	93
	R2A	435 [^] -6 1.0 R.2	<i>Mycobacterium</i> sp.	94
	R2A	439 [^] -2 R.3	<i>Pseudomonas</i> sp.	100
	R2A	439 [^] -3 R.1	<i>Pseudomonas</i> sp.	100
	R2A	438 [^] -7 R.1	<i>Pseudomonas</i> sp.	99
	R2A	435 [^] -6 1.0 R.3	<i>Pseudomonas</i> sp.	99
	R2A	437 [^] -3 1.0 R.1	<i>Pseudomonas</i> sp.	100
	R2A	436-5 1.0 R.3	<i>Pseudomonas</i> sp.	95
	NA	432 1.0 N.2	<i>Pseudomonas</i> sp.	95
	NA	433 1.0 N.4	<i>Pseudomonas</i> sp.	99
	NA	431 [^] -1 1.0 N.1	<i>Pseudomonas</i> sp.	100
	NA	433 [^] -1 1.0 N.2	<i>Pseudomonas</i> sp.	99
	NA	431 [^] -2 N.1	<i>Pseudomonas</i> sp.	99
	NA	434 [^] -2 N.3	<i>Pseudomonas</i> sp.	99
	NA	438 [^] -3 1.0 N.1	<i>Pseudomonas</i> sp.	99
	NA	440-2 N.2	<i>Pseudomonas</i> sp.	99
	NA	431 1.0 N.1	<i>Sphingobium</i> sp.	99
	R2A	440 [^] -2 R.3	<i>Sphingobacterium</i> sp.	99
E	NA	747 [^] -3 N.4	<i>Acinetobacter</i> sp.	99
	NA	746 1.0 N.4	<i>Acinetobacter</i> sp.	96
	R2A	750 [^] -1 R.1	<i>Acinetobacter</i> sp.	95
	R2A	754 1.0 R.3	<i>Bacillus</i> sp.	99
	NA	760 [^] -4 NA	<i>Bacillus</i> sp.	99
	R2A	752-4 1.0 R.3	<i>Cupriavidus</i> sp.	99
	NA	752-4 N.1	<i>Cupriavidus</i> sp.	99
	NA	756-3 1.0 N.3	<i>Cupriavidus</i> sp.	99
	NA	756 [^] -2 1.0 N.2	<i>Enterobacter</i> sp.	100
	NA	756 [^] -2 1.0 N.3	<i>Enterobacter</i> sp.	99
	R2A	756 [^] -4 1.0 R.2	<i>Enterobacter</i> sp.	99
	NA	755 1.0 NA	<i>Hermiimonas</i> sp.	99
	R2A	759 [^] -7 R2A	<i>Pantoea</i> sp.	99
	NA	752-2 1.0 N.1	<i>Pedobacter</i> sp.	97

NA	748 [^] -1 N.1	<i>Pseudomonas</i> sp.	100
NA	749 [^] -1 N.5	<i>Pseudomonas</i> sp.	99
NA	750 [^] -1 N.1	<i>Pseudomonas</i> sp.	99
NA	748 [^] -2 1.0 N.4	<i>Pseudomonas</i> sp.	99
R2A	749 [^] -1 R.4	<i>Pseudomonas</i> sp.	100
R2A	747 [^] -2 R.3	<i>Pseudomonas</i> sp.	98
R2A	746 [^] -3 R.4	<i>Pseudomonas</i> sp.	99
R2A	751 [^] -3 R2A	<i>Pseudomonas</i> sp.	96
R2A	752-3 1.0 R.3	<i>Sphingobium</i> sp.	99

^a Greenhouse S 40°49'31.30"N, 76°48'18.17"W; greenhouse E, 40°13'21.56"N, 76°16'13.60"W

^b NA was used full strain and R2A was used according to manufacturer.

^c Based on 16S rDNA sequences compared to sequences in the GenBank database and Seqmatch from Ribosomal Database Project. Sequences were aligned using Sequencher.



Figure 3-3: Bacterial isolates representing *in vitro* effects assessed in the study A) Bacterium isolate shown to inhibit *Pythium* mycelia growth B) Bacterium isolate attaching to *Pythium* hyphae C) Bacterium isolate stimulated *Pythium* mycelia growth compared to control plate (only *Pythium*)

In vitro* inhibitory effect on *Pythium

Twenty four bacterial isolates inhibited the growth of at least one of the *Pythium* species. The group was dominated by gram negative (19 isolates) *Proteobacteria* and *Bacteroidetes* phyla, while five were gram positive *Actinobacteria* and *Firmicutes* phyla. Among the antagonistic bacteria fourteen isolates were identified as *Pseudomonas*, four as *Bacillus*, and one isolate of each of the following: *Microbacterium*, *Sphingobacterium*, *Enterobacter*, *Sphingobium*, and *Acidovorax*. Most of the isolates inhibited *P. irregulare* (21 isolates) while fifteen isolates inhibited *P. cryptoirregulare*, and six isolates inhibited *P. aphanidermatum*. Some bacterial isolates were species-specific. *Chryseobacterium* sp., *Sphingobacterium* sp., and *Acidovorax* sp. inhibited only *P. irregulare*. Some bacterial isolates exhibited multiple effects, attaching to one *Pythium* species while inhibiting the growth of another *Pythium* species. According to the phylogenetic tree results, most of the *Pseudomonas* species isolates were closely

related but not identical (Fig. 3-4). *Pseudomonas* spp. isolated from different greenhouses grouped together. *Bacillus* spp. isolated from greenhouse S were closely related while the isolates from greenhouse E seemed to be the same isolate (Fig. 3-4).

Table 3-2: Bacterial isolates that inhibited *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* *in vitro*.

Strains ^a	Best match ^b	<i>In vitro</i> inhibition \pm SE ^c (%)		
		<i>P. aphanidermatum</i>	<i>P. irregulare</i>	<i>P. cryptoirregulare</i>
432 [^] -2 1.0 NA*	<i>Acidovorax</i> sp.		3.9 \pm 0.69	
439 [^] -3 R.1*	<i>Pseudomonas</i> sp.		8.4 \pm 0.60	7.7 \pm 1.28
439 [^] -2 R.3*	<i>Pseudomonas</i> sp.		13.3 \pm 0.79	
432 1.0 N.2*	<i>Pseudomonas</i> sp.	17.2 \pm 2.32		12.8 \pm 1.33
748 [^] -1 N.1*	<i>Pseudomonas</i> sp.		16.6 \pm 0.96	
431 [^] -1 1.0 N.1	<i>Pseudomonas</i> sp.	11.2 \pm 0.93	8.5 \pm 0.64	8.9 \pm 1.19
433 [^] -1 1.0 N.2	<i>Pseudomonas</i> sp.	9.8 \pm 1.08	5.9 \pm 0.58	11 \pm 1.47
750 [^] -1 N.1*	<i>Pseudomonas</i> sp.		20.7 \pm 1.26	8.6 \pm 0.76
749 [^] -1 N.5	<i>Pseudomonas</i> sp.		29 \pm 1.69	
437 [^] -3 1.0 R.1	<i>Pseudomonas</i> sp.		2.1 \pm 0.61	3.7 \pm 0.67
433 1.0 N.4	<i>Pseudomonas</i> sp.	7.7 \pm 0.78		9.4 \pm 1.25
749 [^] -1 R.4*	<i>Pseudomonas</i> sp.		19.3 \pm 1.46	2.9 \pm 0.44
438 [^] -7 R.1*	<i>Pseudomonas</i> sp.		1.7 \pm 0.52	1.6 \pm 0.79
435 [^] -6 1.0 R.3	<i>Pseudomonas</i> sp.		1.7 \pm 0.68	
747 [^] -2 R.3	<i>Pseudomonas</i> sp.		21.3 \pm 1.19	
756 [^] -4 1.0 R.2*	<i>Enterobacter</i> sp.		20 \pm 2.14	7.8 \pm 0.81
431 1.0 N.1	<i>Sphingobium</i> sp.	8.6 \pm 1.24	10.2 \pm 0.74	6.3 \pm 0.83
440 [^] -2 R.3	<i>Sphingobacterium</i> sp.		6.1 \pm 0.60	
439 [^] -2 N.4	<i>Chryseobacterium</i> sp.		5.6 \pm 1.32	
434 1.0 N.2	<i>Microbacterium</i> sp.		15.3 \pm 1.06	3.6 \pm 1.07
760 [^] -4 NA	<i>Bacillus</i> sp.	14 \pm 2.04		4.4 \pm 0.87
754 1.0 R.3	<i>Bacillus</i> sp.		6.9 \pm 0.97	2.7 \pm 0.69
439 [^] -2 N.6*	<i>Bacillus</i> sp.		5.4 \pm 0.48	16.3 \pm 1.43
440 [^] -4 R.3	<i>Bacillus</i> sp.		6.1 \pm 0.59	

^a Water sample identification number. Bacterial isolates marked with * exhibited more than one type of *in vitro* effect.

^b Results were based on 16S rDNA sequences compared to sequences in GenBank database and Seqmatch from Ribosomal Database Project.

^c Inhibition was calculated $([(R_1 - R_2) / R_1] \times 100)$, where R_1 is the maximum radius of *Pythium* colonies in bacteria-free control plates and R_2 is the radius of the colonies on the plates containing bacteria).

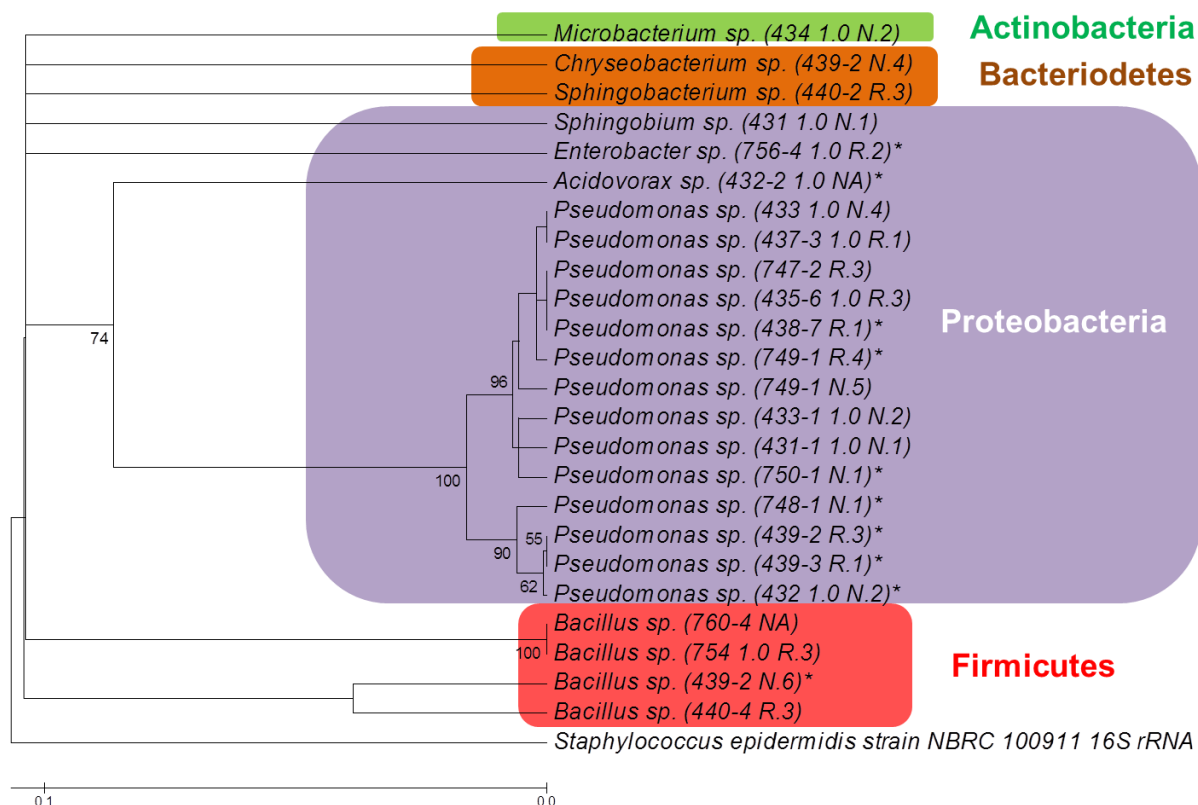


Figure 3-4: Relatedness of 24 bacteria isolates from recycled irrigation water inhibiting growth of *Pythium* *in vitro*. Relatedness was based on 16S rRNA gene. Maximum likelihood method was conducted using MEGA 5.0 [44]. Bacterial isolates marked with * showed multiple effects on different *Pythium* species. Scale bar, 10% divergence.

In vitro* attaching effect on *Pythium

Twenty one bacterial isolates attached to *Pythium* hyphae *in vitro*. This group was dominated by *Proteobacteria*. Seventeen isolates were from the subclass *Gammaproteobacteria* and one from *Betaproteobacteria*. Most of the bacterial isolates decreased the growth of *Pythium* when compared to control plates (data not shown). Most bacterial isolates were identified as *Pseudomonas* spp. (13) from which 12 specifically attached to *P. aphanidermatum* hyphae. Three isolates were identified as *Enterobacter* and 3 as *Acinetobacter*. Some bacterial isolates were *Pythium* species-specific. *Pantoea* sp. attached to *P. aphanidermatum* while *Acidovorax* sp. only attached to *P. cryptoirregulare*. Some bacterial isolates had multiple effects (attached to or inhibited *Pythium*) depending on the *Pythium* species. According to the phylogenetic tree results, *Acinetobacter* spp. isolates seemed to be the same isolate

(Fig. 3-5). They were all isolates from greenhouse E. *Enterobacter* spp. isolates were closely related but not the same isolate (> 95% relatedness). They were all isolates from greenhouse E. *Pseudomonas* spp. isolates were very diverse and only 2 out of 13 isolates seemed to be the same isolate (Fig. 3-5).

Table 3-3: Bacterial isolates that attached (B) to *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* hyphae

Strains ^a	Best match ^b	<i>P. aphanidermatum</i>	<i>P. irregulare</i>	<i>P. cryptoirregulare</i>
432~2 1.0 NA*	<i>Acidovorax</i> sp.			B
746 1.0 N.4	<i>Acinetobacter</i> sp.		B	
750~1 R.1	<i>Acinetobacter</i> sp.	B	B	B
747~3 N.4	<i>Acinetobacter</i> sp.	B		
756~2 1.0 N.2	<i>Enterobacter</i> sp.	B	B	B
756~2 1.0 N.3	<i>Enterobacter</i> sp.		B	B
756~4 1.0 R.2*	<i>Enterobacter</i> sp.	B		
759~7 R2A	<i>Pantoea</i> sp.	B		
439~2 R.3*	<i>Pseudomonas</i> sp.	B		
439~3 R.1*	<i>Pseudomonas</i> sp.	B		
432 1.0 N.2*	<i>Pseudomonas</i> sp.		B	
749~1 R.4*	<i>Pseudomonas</i> sp.	B		
746~3 R.4	<i>Pseudomonas</i> sp.	B		
748~2 1.0 N.4	<i>Pseudomonas</i> sp.	B		
748~1 N.1*	<i>Pseudomonas</i> sp.	B		
438~7 R.1*	<i>Pseudomonas</i> sp.	B		
438~3 1.0 N.1	<i>Pseudomonas</i> sp.	B		
751~3 R2A	<i>Pseudomonas</i> sp.	B	B	
431~2 N.1	<i>Pseudomonas</i> sp.	B		
434~2 N.3	<i>Pseudomonas</i> sp.	B	B	B
750~1 N.1*	<i>Pseudomonas</i> sp.	B		

^a Water sample identification number. Bacterial isolates marked with * exhibited more than one type of *in vitro* effect.

