BERMUDAGRASS ROOT ROT COMPLEXES ASSOCIATED WITH PLANT-PARASITIC NEMATODES AND *PYTHIUM* SPECIES ON GOLF COURSES IN FLORIDA

By

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To my beloved parents for making me be who I am, my husband for supporting me all the way, and my son for giving me courage to overcome difficulties

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LIST OF ABBREVIATIONS

AZI1	Azelaic acid induced 1 gene
CMA	Corn meal agar
GABA	Gamma-aminobutyric acid
GLM	Generalized linear model
ITS	Internal transcribed sequence
NCBI	National Center for Biotechnology Information
PART	A *Pythium* selective media contain ingredients pimaricin, ampicillin, rifampicin, thiamine and corn meal agar
PCR	Polymerase chain reaction
PPI	*Pythium* percent infection
RA	Rosmarinic acid
TAE	Tris-