^b Results were based on 16S rDNA sequences compared to sequences in the GenBank database and Seqmatch from Ribosomal Database Project.

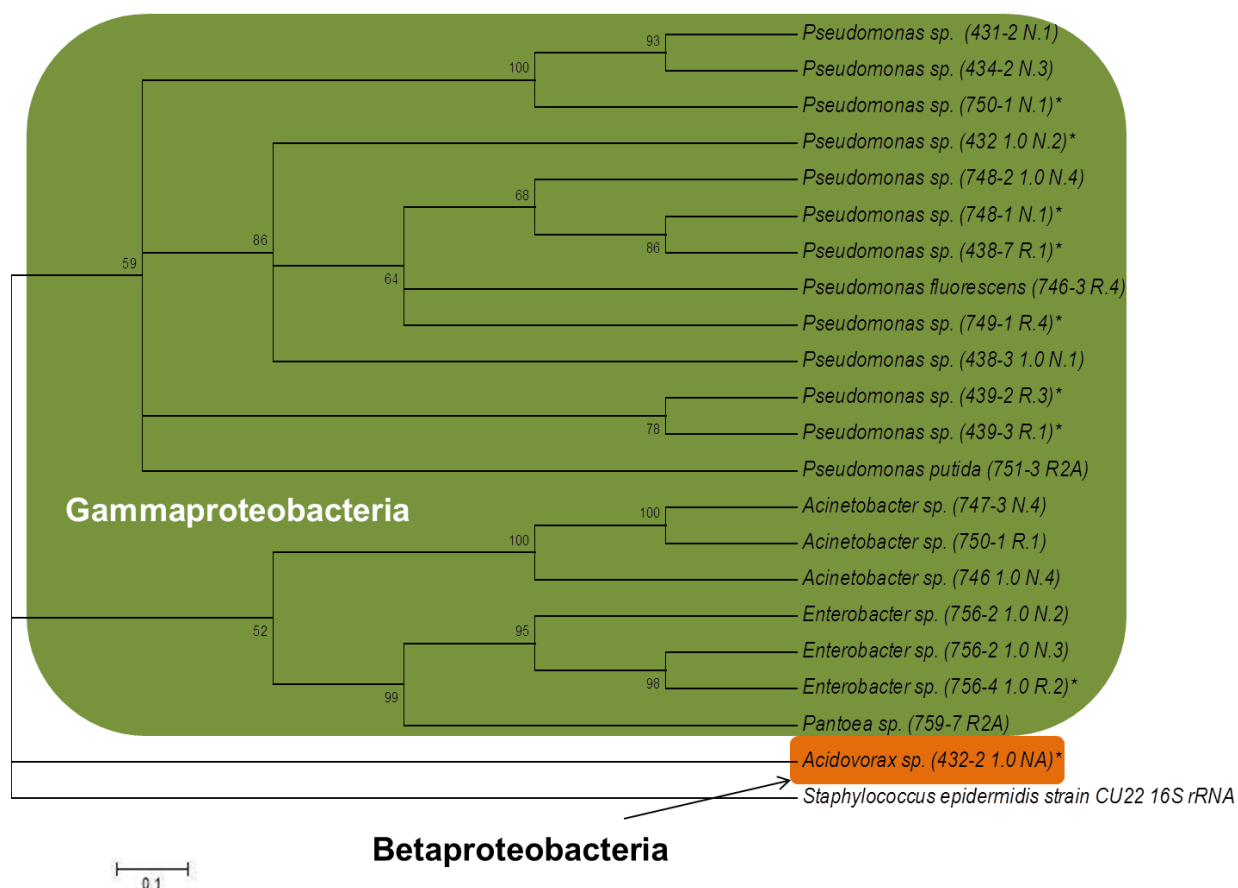


Figure 3-5: Relatedness of 21 bacterial isolates from recycled irrigation water that attached to *Pythium* hyphae *in vitro*. Relatedness was based on 16S rRNA gene. Maximum likelihood method was conducted using MEGA 5.0 [44]. Bacterial isolates marked with * showed multiple effects on different *Pythium* species. Scale bar, 10 % divergence.

In vitro* growth enhancing effect on *Pythium

Thirteen bacterial isolates enhanced the growth of *Pythium in vitro*. Bacterial isolates (69%) were gram negative *Proteobacteria* and *Bacterioidetes*. Gram positive bacteria isolates (13%) were *Firmicutes* and *Actinobacteria*. Some bacterial isolates had species-specific effects. *P. aphanidermatum* growth was enhanced by 77% of the isolates, 62% enhanced the growth of *P. cryptoirregulare*, while 38% enhanced the growth of *P. irregulare*. One bacterium, identified as *Bacillus* spp., had multiple effects. It enhanced the growth of *P. aphanidermatum* and inhibited the growth of *P. cryptoirregulare* and *P. irregulare*. According to the phylogenetic tree results, *Pseudomonas* spp. isolates may be the same isolate due to the degree of similarity on the 16S rRNA gene (Fig. 3-6) while *Cupriavidus*, *Mycobacterium*, and *Bacillus* spp.

were closely related but different isolates.

Table 3-4: Bacterial isolates that enhanced *P. aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* hyphal growth *in vitro*.

Strain ^a	Best match ^b	<i>In vitro</i> enhancing (%) results ^c		
		<i>P. aphanidermatum</i>	<i>P. irregulare</i>	<i>P. cryptoirregulare</i>
752-3 1.0 R.3	<i>Sphingobium</i> sp.	32.6 ± 6.37	36.5 ± 2.78	7.1 ± 1.24
431 1.0 N.2	<i>Bosea</i> sp.		13.9 ± 1.08	5.8 ± 1.18
752-4 N.1	<i>Cupriavidus</i> sp.			6.7 ± 0.99
756-3 1.0 N.3	<i>Cupriavidus</i> sp.	16.2 ± 2.60		
752-4 1.0 R.3	<i>Cupriavidus</i> sp.	16.2 ± 2.60	19.3 ± 1.47	
755 1.0 NA	<i>Hermiimonas</i> sp.		18.6 ± 1.14	34.6 ± 4.81
440-2 N.2	<i>Pseudomonas</i> sp.	11.3 ± 1.66		14.2 ± 1.69
436-5 1.0 R.3	<i>Pseudomonas</i> sp.	15.8 ± 2.51		4.7 ± 0.73
752-2 1.0 N.1	<i>Pedobacter</i> sp.	22.3 ± 2.05	8.7 ± 0.86	
435^6 1.0 R.2	<i>Mycobacterium</i> sp.	10.8 ± 1.95		12.5 ± 2.17
437-5 1.0 R.1	<i>Mycobacterium</i> sp.	15.5 ± 3.0		
439^1 NA	<i>Bacillus</i> sp.	4.1 ± 0.85		12.1 ± 1.30
439^2 N.6*	<i>Bacillus</i> sp.	6.3 ± 1.10		

^aWater samples identification number. Bacterium isolate marked with * exhibited mixed *in vitro* results according to *Pythium* specie (enhanced or inhibiting their growth).

^bResults were based on 16S rDNA sequences compared to sequences in GenBank database and Seqmatch from Ribosomal Database Project.

^cValues represent percentage of growth enhanced. Calculation was done using growth assessment equation [34] with modifications, $(R_2 - R_1) / R_2 \times 100$ where R2 is the maximum radii of *P. aphanidermatum*, *P. cryptoirregulare*, and *P. irregulare* colonies in bacteria-inoculated plates and R1 is the radii of *P. aphanidermatum*, *P. cryptoirregulare*, and *P. irregulare* colonies in bacteria-free control plates.

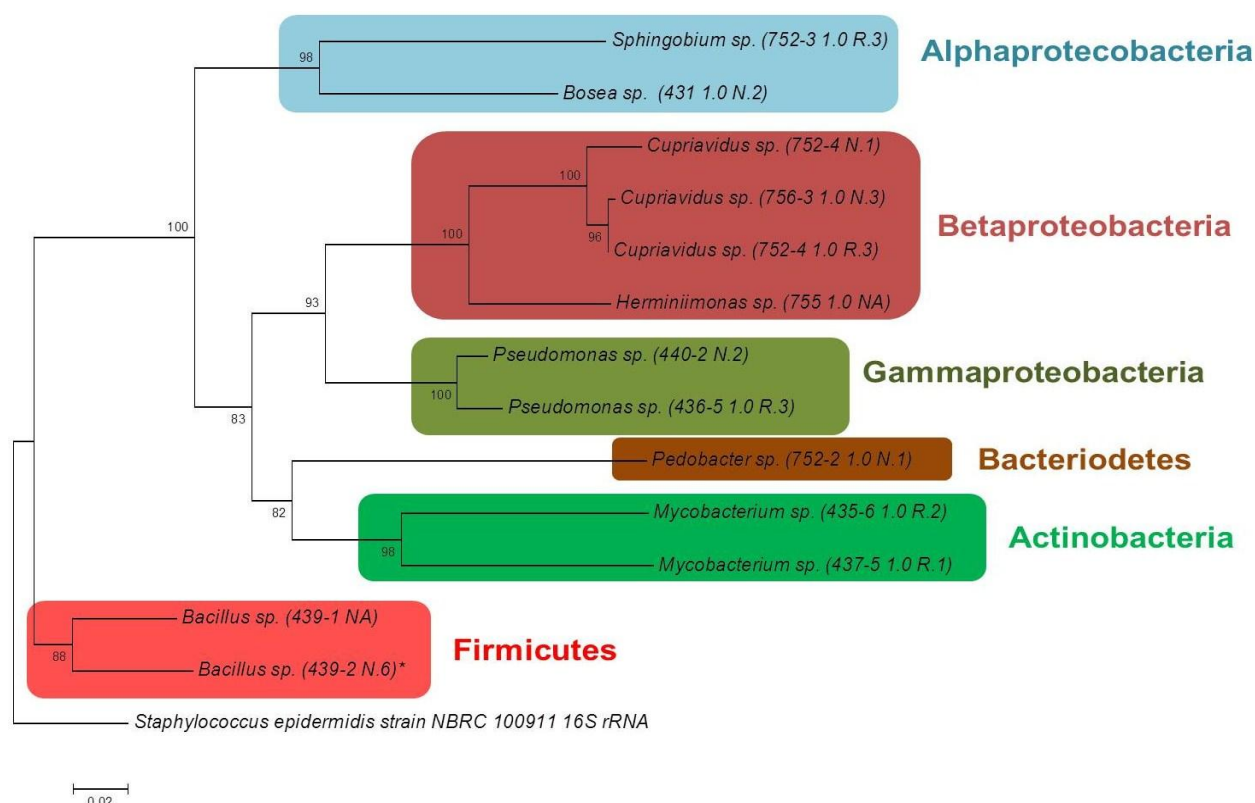


Figure 3-6: Relatedness of 13 bacteria isolates from recycled irrigation water that enhanced the growth of *Pythium* hyphae *in vitro*. Relatedness was based on 16S rRNA gene. Maximum likelihood method was conducted using MEGA 5.0 [44]. Bacterium isolate marked with * showed multiple effects on different *Pythium* species. Scale bar, 2% divergence.

Greenhouse experiment

Disease symptoms caused by *P. aphanidermatum* developed two weeks after inoculating the units with the pathogen. Initial symptoms included stunting and water soaked lesions on stems close to the soil line. Lesions on the stem progressed and eventually killed the plants. The experimental units to which *P. aphanidermatum* was added into the water had the highest incidence of plant death. By week 3, experimental units with *Pythium* applied to the water had higher disease incidence (> 40%) than units where *Pythium* was applied to the potting mix (20%). The systems with 100% mortality were those where all three bacterial isolates were present and *Pythium* had been added to the water reservoir. The systems with the least mortality (<40%) were those in which *Pythium* was applied to the potting mix and no bacteria were added to

the system). *P. aphanidermatum* was detected in the units from the time of inoculation until one week after the plants were dead.

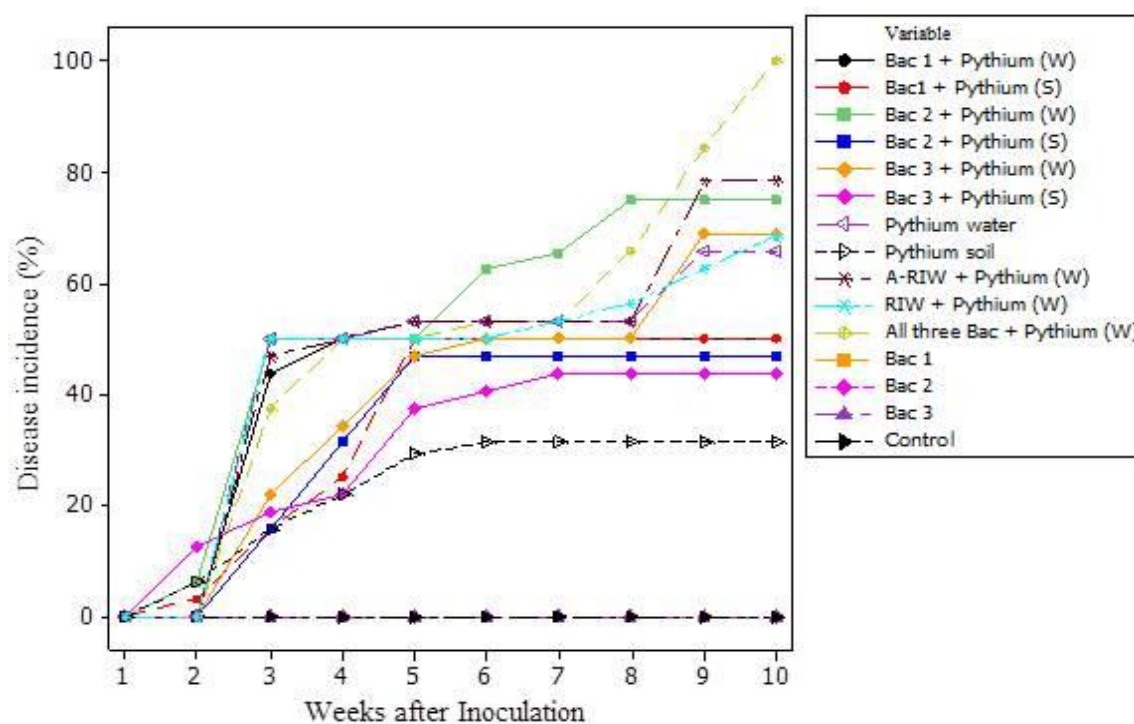


Fig. 3-7: *Pythium aphanidermatum* disease incidence progress curves on geranium (*Pelargonium X hortorum* cv. 'White Orbit') in ebb and flow experimental units in the presence of bacterial isolates originally from recycled irrigation water. Bac.1 (*Sphingobium* sp., 431 1.0 N.1); Bac. 2 (*Pseudomonas* sp., 438⁻³ 1.0 N.1); and Bac. 3 (*Cupriavidus* sp., 756⁻³ 1.0 N.3).

Area under the disease progress curves (AUDPC) values were highest in the experimental units with *Pseudomonas* spp. (Bac. 2) with *P. aphanidermatum* added to water and the in the unit with all three bacteria and *P. aphanidermatum* in water. The lowest AUDPC value was in the unit with *P. aphanidermatum* added to potting mix (soil). The addition of bacteria did not decrease *Pythium aphanidermatum*-caused disease in geraniums under greenhouses conditions.

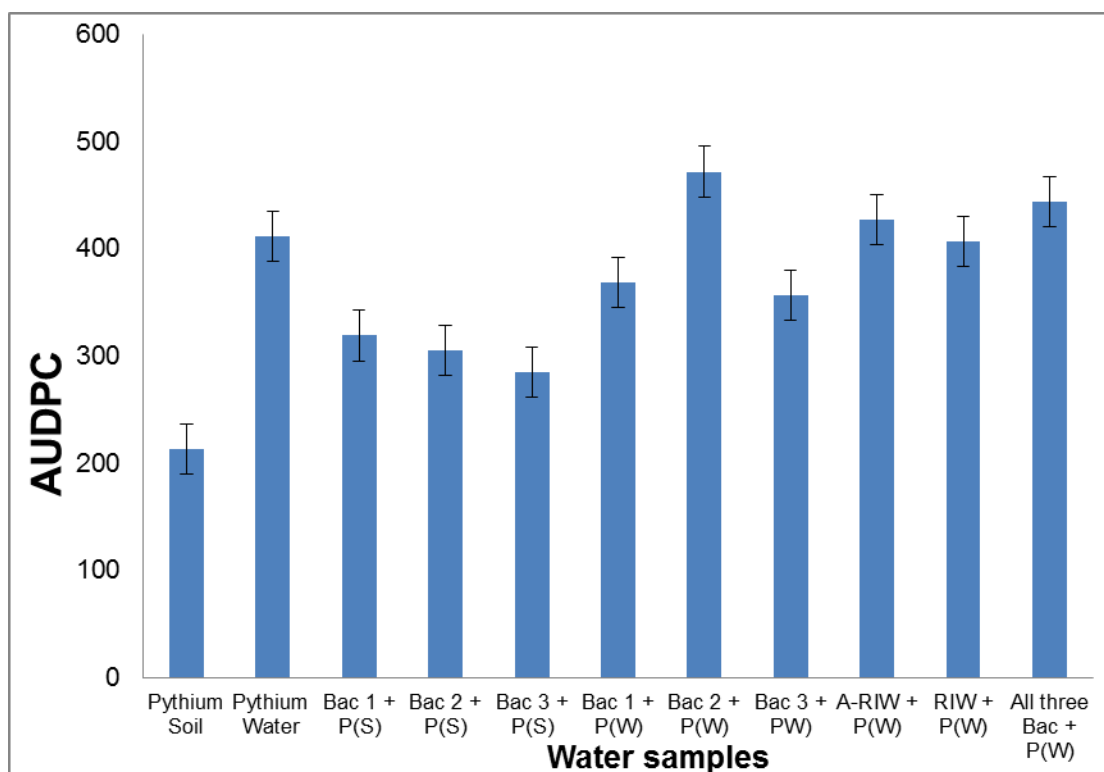


Fig 3-8: Mean area under the progress curve (AUDPC). Water samples assessed were: *Pythium* soil (*P. aphanidermatum* only added to potting mix); *Pythium* water (*P. aphanidermatum* only added to water reservoir); Bac. 1 + P(S) (*Spingobium* sp., 431 1.0 N.1 added to water reservoir and *P. aphanidermatum* added to potting mix); Bac. 2 + P(S) (*Pseudomonas* sp., 438[^]3 1.0 N.1 added to water reservoir and *P. aphanidermatum* added to potting mix); Bac. 3 + P(S) (*Cupriavidus* sp., 756[^]3 1.0 N.3 added to water reservoir and *P. aphanidermatum* added to potting mix); Bac.1 + P(W) (*Spingobium* sp., 431 1.0 N.1 and *P. aphanidermatum* added to water reservoir); Bac. 2 + P(W) (*Pseudomonas* sp., 438[^]3 1.0 N.1 and *P. aphanidermatum* added to water reservoir); Bac. 3 + P(W) (*Cupriavidus* sp., 756[^]3 1.0 N.3 and *P. aphanidermatum* added to water reservoir); A-RIW + P(W) (Autoclaved recycled irrigated water and *P. aphanidermatum* added to water reservoir); RIW + P(W) (recycled irrigated water and *P. aphanidermatum* added to water reservoir); All three Bac. +P(W) (Bac. 1, Bac. 2, Bac. 3 and *P. aphanidermatum* added to water reservoir).

Discussion

The recycled irrigation water from two commercial greenhouses was sampled over a three year period to determine the quantities of heterotrophic bacteria present and to identify those bacterial isolates with *in vitro* effects on *Pythium aphanidermatum*, *P. cryptotirregularare* or *P. irregulare*. Water from both greenhouses had high total populations of bacteria. Although crop production in the two commercial greenhouses are managed similarly, they are geographically separated, and have different sources of irrigation water, they shared similar phyla in the water that is being recycled during

crop production.

Our hypothesis was that naturally occurring heterotrophic bacteria would be common in water and would be present even under different greenhouses throughout the year. Further, we suspected that there would be bacteria present that could suppress disease development under greenhouse conditions. Most of the genera identified were isolated on both media. It is known that some genera can switch from copiotrophic to oligotrophs growth depending on the growing conditions [25]. Microbial growth in nature is often characterized as “nutrient limited” [41] which explains the mechanisms of some microorganisms to switch depending on nutrient availability in the environment.

Disease suppression of *Pythium* by bacteria has been associated with microbes [3] and management practices influence biological processes in agricultural ecosystems. Multiple microbial interactions provide enhanced biocontrol in many cases in comparison with biocontrol agents used singly [50]. Although RIW contained high numbers of heterotrophic bacteria (up to 10^9 cfu/ml) only a small group of microorganisms were identified as having an *in vitro* effect on the growth of any of the three *Pythium* species. Previous studies on the mechanisms of plant-associated microbes that suppress infection of plants come from studies of the *Gammaproteobacteria* and *Firmicutes* [6]. In this study, most of the heterotrophic bacteria isolated with antagonistic properties were identified as *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. The most abundant genus was *Pseudomonas* spp. Most of the bacteria genera isolated in this study had previously been identified as containing biocontrol agents (*Microbacterium*, *Bacillus*, *Sphingobacterium*, *Chryseobacterium*, *Enterobacter*, *Sphingobium*, and *Acidovorax*) [1, 4, 8, 16, 18, 32, 40, 42]. The relatedness of the isolates in the phylogenetic tree showed that *Pseudomonas* isolates were highly diverse and isolated from both greenhouses. Four taxa represented the bacterial isolates with inhibitory properties against *Pythium* growth (*Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*). Most groups were previously identified as having biocontrol agents against *Pythium* [9, 17, 29]. Some bacterial isolates were able to attach to *Pythium* hyphae and

slowed their growth *in vitro*. Most of those isolates were identified as *Proteobacteria* mainly with affinity toward *P. aphanidermatum*. Other studies found that Enterobacteriaceae attached to surfaces, including hyphae [27]. A common feature of *E. cloacae*-*P. ultimum* *in vitro* was the ability of the bacterium to attach to hyphae [32]. This mechanism was associated with hyphal growth inhibition. We observed the same association *in vitro* as *Pythium* grew slower in the presence of attached bacterial isolates. There are no previous reports of whether such attaching observed *in vitro* has an effect on disease development under greenhouse or field conditions. The phylogenetic tree results showed that *Pseudomonas* isolates were highly diverse. All *Pseudomonas* isolates grouped together regardless of which greenhouse they were recovered. This agrees with previous results reporting that *Pseudomonas* species are widespread in the environment [47], including aquatic ecosystems.

A surprising finding was the identification of bacterial isolates that stimulate the growth of the three *Pythium*. It is not known to what extent bacterial isolates enhance the survival of *P. aphanidermatum*, *P. cryptoirregulare* and *P. irregulare* in recycled irrigation water reservoirs. One of the bacterial isolates was identified as *Cupriavidus* spp. This genus had been isolated from heavy metal rich soils [20]. Other genera were *Bosea*, *Herminiimonas*, *Sphingobacterium*, and Actinobacteria. The genera *Mycobacterium* and *Bosea* have been recovered from Antarctic lakes [35] and *Herminiimonas* from water containing arsenic contaminated sludge and lichen colonized rocks [24, 31]. *Sphingobacterium* species have been studied for their ability to degrade lignin [45]; Actinobacteria have been studied as a root-associated bacteria [12] from agricultural crops while *Mycobacterium* has been isolated from soil and water [33]. Most of the genera seemed to have in common the ability to survive in extreme ecosystems. In the present research, *Bacillus* was identified for its ability to enhance the growth of *P. aphanidermatum* and inhibit growth of *P. irregulare* and *P. cryptoirregulare*. Previous study found that the movement of *Bacillus subtilis* along killed hyphae of *Pythium ultimum* was restricted in soil [51], whereas the movement of the bacterium along the hyphae on agar depended in a combination of live bacterium-fungus chosen [26]. It is possible that the outcome of the interaction depends on the

need of the bacterium for hyphae in nature. *Pythium* may provide the bacterium with a physical surface on which to disperse to other parts of the location as suggested for filamentous fungi [21]. The migration of the bacteria to the hyphae *in vitro* and its colonization are thought to be mediated by chemotactic substances due to the similar effect on hyphae growth by zarilamide [10]. This may explain why some bacteria isolated in this study enhanced the growth of *P. aphanidermatum*, *P. cryptoirregulare* or *P. irregulare*. Although it is not clear what mediates the attraction and attachment interaction, these interactions deserve attention because it is possible that such interactions determine whether a biocontrol agent is effective or ineffective in the field. Interestingly, the phylogenetic composition of the enhancing bacterial isolates (Fig. 3-6) differs markedly from the inhibiting and attaching bacterial isolates group. The bacterial diversity of the enhancing bacterial isolate group was high compared to the bacterial inhibiting and attaching isolates identified in the study (Fig. 3-4 and 3-5).

In our greenhouse experiment none of the bacterial isolates enhanced or decreased *Pythium aphanidermatum*-caused disease in geranium under greenhouse conditions. When the pathogen was placed in the potting mix, symptom development on plants and disease progress in the ebb and flow units was significantly slower as compared to that in units where *Pythium* was added to the water reservoirs. The most severe symptoms were observed in the experimental units inoculated with any of the three bacterial isolates and *Pythium* directly in the water reservoir. This suggests that the bacteria-*Pythium* interaction is complex and cannot be predicted by *in vitro* tests. There was a tendency for *Pseudomonas* sp. (Bac. 2) to increase disease incidence when compared to the units without the bacterium. It is possible that the bacteria influence *Pythium* growth to their advantage and as a survival mechanism. This is an important area of research because it raises questions about the outcome of the introduction of bacteria into new environments.

In the present research, three bacteria were selected from a complex, naturally occurring microbial community that had been found in recycled irrigation water from commercial greenhouses and then introduced into what was probably a much simpler, less microbially-diverse environment in an experimental greenhouse. The *in vitro*

effects of the individual bacteria on *Pythium* did not coincide with their effects under greenhouse conditions. Further investigations are needed to determine whether a more complex microbial community would have different effects on *Pythium* and on disease development.

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CHAPTER 4

Shift of Bacterial Community in Recycled Irrigation Water

Abstract

Bacterial community composition in recycled irrigation water samples from two commercial greenhouses amended with *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* mycelia were monitored using automated ribosomal intergenic spacer analysis (ARISA). Comparison of ARISA profiles showed differences in bacterial communities over time (day 0, day 2, and day 7) and between non-amended and amended water samples. Two hundred twenty bacterial operational taxonomic units (OTU's) were identified by ARISA. OTU number was higher in water samples from greenhouse S, than in water samples from greenhouse E. OTU number changed over time in samples amended with the different *Pythium* species mycelium when compared to non-amended samples. ARISA results suggest that the composition of the bacterial communities in the two greenhouses differed, although there was overlap between water samples. Nonmetric multidimensional scaling (NMDS) ordination analysis clustered samples according to greenhouse, *Pythium* species added to water, and time. The capacity of the three *Pythium* species to influence bacterial populations was assessed using quantitative PCR. Quantification of the 16S rRNA targeting γ -Proteobacteria indicated that time and *Pythium* had significant effects on the amount of that gene detected ($p < 0.0001$) in recycled irrigation water samples. Our results suggest that the presence of *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* mycelium in irrigation water influences the composition of the bacterial community in water.

Introduction

Our knowledge of microbial communities in different ecosystems has increased substantially with the use of culture-independent, genetic fingerprinting techniques [2, 14, 23]. To date, this methodology has not been used to examine the microbial communities of irrigation water that is being reused during the production of plants in

greenhouses. Automated ribosomal intergenic spacer analyses (ARISA) [19] have been used to study terrestrial and aquatic bacterial populations as well as spatial and temporal dynamics of bacterial communities [15, 34, 42] elsewhere. ARISA was used to analyze the size and fluorescence intensity of labeled DNA fragments. DNA fragments of different sizes and its relative peak areas are commonly classified as OTU, although it is known that PCR-fingerprinting methods do not yield a true original DNA ratio because of nonspecific amplification [40]. However, it is generally accepted that variation in relative fragment peak area should not be affected by PCR bias because they apply to all samples [40]. Despite the lack of information on OTU identity, ARISA provides robust insight into bacterial community dynamics at different spatial and temporal scales [41]. Nevertheless, OTU can still be used to screen samples for OTU presence and a previous study used OTU to quantify bacterial diversity in terms of richness: the number of detectably different 16S-23S rRNA spacer sequence lengths or OTUs, in a standardized sample [21]. This method allowed them to detect patterns of diversity [21]. ARISA profiles does not provide specific identification of individual OTU. Therefore we used quantitative PCR to estimate in which sample γ -Proteobacteria was more abundant. The increased of 16S rRNA gene specific to γ -Proteobacteria served to determine how this class respond to the presence of *Pythium* species. Many species within these taxa (such as *Pseudomonas* spp.) are known to be suppressive to *Pythium* diseases [9, 37, 45]. In addition, gene abundance can be used to study the distribution of phylogenetically distinct bacteria in natural environments. This is of primary importance to an understanding of ecological dynamics [16].

Aquatic bacterial communities are influenced by multiple ecological factors [28]. Food availability and resources are known to influence bacterial abundance and activity [18, 27, 31, 38]. Recycled irrigation water reservoirs contain bacterial communities (see chapter 2). Bacteria have the potential to impact higher trophic levels [3] including the survival of *Pythium* species, especially with their role of nutrient decomposition. From a different view point, we have little information on the dynamics of bacterial communities residing in recycled irrigation water reservoirs as influenced by the presence of *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* in this environment. A previous study demonstrated that a shift of bacteria composition

could be related to the presence of *P. ultimum* [24]. Because individual bacterial taxa may differ in their response to shift in resource availability, the interactions between *Pythium* and the bacteria community in recycled irrigation water should be examined. Of particular interest is the shift of γ -Proteobacteria in the aquatic system. Several species of *Pseudomonas*, members of the γ -Proteobacteria, are known biocontrol agents with deleterious effects against *Pythium* spp. and have been employed to suppress *Pythium* root rot in hydroponic systems [33].

This study aims to show the changes in the bacterial population occur in response to the presence of *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* in recycled irrigation water over time (day 0, day 2, and day 7). The bacterial community was assessed using the culture-independent fingerprinting technique Automated Ribosomal Intergenic Spacer Analysis (ARISA). In addition, we used quantitative PCR (q-PCR) to provide an indication of γ -Proteobacteria increase when *Pythium* mycelium was added to the water. We used absolute quantification to determine the abundance of target DNA sequences to monitor changes in the quantity of γ -Proteobacteria in recycled irrigation water not amended and amended with *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* mycelia. A standard curve based on serial dilutions of template DNA against C_t was used to determine DNA.

Materials and Methods

Pythium inoculum

Pythium aphanidermatum, *P. irregulare*, and *P. cryptoirregulare* were grown on potato dextrose agar (PDA) plates. After 48 hours actively growing mycelia from the edge of the plate was transferred to 20% V8 juice broth in petri plates and incubated at 21°C for 2 days. *Pythium* mycelia was cut with a 5 mm diameter cork borer and used as amendment into recycled irrigation water samples (RIW).

Water samples

Recycling irrigation water (RIW) samples were collected from two commercial greenhouses in Pennsylvania (greenhouse S, 40°49'31.30"N, 76°48'18.17"W; and 76,000 L tank in greenhouse E, 40°13'21.56"N, 76°16'13.60"W). Water was collected in sterile, 1 L glass jars from cement tanks and transported to the laboratory. For each sample, 100 ml of RIW was transferred into a 125 ml Erlenmeyer flask. *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare* mycelium was added to a flask. Flasks to be sampled on day 0, day 2, and day 7 were prepared individually. In addition, flasks of RIW not amended with *Pythium* mycelia were prepared. All flasks were incubated at 21°C on a shaker until processed. The samples were processed by filtering the water through a 0.2 µm pore size membrane filter. Care was taken to discard *Pythium* mycelia and minimized carry over into the filter. Filters were stored in cryovial at -80°C until analyzed. RIW samples were processed on day 0 (< 8 hours after incubation), day 2 (48 hours after incubation), and day 7 after incubation.

Isolation of bacterial DNA and quantification

Genomic DNA extraction from the bacteria on the membrane filters was done using RapidWater DNA Isolation kits (MoBio Laboratories Inc., CA USA) following the manufacturer's instructions. Extracted DNA was quantified using Nano Drop spectrophotometer (Thermo Scientific), DNA dilutions were prepared to obtain 10 ng µl⁻¹ and these were stored at -80°C until further use.

PCR amplification of the ITS region

PCR reactions were conducted in triplicate to amplify the intergenic region between the small (16S) and large subunit (23S) rRNA genes of the rRNA operon characterized by significant variability in length and nucleotide sequence among bacterial genotypes [15]. PCR amplification was carried out using universal primer ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and eubacterial primer ITSReub (5'-GCCAAGGCATCCACC-3') [7] with modifications. The reverse primer was labeled with the fluorochrome VIC dye (Applied Biosystems, Foster City, CA). These primers

amplified the intergenic space between the 16S and 23S ribosomal subunit from position 1423 on the 16S ribosomal RNA (rRNA) subunit to position 38 on the 23S rRNA [22]. PCR reactions were performed in a 25 μ l volume containing 5 μ l of GoTaq Green (5X) (Promega), 0.5 μ l GoTaq DNA polymerase (1.25U), 1 μ l of dNTP mix (10 μ M), 2 μ l of forward and labeled reverse primers (10 μ M), 2 μ l of DNA template (10 ng μ l⁻¹), and 12.5 μ l of sterile water. PCR cycling was carried out in a PTC-100TM or PTC-200TM Programmable Thermal Controller (MJ Research) as follow: 95°C for 3 min, and 25 cycles of 95°C for 30 s followed by, 56°C for 1 min, 72°C for 1.5 min, and an extension step of 72°C for 5 min [22]. All PCR products were visualized by electrophoresis in 1.5% agarose gel. After amplification, PCR products were purified using DNA Clean & Concentrator-TM-25 (Zymo Research, Irvine, California) and stored at -80°C until further use.

Automated ribosomal intergenic spacer analysis (ARISA)

Two microliters of PCR product (10 ng μ l⁻¹) were placed in each well of a 96-well plate mixed with 0.4 μ l of an internal size standard (G 1200) and 12.1 μ l HiDi formamide (Applied Biosystems, Foster City, CA) and analyzed by automated capillary electrophoresis [7] on the ABI Hitachi 3730XL DNA analyzer (Applied Biosystems, Foster City, CA) at the Pennsylvania State University Genomic Core Facility, University Park, PA. ARISA profiles were analyzed using GeneMarkerTM software (SoftGenetics, State College, PA). Peaks above a threshold of 50 fluorescence units and between 150-900 bp in length were taken into consideration to exclude background fluorescence. The number of peaks outside this range was negligible in all samples. Peaks represented fragments of different sizes, and peaks areas represented the relative proportion of the fragments [10]. Each DNA fragment within a sample was designated an OTU and used for diversity indices. Diversity indices are mathematical measures of species in a community used to understand community structures. Richness (S) represents the number of species, or OTU for ARISA, present in the sample. Shannon-Wiener index (H) was used to measured species diversity in ARISA community. Richness does not take into account abundance of specie while diversity does. Evenness (E) is used to study how close in numbers each species are in the

environment [26]. All diversity indices were calculated according to Legendre et al. 2001 [32] using BiodiversityR [29]. In addition, only peaks present in two of three replicates were included in the analyses. GeneMarker output files were reformatted using R binning scripts [40]. To account for size calling imprecision, samples were binned with automatic and interactive binner as previously described [40]. Although it is well known that a single bacterial species may produce more than one peak, peak numbers in ARISA profiles have been used as an indicator of bacterial diversity in a complex community [19].

Genus-specific quantitative PCR (q-PCR)

To investigate the effect that the presence of *P. aphanidermatum*, *P. cryptoirregulare*, or *P. irregulare* mycelia had on the γ -Proteobacteria population in water, qPCR was performed with genus-specific primers according to the protocol of Bacchetti with some modifications [16]. The γ -Proteobacteria 16S rRNA gene was quantified using q-PCR. Non-amended and amended water samples, after 0, 2, and 7 days of incubation were used to characterize the changes in the amount of gene detected over time. All qPCR reactions were performed using 12.5 μ l of 2x Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5 μ l each of primers (10mM), and 2.5 μ l of DNA template. Absolute quantification analysis was used in this study. Product specificity was confirmed by using non-target DNA template in all q-PCR reactions and standard samples with known DNA amounts. Standards were made from 10-fold dilutions containing a bacterium isolate recovered from recycled irrigation water samples identified as *Pseudomonas* sp. All samples were run in triplicate. Standard curves were prepared from serial dilution of the known DNA quantity from *Pseudomonas* sp. At the end of the PCR reaction, dissociation curves were run to ensure that the targeted gene was the only gene amplified and that the primer-dimers were absent. q-PCR provides useful information to determine the abundance of the target DNA sequences in a given environment. Absolute transcript copy numbers for the gene was calculated with the ABI 7300 system SDS software version (Applied Biosystems, Foster City, CA). The correlation coefficient (R^2) for the assay was approximately 0.98 (\pm 0.03).

Data analysis

ARISA profile peaks area was transformed prior to applying multivariate tests. A similarity matrix between samples was calculated using Bray-Curtis distance matrix [5] and used to performed multivariate analyses. Analysis of similarity (ANOSIM) was used to test for significant community differences composition among *Pythium*-amended and non-amended samples. *R*- statistic ranging from -1 to 1 generated by ANOSIM indicates the degree of separation between groups of samples [12]. A score of 1 indicates complete separation between groups, a score of 0 indicates no separation, and negative values are interpreted as higher dissimilarity within samples than between samples [8]. Diversity indices including richness (S), diversity (H), and evenness (E) were calculated using BiodiversityR [29]. A nonmetric multidimensional (NMDS) ordination scale was used to visualize the similarity between samples in a two-dimensional plot [13]. Repeated measures ANOVA was used to test the impact that time and *Pythium* species presence had on the amount of γ -Proteobacteria in the recycled irrigation water.

Results

ARISA fingerprinting

From a pool of 220 different operational taxonomic units (OTUs) occurring in the entire data set (90 samples), between 2 and 130 OTUs were obtained per water sample. Operational taxonomic unit (OTU) numbers, or richness, was higher in greenhouse S water samples than those from greenhouse E (Fig. 4-1). OTU's number for samples from greenhouse S was similar (table 4-1) while OTU richness was different for water amended with *P. irregulare* (PI = 100 ± 40) and *P. aphanidermatum* (PA = 94 ± 37) as compared to non-amended water samples (NP = 44 ± 9.8). The sample with the lowest OTU (Fig. 4-2) was the non-amended (NP) sample from greenhouse S while the lowest OTU in greenhouse E (Fig. 4-3) were samples amended with *P. cryptoirregulare* (PC) and NP. In contrast, the highest OTU from greenhouse S was that amended with *P. irregulare* (PI) and in greenhouse E, that

amended with *P. aphanidermatum* (PA). OTU richness differed with it being greater in greenhouse S than in E (table 4-1). Shannon's index used to measure species diversity was highest in PA on greenhouse S and PI on greenhouse E. In addition, evenness, used to study how close in numbers each species are in each sample, was highest in PA on both greenhouses.

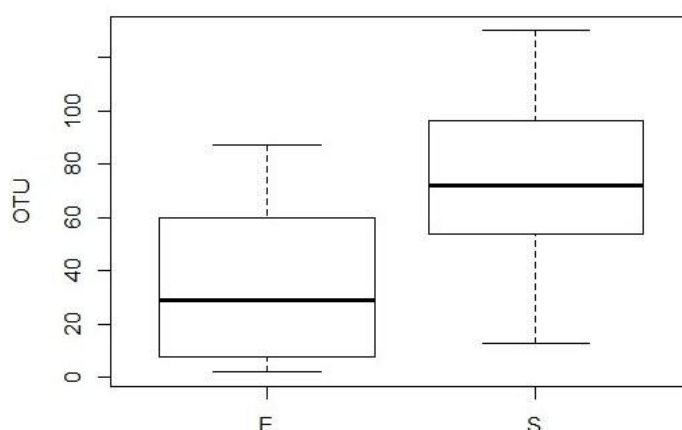


Figure 4-1: Operational taxonomic unit (OTU) numbers in commercial greenhouse S and E recycled irrigation water as measured using automate ribosomal intergenic spacer analysis per greenhouse. Thick bar in the boxes represents the sample median and the end whiskers represent the minimum and maximum value of all the data.

OTU partitioning among the days sampled showed that, from a total of 220 different OTUs detected in ARISA profiles from both greenhouses, only 30 OTU's were exclusively present in greenhouse S. Water samples from greenhouse S amended with *Pythium aphanidermatum* (PA), *P. irregulare* (PI), and *P. cryptoirregulare* (PC) did not contained unique OTU's while non-amended (NP) had 3 unique OTU's. For greenhouse E, NP had 11 unique OTU's, PI had 5, and PA had 2 unique OTU's. In addition, 18 OTU's were observed in amended samples (PA, PI, and PC) but not in NP samples. Overall, OTU's richness profiles, or number of OTU per sample, changed in *Pythium*-amended samples and changes were *Pythium*-specific (Fig. 4-2). Day 2 had the highest amount of OTU's when compared to day 0 and day 7. Peak intensity (Fig. 4-2, and 4-3; represented by amplitude) was variable among greenhouses. It was highest in PC and day 0 on greenhouse S and in PI and day 7 on greenhouse E.

Table 4-1: Diversity indices for greenhouse S and greenhouse E. Diversity of operational taxonomic units (OTU's) from the data of ARISA profile analysis

Sites ^a	S ^b	H ^c	E ^d
NP	154 ± 4.8	3.99 ± 0.12	0.36 ± 0.05
PA	156 ± 25.1	4.10 ± 0.33	0.39± 0.02
PC	150 ± 29.0	3.95 ± 0.63	0.35 ± 0.12
PI	133 ± 41.3	3.58 ± 1.23	0.27 ± 0.22
NP	44 ± 9.8	2.63 ± 0.58	0.36 ± 0.05
PA	94 ± 37.0	3.29 ± 0.88	0.39± 0.02
PC	71 ± 37.0	3.10 ± 0.69	0.35 ± 0.12
PI	100 ± 40.0	3.45 ± 0.73	0.27 ± 0.22

^a NP = No *Pythium* added to water samples; PA = *P. aphanidermatum* added to water samples; PC = *P. cryptoirregulare* added to water samples; PI = *P. irregulare* added to water samples

^b S, richness = number of species (OTU's) in ARISA samples. Results were calculated using operational taxonomic units (OTU's) of all water samples from greenhouse S.

^c H, diversity = $\sum (P_i \cdot \ln(P_i))$ = Shannon's diversity index

^d E = evenness = $H/\ln S$

Diversity index were calculated using BiodiversityR package with R program [29].

Community similarity

OTU number and peak intensity (amplitude) of each sample were used to calculate Bray Curtis index matrix. The matrix was used to study similarities and differences in the bacterial community in *Pythium*-amendment and non-amendment water samples using ANOSIM. ARISA profiles of samples from greenhouse S after 0 and 7 days of incubation differed although they overlapped in the bacterial structure (ANOSIM, $R=0.39$ $p=0.001$) while ARISA profiles from greenhouse E after 0 and 2 days of incubation exhibited greater differences (ANOSIM, $R=0.54$ $p=0.01$). Bacterial community changed in *Pythium*-amended samples and changes were distinct among greenhouse samples.

Nonmetric multidimensional plots

NMDS was used to determine bacterial community composition in *Pythium* mycelium amended and non-amended cultures at different times. MDS are interpreted using the distance between ordination points; when points are in close proximity, they

are considered to have similar community compositions [14]. ARISA fingerprint results were clustered using the XLStat (AddinSoft SARL) program with Bray Curtis for ARISA community profiles. All three plots had a stress values less than 0.2, meaning that the plots provide a reliable representation of the data [11, 14]. Stress values indicate the goodness-of-fit of the 2-dimensional representation compared to the original multi-dimensional matrix.

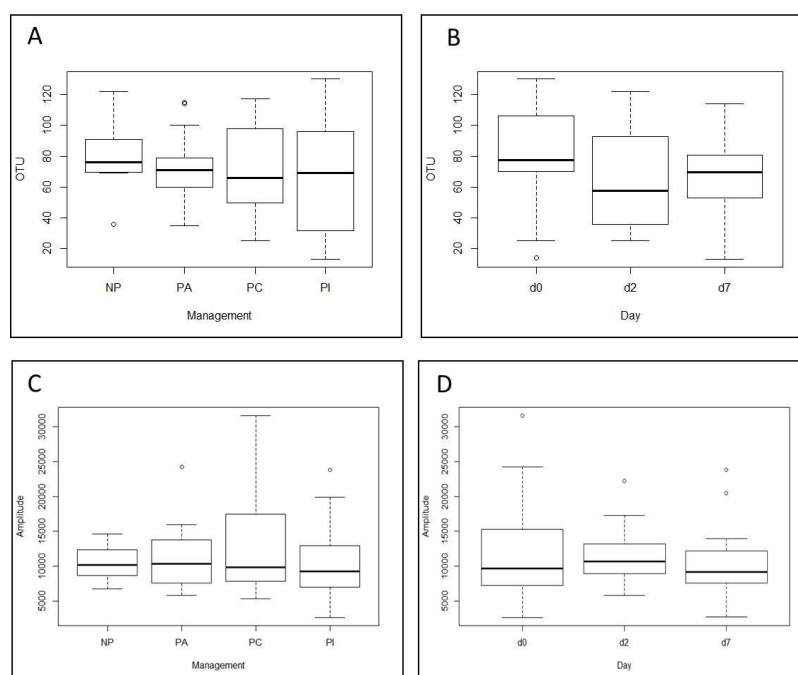


Figure 4-2: Analyses of bacterial communities in recycled irrigation water from greenhouse S. Results were calculated using water samples from greenhouse S with operational taxonomic unit (OTU) and total peak intensity or area of the peaks (Amplitude) of the different OTU's in ARISA profiles. A) OTU numbers for managements (treatments) NP (No *Pythium*), PA (*P. aphanidermatum*), PC (*P. cryptoirregulare*), and PI (*P. irregulare*) B) OTU numbers for samples that were incubated 0 (d0), 2 (d2), and 7 (d7) days. C) Amplitude represents total peak intensity for all OTU's in samples NP, PA, PC, and PI D) Results of the amplitude quantities for each time sample. Thick bar in the boxes represents the sample median, the end whiskers represent the minimum and maximum value of all the data and circles represent outliers.

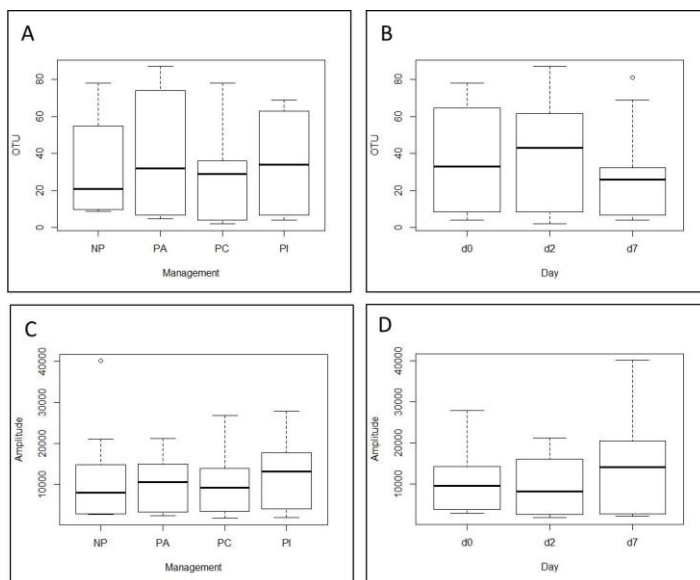


Figure 4-3: Results were calculated using water samples from greenhouse E with operational taxonomic unit (OTU) and total peak intensity or area of the peaks (Amplitude) of the different OTU's in ARISA profiles. A) OTU numbers for managements NP (No *Pythium*), PA (*P. aphanidermatum*), PC (*P. cryptoirregulare*), and PI (*P. irregulare*) B) OTU numbers for time samples by day 0 (d0), day 2 (d2), and day 7 (d7) C) Amplitude represents total peak intensity for all OTU's in samples NP, PA, PC, and PI D) Results of the amplitude quantities for each time sample. Thick bar in the boxes represents the sample median, the end whiskers represent the minimum and maximum value of all the data and circles represent outliers.

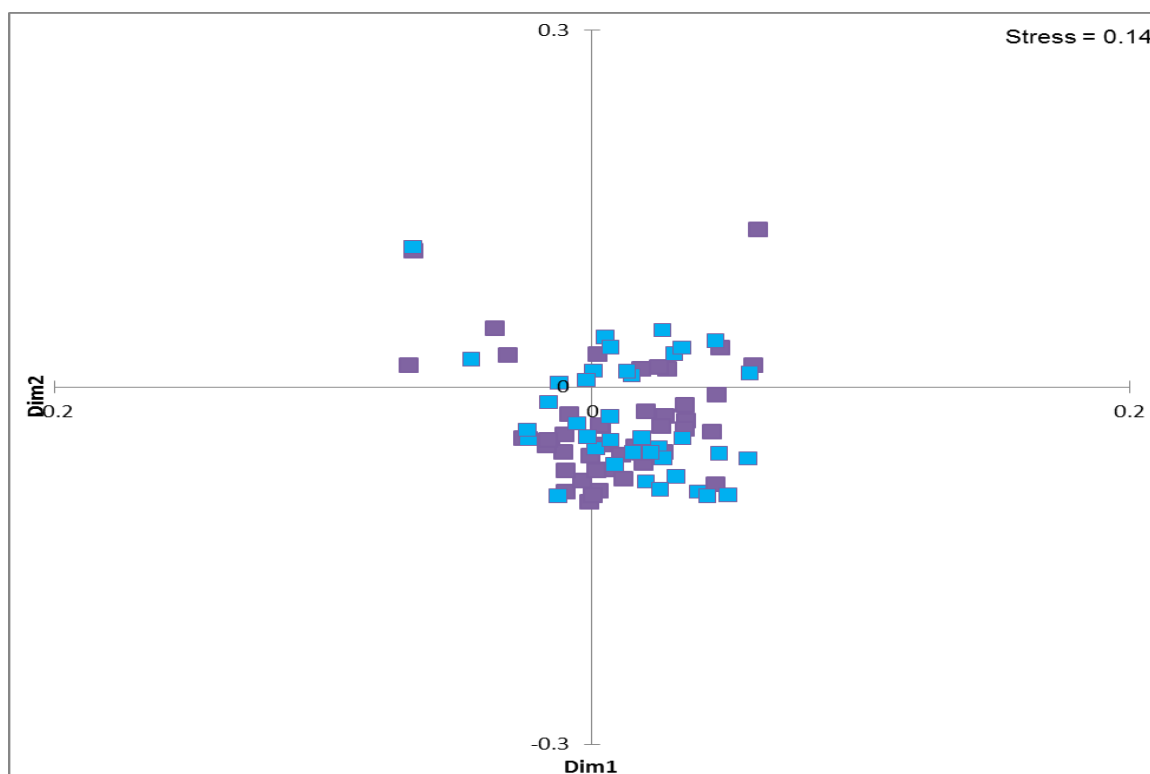


Figure 4-4: Nonmetric multidimensional scaling (NMDS) plot was constructed using all ARISA profiles from greenhouse S and E samples and analyzed in XLStat (AddinSoft SARL) using total area of peaks and total amount of OTU. Similarity in bacterial communities was determined using Bray-Curtis similarity index. Purple squares are for greenhouse S water samples and blue squares are for greenhouse E water samples. Distance between squares represents dissimilarity in bacterial community, samples that have similar species composition plot close together in the graph. Stress value represents the goodness-of-fit of the 2-dimensional plot. Stress value < 0.15 represents a good and reliable representation of the data [11].

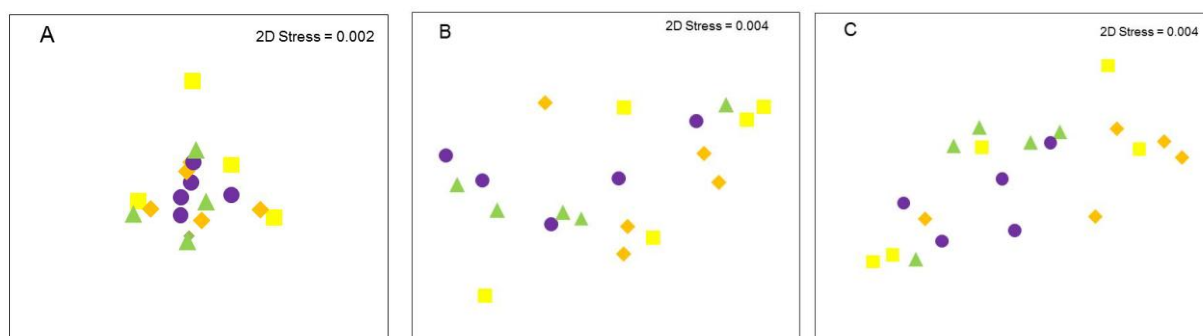


Figure 4-5: Nonmetric multidimensional scaling (NMDS) plot was constructed using all ARISA profiles from greenhouse S for time samples day 0, 2, 7 and analyzed in XLStat (AddinSoft SARL) using total area of peaks and total number of OTU. Graphs are as follow: A) Results for day 0; B) Results for day 2; and C) Results for day 7. Similarity in bacterial communities was determined using Bray-Curtis similarity index. Different symbols represent non-amended water samples (NP) = purple (●), amended with *P. aphanidermatum* (PA) = orange (◆), amended with *P. cryptoirregulare* (PC) = yellow (■), and amended with *P. irregulare* (PI) = olive green (▲). Distance between symbols represents dissimilarity in bacterial community, samples that have similar species composition plot close together in the graph. Stress value represents the goodness-of-fit of the 2-dimensional plot. Stress values < 0.004 are ideal results that provides a reliable representation of the data [11].

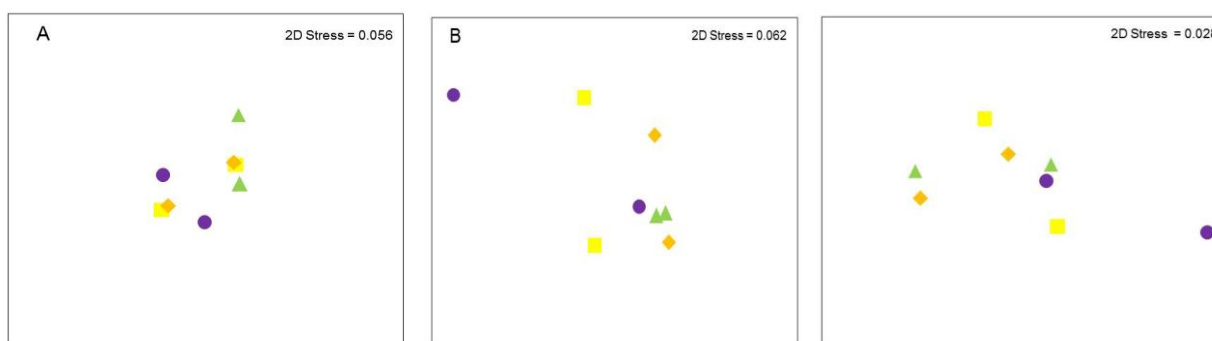


Figure 4-6: Nonmetric multidimensional scaling (NMDS) plot was constructed using all ARISA profiles from greenhouse E for time samples day 0, 2, 7 and analyzed in XLStat (AddinSoft SARL) using total area of peaks. Graphs are as follow: A) Results for day 0; B) Results for day 2, and C) Results for day 7. Similarity in bacterial communities was determined using Bray-Curtis similarity index. Different symbols represent non-amended water samples (NP) = purple (●), amended with *P. aphanidermatum* (PA) = orange (◆), amended with *P. cryptoirregulare* (PC) = yellow (■), and amended with *P. irregulare* (PI) = olive green (▲). Distance between symbols represents dissimilarity in bacterial community, samples that have similar species composition plot close together in the graph. Stress value represents the goodness-of-fit of the 2-dimensional plot Stress value < 0.06 is an ideal results that provides a reliable representation of the data [11].

Table 4-2: Analysis of similarity (ANOSIM) between bacterial communities in recycled irrigation water for ARISA profiles based on Bray-Curtis similarity matrix

Samples ^a	Greenhouse S ^b		Greenhouse E ^c	
	R-statistic	P-value	R-statistic	P-value
d0-d2	0.12	0.94	0.54	0.01
d0-d7	0.39	0.001	0.34	0.98
d2-d7	0.09	0.12	0.35	1

^a Samples were collected at d0 in less than 8 hours of incubation after amendment with *Pythium* mycelia, at d2 after 48 hours of incubation after amendment, and d7 after 7 days of incubation after amendment. An R-statistic of zero means there is no differences between groups. R values range from -1 to 1, with positive values indicating a difference between groups, and negative values indicating a greater difference within groups than between groups. The significance of the R-statistic was tested by randomization with 1000 permutations.

^b Results are for greenhouse S

^c Results are for greenhouse E

Quantitative PCR (q-PCR)

Over time, the addition of *Pythium* to recycled irrigation water samples had a significant effect ($p < 0.0001$) in the amount of 16S rRNA gene of γ -Proteobacteria present in water samples (d2 and d7) from both greenhouses S and E compared to the amount in non-amended samples (no *Pythium* mycelia added to the water). The amounts of 16S rRNA gene were significantly different ($p < 0.0001$) for sample from

both greenhouses after 2 days of incubation. The addition of mycelium of all three *Pythium* species resulted in an increase in the amount of γ -Proteobacteria population when compared to non-amended water samples, based on the 16S rRNA gene concentrations. *P. aphanidermatum* had the greatest impact on the increase of γ -Proteobacteria in water samples from greenhouse S (Fig. 4-6a) while *P. cryptoirregulare* had the greatest impact in water samples from greenhouse E (Fig. 4-6b).

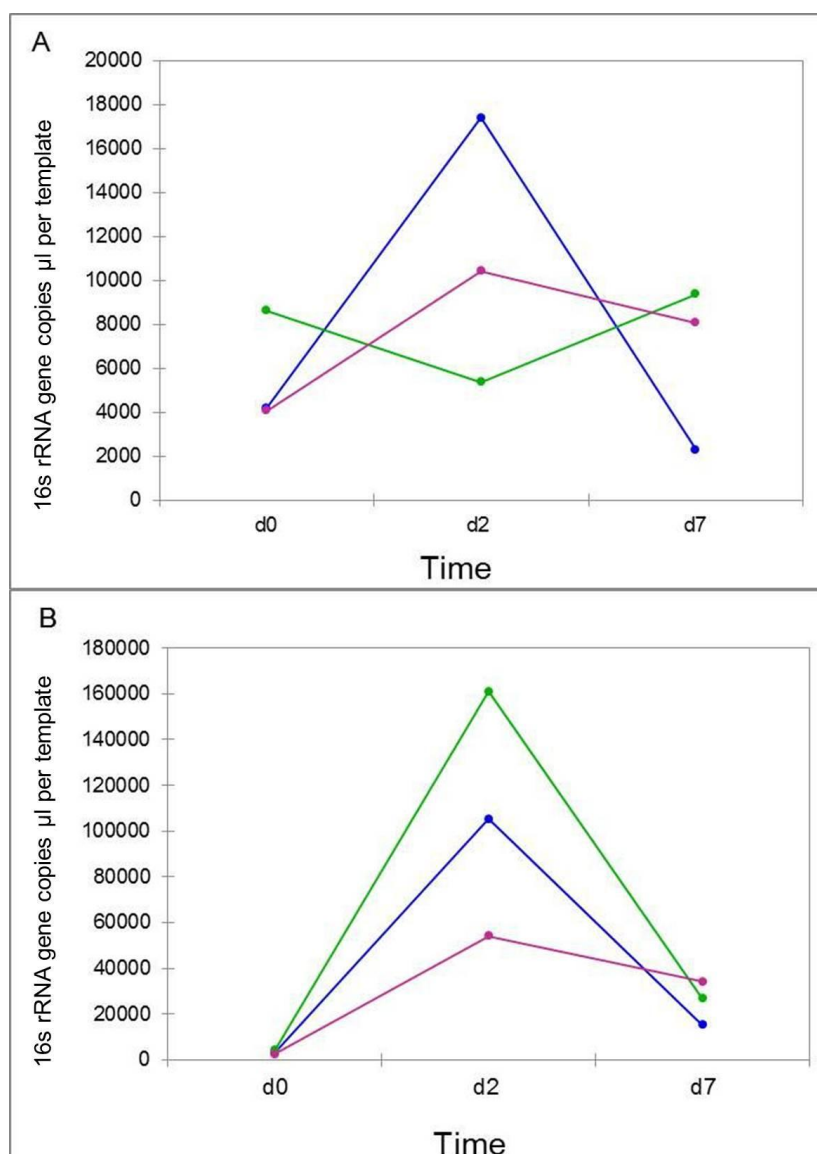


Figure 4-7: q-PCR results for the amount of 16S rRNA γ -Proteobacteria genes in water samples detected following 0 (d0), 2 (d2) and 7 (d7) days of incubation. Blue line = *P. aphanidermatum*, green line = *P. cryptoirregulare*, pink line = *P. irregulare*. A) Greenhouse S B) Greenhouse E. Graphs were constructed using repeated measure ANOVA using XLStat (AddinSoft SARL). Non-amended results (no *Pythium* mycelia added to water) were negligible and were not added to the graph (Greenhouse S, $p < 0.0001$ and greenhouse E, $p = 0.002$).

Discussion

ARISA has been used to characterize the diversity of bacterial communities under various conditions [1, 7, 19, 20, 22, 25]. The aim of the current study was to employ ARISA to examine bacterial community composition and temporal changes in those communities in recycled irrigation water in the presence of *P. aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare*. Our results did not reveal significant changes in richness overtime for amended samples compared to non-amended samples. The lack of changes in richness, based on OTU's numbers, in the amended water samples could be the result of a "replacement effect" whereby the diversity level is stable due to continuous displacement of bacteria taxa by other microbes [35]. In a previous study diversity values remained similar, even when communities underwent changes, showing microbial diversity may be a poor indicator of community fluctuations in an ecosystem [36]. ARISA profiles from greenhouse S (ANOSIM, $R=0.39$ $p=0.001$) and from greenhouse E (ANOSIM, $R=0.54$ $p=0.01$) changed over time following 0, 2, and 7 days of incubation when *Pythium* mycelium was present as compared to when *Pythium* was not present. In addition, ARISA profiles from greenhouse E following 0 and 2 days of incubation differed in their composition. ANOSIM applications in microbial ecology include testing for spatial and temporal changes in microbial assemblages [39]. These ARISA profiles are interpreted as indicating that there is more variation between samples than within samples. According to ANOSIM results we can conclude that bacterial communities differed between days and greenhouses when *Pythium* was added to water. Bacterial communities in water samples from greenhouse S that were amended with *Pythium* mycelium were statistically different on day 0 and day 7 while greenhouse E sample were statistically different on day 0 and day 2. We observed that greenhouse S had higher number of bacteria when we cultured water in NA and R2A media (Chapter 3). This may explained the results per day for both greenhouses. Differences may be due to bacterial population quantities in each greenhouse. Changes in ARISA peak intensity are correlated to the dynamics of the bacterial populations represented by specific peaks [6]. Intensity of ARISA peaks increased in *Pythium*-amended water samples suggesting that the presence of *Pythium* mycelia may have an impact on bacterial populations. This was confirmed with

q-PCR results where γ -Proteobacteria genes increased exponentially in amended samples when compared to non-amended water samples. The abundance of γ -Proteobacteria peaked in amended water samples analyzed after 2 days of incubation. We do not know the identity of the bacteria responsible for peaks with the highest intensity in the ARISA profile. Although bacterial community richness (OTU) differed between the two greenhouses, indications are that changes in the samples were similar to one another when samples were amended with *Pythium* mycelium. ARISA fingerprinting patterns of water bacterial communities non-amended, amended with *Pythium* mycelia were grouped separately by nonmetric multidimensional spacing suggesting that the presence of *Pythium* could determine the bacterial populations.

It is known that the suppression of *Pythium* can be mediated by microbial communities [30] and that γ -Proteobacteria, in particular, have been associated with this suppression [4, 17, 43, 44]. Results here indicate that γ -Proteobacteria populations increase when *P. aphanidermatum*, *P. cryptoirregulare*, or *P. irregulare* mycelium is present. This contrasts with the results of Hagn et al. 2008, that did not show an increase in numbers of this taxon [24] in the presence of *P. ultimum*. They used cloning to enumerate the taxa whereas q-PCR was used in the present study. Furthermore, *Pythium*-amended samples in q-PCR samples were statistically different from samples with no *Pythium*. This study represents the first survey of bacterial populations in recycled irrigation water associated with *P. aphanidermatum*, *P. cryptoirregulare*, and *P. irregulare*. Our findings reveal a bacterial specificity and variation associated to *Pythium* specie. In other words, each *Pythium* species seemed to play role structuring bacterial communities in recycled irrigation water.

Acknowledgments

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CHAPTER 5

Pythium species in recycled irrigation water reservoirs

Abstract

In the present study we surveyed water tanks from two commercial greenhouses to determine the identity of *Pythium* species present. *Pythium* isolates were recovered by baiting with blades of creeping bentgrass (*Agrostis stolonifera* L. 'Penn Eagle') and plating onto semi-selective media (NARF). Baits deployed for 7 days at the top and bottom of each tank were recovered for processing and fresh baits were deployed. Twenty seven such deployments were completed in one greenhouse and 34 in a second greenhouse in 2011. One hundred forty one isolates were examined microscopically and then, using the internal transcribed spacer (ITS) DNA sequences, compared to DNA sequences in GenBank. Four *Pythium* species were recovered and identified as *P. helicoides*, *P. catenulatum*, *P. chamaeohyphon*, and *P. middletonii*. Many of the isolates (50%) were closely related to more than one species based on sequences and could not be delineated microscopically. These isolates were categorized as Group 1 (DNA sequences closest to *P. dissotocum*, *P. diclinum*, *P. lutarium*, and *P. coloratum*), Group 2 (DNA sequences closest to *P. adhaerens*, *P. chondricola*, and *P. porphyrae*), Group 3 (DNA sequences closest to *P. flevoense*, *P. capillosum*, and *P. pectinolyticum*), Group 4 (DNA sequences closest to *P. aploverotum*, *P. aquatile*, *P. pachycaule*, *P. oopapillum* and *P. sukuense*), and Group 5 (DNA sequences closest to *P. rostratum* and *P. rostratifingens*). In addition, 27 isolates were not similar to any known species based on their DNA sequences and were different from one another. These were classified as *Pythium* spp. The results indicate that there were at least 10 distinct species present in recycled irrigation water tank reservoirs in two commercial greenhouses in Pennsylvania during the survey period.

Introduction

Pythium is worldwide distribution and contains species that are saprophytes as well as pathogens of plants, fungi, and mammals. The genus consists of approximately

120 species [7]. Identification based on morphological characteristics is challenging and requires extensive experience [18]. Morphological keys [4, 21] help to separate closely related species that cannot be differentiated with ITS sequences [11]. Recent surveys for emerging oomycetes pathogens have resulted in the discovery of several new species in natural ecosystems, demonstrating our limited understanding of *Pythium* taxonomy, distribution, and diversity [17]. When *Pythium* is suspected of causing disease they are usually only identified to genus [2]. It is usually assumed that any *Pythium* found is a pathogen and little is known about which species is causing losses or which are associated with particular crops [13]. This hinders our understanding of the diversity of species within different ecosystems as well as the dynamics of particular species across natural and agricultural sites. The identification of *Pythium* to species is important to clarify host ranges, geographical distributions, and efficacy of various management strategies [9] as well as to increased our understanding of the role that different environmental conditions play in the survival of the various species in this genus.

Current methods for the detection of *Pythium* include direct plating of soil, water, and plant tissue on semi-selective media, baiting [5, 8, 13] with susceptible plant material, and cultural-independent techniques [10, 17]. Comprehensive taxonomic phylogenetic analyses of the *Pythium* genus have been done by Lévesque and De Cook [11], Martin [12], and Villa [23]. *Pythium* species are easily isolated from water using baiting material. Several semi-selective media have been described for the isolation of *Pythium* species including NARF (containing nystatin, ampicillin, rifampicin, and fluazinam) [14]. A previous survey of greenhouses in Pennsylvania found that *P. aphanidermatum*, *P. irregulare*, *P. dissotocum*, *P. myriotylum*, *P. heterothallicum*, and *P. ultimum* were associated with infected plants [6]. Water samples from greenhouses were tested in preliminary work and *P. irregulare* and *P. sylvaticum* were recovered but other plant pathogenic species were not isolated from the water.

This study was conducted to identify *Pythium* species found in recycled irrigation water reservoir tanks in two commercial greenhouses in Pennsylvania in order to gain insight on plant pathogenic and non-pathogenic species which may be permanent

residents and which may be transient in recycled water irrigation tanks.

Material and Methods

Pythium isolation

In 2011, blades of creeping bentgrass (*Agrostis stolonifera* L. 'Penn Eagle') sandwiched between pieces of fiberglass screen (leaf traps) were submerged in cement block tanks of recycling irrigation water in two commercial greenhouses in Pennsylvania. The tanks were baited only when they were in active use for irrigating potted plants on flooded floors and benches in the greenhouses. Samples were obtained from 27 weeks (March-June and September-December, 2011) of baiting one tank (designated C) in greenhouse S (40°49'31.30"N, 76°48'18.17"W) and from 34 weeks (March-December, 2011) of baiting one to four tanks (designated L, R, SB, and G) in greenhouse E (40°13'21.56"N, 76°16'13.60"W). Well water at each site was used to initially fill and to maintain the proper water levels in the tanks. Coarse screens (greenhouse S) or coarse fabric filters (greenhouse E) removed most particulates from water returning the tanks after each irrigation. Two leaf traps were deployed per tank. One trap was attached to an anchor at the bottom (designated B) of the tank (> 3 meters) and the other was allowed to float at the surface (designated T) of the water tank reservoir (< 1 meter). After being deployed for 7 days, the leaf traps were sent, via overnight express, to the laboratory by the grower and fresh leaf blade-containing traps were deployed. Late in the year, apparently healthy, white, young roots from poinsettias (*Euphorbia pulcherrima*) were added to the traps to determine whether different species of *Pythium* would colonize them as compared to the leaf blades in the traps. Each sample was labeled with the greenhouse, date, tank, and trap location designation. For example, a sample from greenhouse S, tank C, on March 22, 2011, from the bottom was labeled S3.22.11CB while the sample from the trap floating on the top was S3.22.11.CT. Leaves were plated on NARF (clarified 20% V8 juice agar amended with nystatin, ampicillin, rifampicin, and fluazinam) [14] in 60 X 15 mm petri plates. Plates were incubated in the dark at 21°C. Mycelium was transferred into new

NARF plates to obtain a pure culture and to water agar (WA) plate for microscopic observations.

Morphological identification

Mycelium was transferred to a petri plate containing 5-10 mm segments of creeping bentgrass (*Agrostis stolonifera* L. 'Penn Eagle') or rye (*Secale cereale* L.) leaf blades that had been boiled in distilled water for 10 minutes and suspended in sterile 10% soil extract. Preliminary identification was based on the key of Van Der Plaats-Niterink and descriptions of species published after 1981.

DNA isolation and PCR for amplification of the ITS1, 5.8, and ITS2 rDNA

Isolates identified as *Pythium* based on microscopic examination were grown in NARF and used for direct PCR amplification. The surface of a colony was scratched twice (2-3 mm each time) using a disposable plastic tip on a 20 µl pipettor that was then titrating twice in the PCR reaction mixture [10]. PCR amplification of the ITS1, 5.8, and ITS2 was carried out using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATAGC-3') [15]. The PCR master mix used to carry out all PCR reactions contained 2 µl of (10x) PCR buffer standard, 0.5 µl of dNTP (10 mM), 1 µl of each ITS1 and ITS4 (5 mM), 0.1 µl of Taq polymerase, 14.4 µl of sterile distilled water, for a total of 20 µl. PCR reactions were carried out in a PTC-100TM or PTC-200TM Programmable Thermal Controller (MJ Research): 94° C min for 5 min; followed by 35 cycles of 94° C for 1 min., 55° C for 1 min., 72° C for 1 min., and a final extension of 72° C for 10 min. PCR products were visualized by electrophoresis in 1% agarose gels stained with Gel EZ-VisionTM Three, DNA dye Loading buffer (AMRESCO, Solon Ohio). The presence of bands in the agarose gel was detected using a UV transilluminator (256-366 nm) to confirmed DNA product amplification. PCR products were subjected to DNA sequencing to identify the isolates.

DNA sequencing

PCR products were purified using ExoAP (New England Biolabs) following the

manufacturer instructions. PCR products were adjusted to 20 ng/μl for the sequencing reaction. The primers used for the initial PCR reaction were used for DNA sequencing. Two microliters of each (1X) ITS1 and ITS4 primers and 2 μl of DNA template were transferred to a 96 well PCR plate (μlttraAmp™). Sequencing was conducted in an ABI Hitachi 3730XL DNA analyzer at the Nucleic Acid Facility, The Pennsylvania State University, University Park, PA. The DNA sequence obtained was edited and compared to those in the NCBI database.

Results

Pythium species identification

A. Greenhouse S

Fifty one *Pythium* isolates were obtained from water tank C. The isolates were categorized as Group 1, Group 2, *P. catenulatum*, *P. chamaehyphon*, *P. middletonii*, and *Pythium* spp. The most frequently isolated species were in Group 1. Isolates in Group 1 and 2 have filamentous non-inflated sporangia. A Group 2 isolate was recovered one time in May from a bottom leaf trap. *P. chamaehyphon* was isolated only from leaf traps deployed in this greenhouse with 5 out of 7 isolates from bottom leaf traps. *P. middletonii* was isolated from top and bottom leaf traps. Isolates identified as *Pythium* spp. were recovered throughout the season in both top and bottom leaf traps. There were 4 isolates and one was not identical to the other three isolates (figure 5-1, B). Based on ITS sequences, most Group 1 isolates, *P. chamaehyphon*, and *P. middletonii* grouped together (Fig. 5-1 and b).

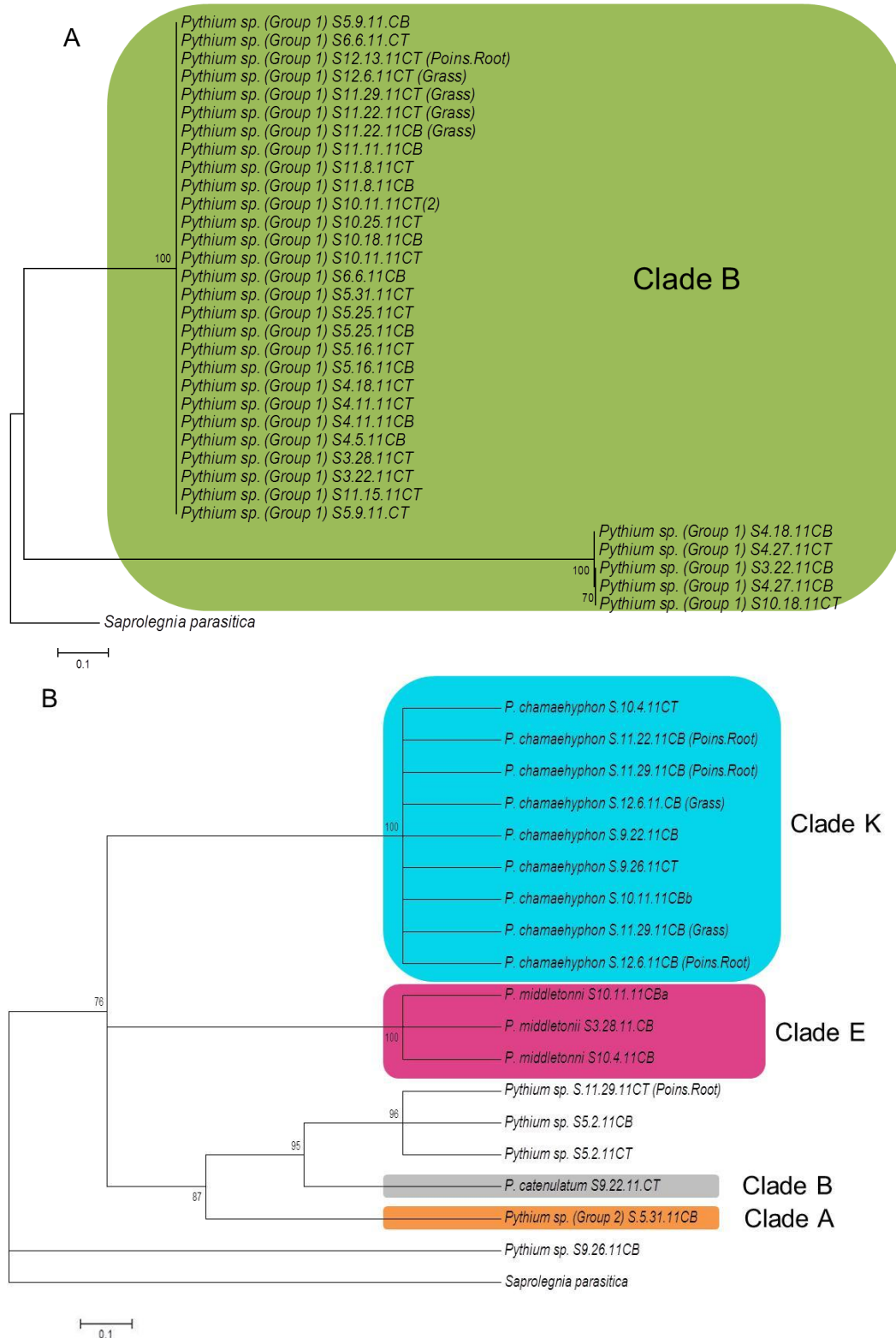


Figure 5-1: Phylogenetic tree constructed with ITS sequences. Maximum likelihood branch lengths are shown. Number on nodes represents bootstrap support value for maximum likelihood. Scale bar indicates number of substitutions per site. (0.1) A) Phylogenetic tree constructed with *Pythium* Group 1 isolates only and B) Phylogenetic tree constructed with all other *Pythium* isolates trapped from tank C reservoir from greenhouse S. *Saprolegnia parasitica* was used as outgroup species.

***Pythium* species identification**

B. Greenhouse E

Ninety isolates were obtained from four water tanks in greenhouse E. *Pythium* Group 1 isolates were the most abundant. Only water tank SB did not contain members of this group. A Group 2 isolate was recovered from tank L at the surface of the water. A Group 3 member was recovered only one time from tank L and it was recovered from a bottom leaf trap. Group 4 isolates were recovered only from tank SB from top and bottom leaf traps. Group 5 isolates were recovered only from tank SB from top and bottom leaf traps. *P. middletonii* was isolated only from greenhouse E, tanks L and G from top and bottom leaf traps. It was the most abundant species isolated from tank G (7 out of 12 isolates). *P. helicoides* was isolated in greenhouse E and was found in tanks G, R, and SB from top and bottom leaf traps. *Pythium* spp. were recovered in both top and bottom leaf traps from tanks L, R, and SB. There were 23 isolates whose ITS sequences were identical to one another (see figure 5-2 to 5-5). According to the relatedness of the ITS sequence, most of the isolates grouped together in Fig. 5-2, 5-3, 5-4, and 5-5. Isolates identified as *Pythium* spp. were highly diverse (Fig. 5-2 and Fig. 5-3).

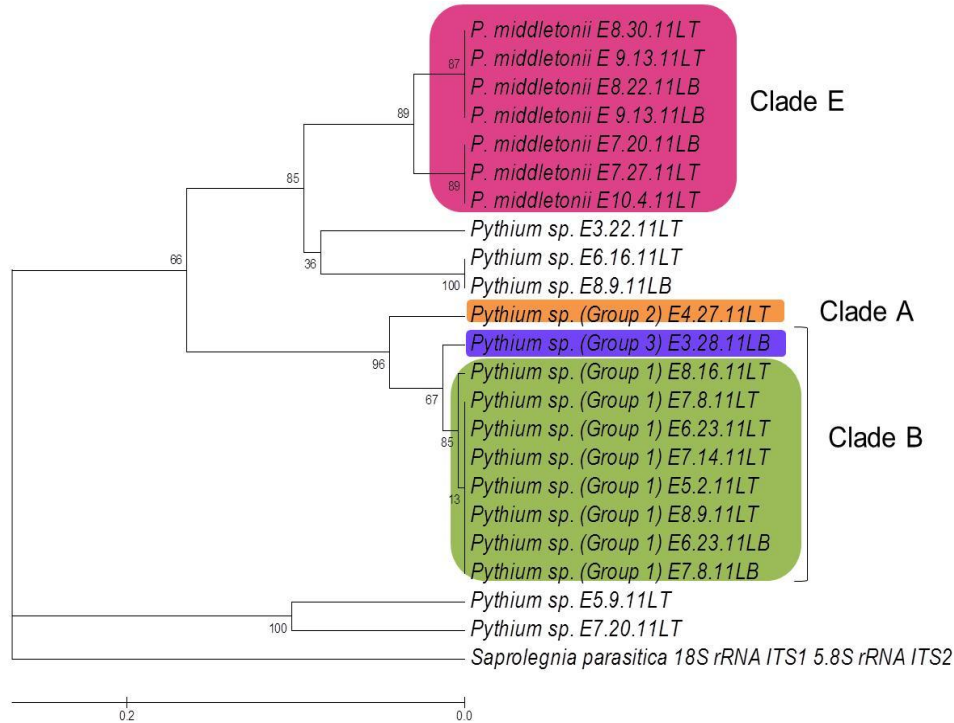


Figure 5-2: Greenhouse E tank L. Phylogenetic tree constructed with ITS sequences. Maximum likelihood branch lengths are shown. Number on nodes represents bootstrap support value for maximum likelihood. Scale bar indicates number of substitutions per site (0.2). Phylogenetic tree constructed with isolates from water tank L. *Saprolegnia parasitica* was used as outgroup species.

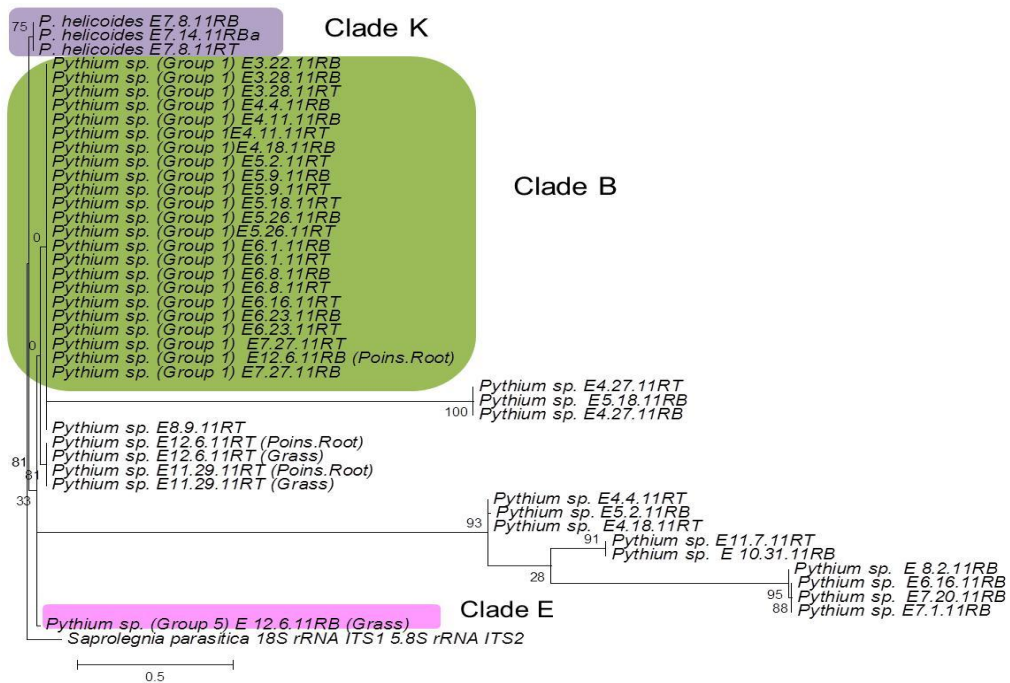


Figure 5-3: Greenhouse E tank R. Phylogenetic tree constructed with ITS sequences. Maximum likelihood branch lengths are shown. Number on nodes represents bootstrap support value for maximum likelihood. Scale bar indicates number of substitutions per site (0.5). Phylogenetic tree constructed with isolates from water tank R. *Saprolegnia parasitica* was used as outgroup species.

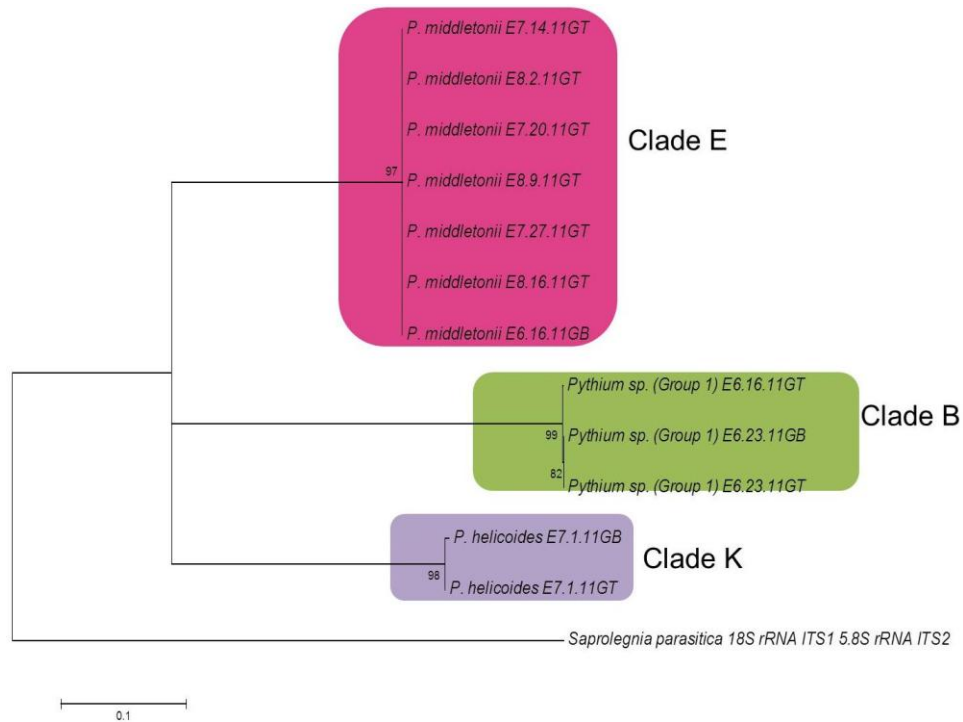


Figure 5-4: Greenhouse E tank G. Phylogenetic tree constructed with ITS sequences. Maximum likelihood branch lengths are shown. Number on nodes represents bootstrap support value for maximum likelihood. Scale bar indicates number of substitutions per site (0.1). Phylogenetic tree constructed with isolates from water tank G. *Saprolegnia parasitica* was used as outgroup species.

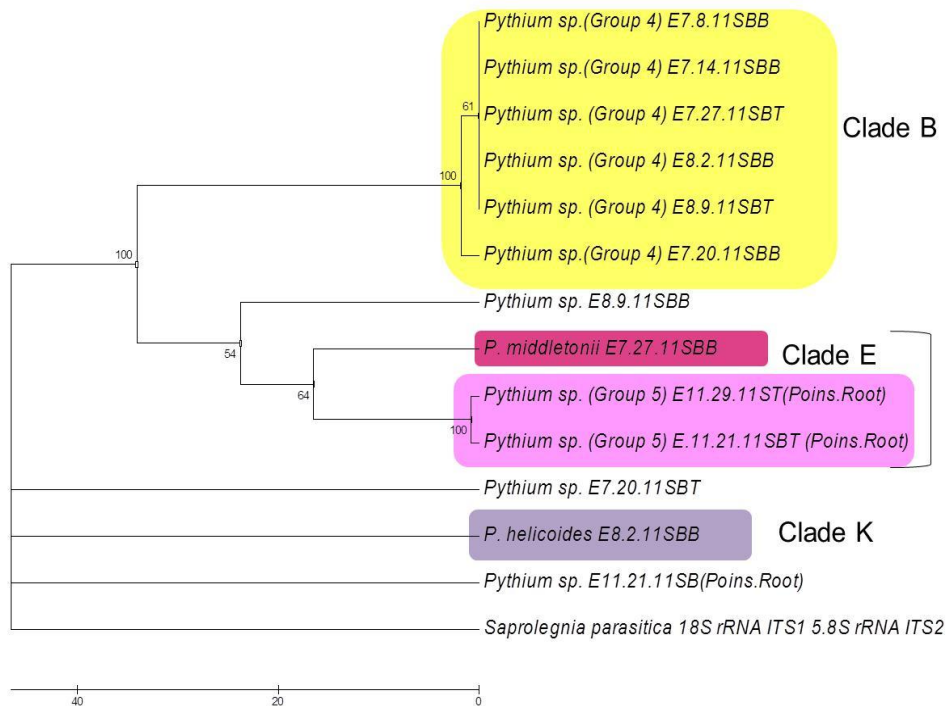


Figure 5-5: Greenhouse E tank SB. Phylogenetic tree constructed with ITS sequences. Maximum likelihood branch lengths are shown. Number on nodes represents bootstrap support value for maximum likelihood. Scale bar indicates number of substitutions per site (20). Phylogenetic tree constructed with isolates from water tank SB. *Saprolegnia parasitica* was used as outgroup species.

***Pythium* species frequency**

A total of 140 isolates were obtained (Table 5-1) from the two greenhouses and compared to GenBank DNA sequences using BLAST. *Pythium* species recovered from the tank in greenhouse S were usually the same (14 out of 22) whether they were from the top or bottom leaf traps. In greenhouse E tank L 3 out of 4 of the isolates from the top traps were the same as those from the bottom. Tank R had 9 out of 14 of the isolates from the bottom the same as those from the top leaf traps. Tank G had no differences among species isolated from top and bottom leaf traps. Tank SB had 1 out of 5 of the isolates from top and bottom leaf traps the same species.

The most abundant isolates throughout the sampling period were members of, Group 1 (67), Group 2 isolates were obtained twice. The Group 3 isolate was obtained in March. Group 4 isolates were obtained from July to early August while Group 5 isolates were found from the end of November to early December. *P. catenulatum* was isolated only one time (September). *P. middletonii* was recovered once in March, but more frequently in July to October leaf traps. *P. helicoides* was recovered from July and August samples. *P. chamaeophon* was recovered from September-November. Some of the isolates did not fit any known species based on the ITS DNA sequence. Their sequences had less than 97% similarity to any *Pythium* species in the database and were not identical to one another. By the middle of December, *Pythium* was not recovered.

Table 5-1: *Pythium* species isolated from all water tanks

	Greenhouse S tank ^d		Greenhouse E tank ^e				
<i>Pythium</i> ^a	Clade ^c	C	L	G	R	SB	Total ^f
Group 1	B	33	8	3	23		67
Group 2	A	1	1				2
Group 3	B		1				1
Group 4	B					6	6
Group 5	E					3	3
<i>P. catenulatum</i>	B	1					1
<i>P. middletonii</i>	E	3	7	7		1	18
<i>P. helicoides</i>	K			2	3	2	7
<i>P. chamaehyphon</i>	K	9					9
<i>Pythium</i> spp.	-	4	5		17		26
Total ^b		51	22	12	43	12	140

^a Species were divided according to ITS sequence identification using NCBI Blast. Some samples could not be identified to due to identical ITS sequences or differences of fewer than 2 bp [11]. These isolates were categorized according to closest species identity: Group 1 (*P. dissotocum*, *P. diclinum*, *P. lutarium*, and *P. coloratum*); Group 2 (*P. adhaerens*, *P. chondricola*, and *P. porphyrae*); Group 3 (*P. flevoense*, *P. capillosum*, and *P. pectinolyticum*); Group 4 (*P. aploeroticum*, *P. aquatile*, *P. pachycaule*, *P. oopapillum*, and *P. sukuiense*); and Group 5 (*P. rostratum* and *P. rostratifingens*).

^b Total number of samples identified per water tank reservoir for each greenhouse.

^c Classification of each species to Clade [11].

^d Results are for water tank reservoir from greenhouses S. Letter C represents the water tank sampled from the greenhouse.

^e Results are for water tank reservoir from greenhouses E. Letters L, G, R, and SB identify each water tank where *Pythium* baiting was conducted.

^f Total number isolated during the baiting period.

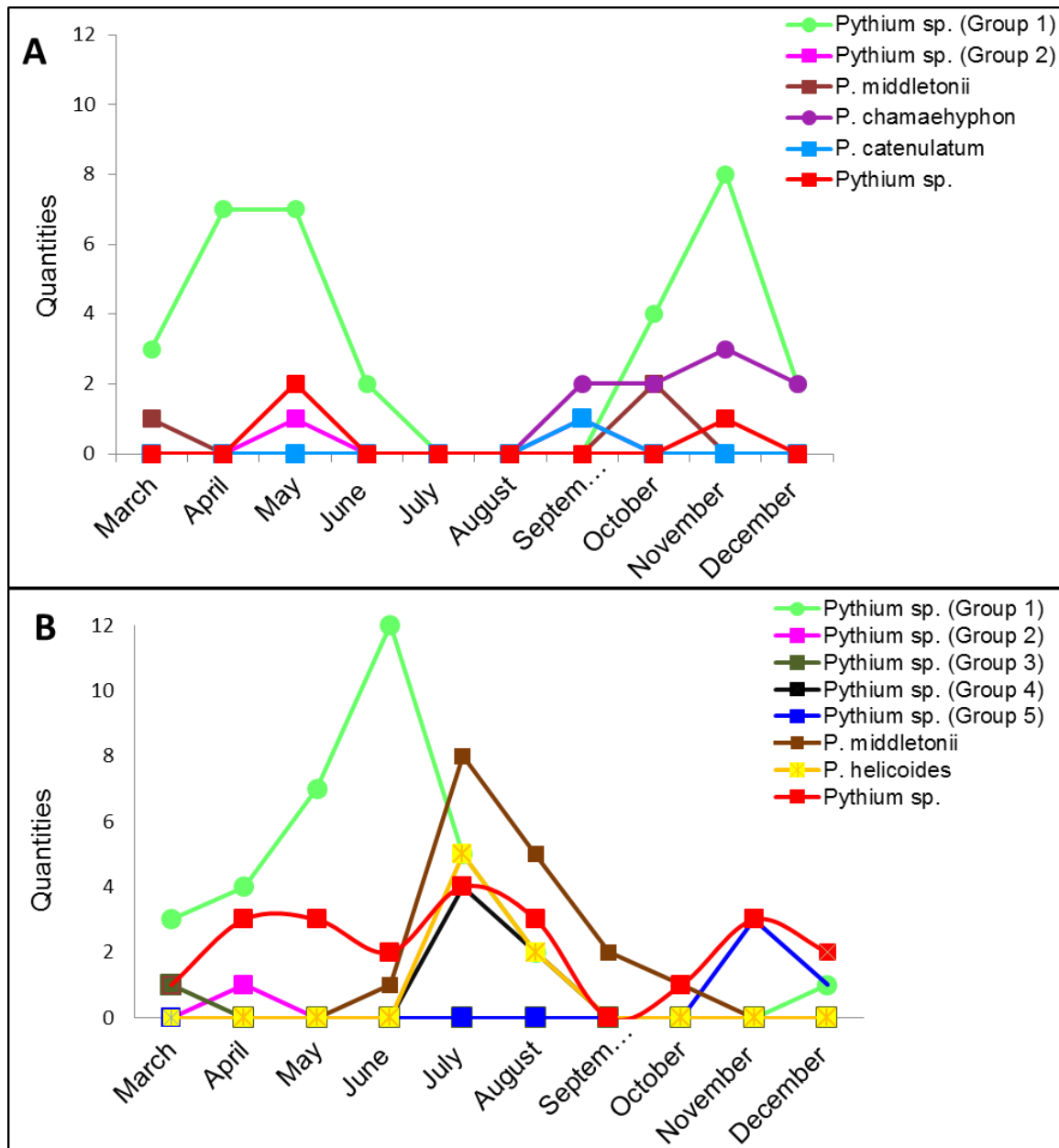


Figure 5-6: Distribution of *Pythium* species according to the month they were isolated. A) *Pythium* isolated from greenhouse S from one water tank B) *Pythium* isolated from four water tanks from greenhouse E.

Discussion

Recycling irrigation water contributes to the spread of the zoosporic *Pythium* and *Phytophthora* species and increase the risk of plant diseases [16, 19, 20]. While some studies have not found the water to be contaminated with *Pythium* inoculum [1] others have detected a large number of *Pythium* propagules in recycling water

irrigation systems [2]. Most studies only identified the isolates to genus and did not determine the frequency of isolation of particular species in water. In this study we found that some *Pythium* species were unique to one greenhouse while other species were present in both greenhouses. Many of the isolates that were frequently obtained had filamentous, non-inflated sporangia. Members categorized as *Pythium* Group 1, Group 2, Group 3, and Group 4 are closely related to *Pythium* Group F [21]. Preliminary research indicates that members of these groups are very weakly or non-pathogenic (data not shown). This filamentous non-inflated sporangia group is a ubiquitous, though minor, pathogen in soilless and hydroponic culture [22]. Species recovered only in one the greenhouses included *P. helicoides*, Group 3, Group 4, Group 5, *P. chamaehyphon*, and *P. catenulatum*. This may indicate that specific conditions are needed for these species to survive.

Some *Pythium* species seemed to be seasonal and were recovered only at certain times of the year (figure 5-6). *P. catenulatum* was only recovered in September while *P. chamaehyphon* was mostly recovered in the Fall as well as *Pythium* Group 5. This last group is closely related to *P. rostratum*, *P. rostratiformis* (Clade E) [11]. *P. rostratum* and *Pythium* species with filamentous non-inflated sporangia generally occur in water [21]. In addition, *Pythium* Group 4, *P. middletonii* and *P. helicoides* were recovered in summer. This is consistent with previous studies that found *P. helicoides* can be a serious pathogen in ebb and flow and hydroponic culture systems when temperatures exceed 40°C [24]. In previous studies, this species was detected in the water approximately one month before disease was observed in host plants [24], indicating that this species may survive well in water or in soilless environments. In addition, *P. middletonii* is considered an aquatic organism [21] and may explain its presence in water tanks. Previous research demonstrated the growth of this species was dependent on available substrate, environmental conditions and chemical composition [3]. From the species isolated, the most abundant species was *Pythium* Group 1. Surprisingly the occurrence of this species was divided into two seasons, spring and fall. Perhaps there is a decrease in population and we were not able to recover the species during the summer. *Pythium* Group 2 and Group 3 were mainly recovered in spring which may reflect a preference for certain temperature. Potential

substrates may strongly influence which *Pythium* species endemic to water environments and which are transients. The group of isolates that were not closely related to any species were recovered during the entire season. This group of isolates is not the same and may represent different species or new species that are adapted to survive in water tank environments. Our knowledge of the variety of species and their survival during the different seasons is limited. We do not know what governs the changes in *Pythium* species.

In this study recycled irrigation water contained many different *Pythium* species most of which were not closely related to any recognized species. ITS sequences are not always sufficient to separate species [11]. Here, the sequences of several isolates closely matched those of 3 or more known species. In order to identify those isolates, other regions of the DNA will need to be sequenced. Currently, the GenBank database contains the *cox II* sequences of most of the known species of *Pythium* and those sequences may clarify the identity of isolates obtained from the greenhouse water tanks. Currently, our knowledge of the identity of *Pythium* species that are endemic to the aquatic environment and those that are transient species in water is limited. Our results indicate that recycled irrigated water harbored many non-pathogenic or weakly pathogenic species that are probably endemic to the aquatic environment. In addition, recycled irrigated water may harbored many different *Pythium* species with some only detected for a short period of time. It is important to note that the *Pythium* species known to cause substantial crop losses in greenhouses in Pennsylvania, *P. aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare*, were not recovered from the water tanks. It is possible that these species were present and undetected because of low population levels or that they are not able to survive for long periods of time in this ecosystem. One limitation with the baiting technique is the bias associated with the susceptibility of the bait material to members of the target genus. Previous work (see Chapter 2) using creeping bentgrass indicates that is a good bait for *P. aphanidermatum* and preliminary research (data not shown) indicates that *P. irregulare* and *P. cryptoirregulare* also readily colonize it. Successful baiting for *Pythium* probably depends to some extent on whether a species produces zoospores. Not all species or all isolates within a given species produce zoospores. Mycelial fragments free in water

or associated with particulates in water can also act as propagules. The greenhouses sampled in the present work use coarse filters to remove particulates from recycled irrigation water it returns to the reservoir. Here, baits were deployed for 7 days and then replaced weekly over many weeks. This increases the likelihood that the majority of *Pythium* species would be detected at some time during the year either through contact with mycelial fragments or zoospores. The lack of recovery of *P. aphanidermatum*, *P. irregulare*, and *P. cryptoirregulare* from the water tanks may indicate their poor adaptation to recycled irrigation water. In addition there may be a deleterious effect by microbial communities that restricts their survival in the ecosystem (see Chapter 2).

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CONCLUSIONS

What we know

The genus *Pythium* consists of a diverse group of species ranging from soil saprophytes [5] to mycoparasites [6] living in diverse ecological niches. Most common species seem to be distributed worldwide although it is unknown if they are ubiquitous in nature or due to movement of plant, soil, and human activities [4]. *Pythium* has 120 species [2] and the number keeps rising with species described constantly. The number of characteristics suitable to identify and described *Pythium* species is relatively limited with no correlation evident with geographic distribution [4]. Some *Pythium* species are adapted to live in water and others to live in soil. Currently, the consensus is that the main source inoculum comes from soil and movement of infected plants. *Pythium* infections of the different crops are well documented but limited information is available concerning which species are most responsible for the disease [3]. Variation in distribution across environments and virulence among isolates are two keys of their adaptation to the different niches they inhabit. Several *Pythium* species may be isolated from a single piece of tissue or from soil surrounding the roots making it difficult to attribute the disease to one species [1]. Recent studies started to determine pathogenicity of *Pythium* species to specific crops: corn and soybean seed [1], wheat [3], rice cultivation [9]. It is a daunting task to identify host range for each *Pythium* species. With the many molecular advances more in depth studies could help clarify and determine which species inhabit and survived in the different niches. The increased discovery of new species in different niches makes it a priority to study and identify species and their survival strategy to narrow management strategies specific to phytopathogenic isolates and not to all species in the environment.

New frontier in *Pythium* studies

Usually, when *Pythium* species are isolated from irrigation water, they are not identified to species. It is unclear whether they are consistently the same species or if species in the water are causing crop losses in the greenhouse. There is limited

information on the survival of the different species inhabiting the greenhouse. Research should target this aspect of the production in the greenhouse in order to have a better understanding of the different species residing in recycled irrigation water reservoirs. In general, water is suspected to be a point of distribution for *Pythium* species. It is unknown if those species are viable for long periods of time. Recycled water may introduce or re-introduce *Pythium* inoculum into new areas of the greenhouse. Nevertheless, it is unknown how long they survive in this environment. Aspects that deserve close attention are the contribution of water to the survival, establishment, and distribution of saprophytic and pathogenic *Pythium* species residing in recycled irrigated water in the greenhouse.

New studies of the ecology of *Pythium* should examine aquatic *Pythium* and their role in pathogenesis in commercial greenhouses, their saprophytic life stages, and their influence in aquatic microbial communities. A better understanding of the survival, distribution, and epidemiology of *Pythium* in water is very important to help control this pathogen. We have limited information on which species are endemic and which are transient in different ecosystems. Not all *Pythium* seemed to survive in all environments (soil, plant material, and water reservoir). Therefore, identification of transient species vs. endemic species will add to our understanding of the life cycle of individual *Pythium* species. Key areas of research should include the distinction of species adapted to live in recycled water vs. soil around the greenhouse. Adaptation to live in water or soil is an area that is underrepresented in the literature. Temporal distribution of *Pythium* may give us a better idea of which species are not causing any problem and which should be closely monitored and controlled. This will add to our understanding of the ecological roles and phylogeny of the genus [8].

The composition of any community is determined by the species that happen to be distributed in the area and can survive its environmental conditions [7]. Information on microbial influence on the behavior and survival of *Pythium* in recycled irrigation water is limited. The development of effective disease management strategies must be based on an understanding of basic differences between soilborne and waterborne *Pythium* species. In addition, the bacterial community in the aquatic ecosystem may

play a role in their survival. It is an exciting era to add to our knowledge to the microbial ecology of this genus. We need to address beneficial interactions they may have with other microbes, including bacteria, in plants, soil, and water. This may prove to be key to understanding why this genus is well adapted to different niches and why they survived in the absence of a susceptible host.

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VITA of María L. Burgos Garay

EDUCATION

Ph.D. Plant Pathology, The Pennsylvania State University, May 2013
B.S. of Microbiology, Interamerican University of Puerto Rico, May 2004

AWARDS

2013 Oomycete Molecular Genetics Travel Fellowship, Asilomar, CA
2010 Specialty Crops Research Initiative Award, Pennsylvania State University, University Park, PA
2010 Lester P. Nichols Memorial Awards, Pennsylvania State University, University Park, PA
2009 Alfred P. Sloan fellowship recipient New York, New York
2008 Floriculture and Nursery Crops Research Initiative Award Specialty Crops Research Initiative Award
2007 Bunton-Waller Graduate Fellowship Pennsylvania State University, University Park, PA
1998 PR-LSAMP (Alliance for Minority Participation), Interamerican University, Bayamon, PR

PUBLICATIONS

1. Burgos-Garay M.L., Moorman, G.W. 2013. Effect of heterotrophic communities on *Pythium* spp. in recycled irrigation water. Oomycete Molecular Genetics Network Meeting. Asilomar, CA.
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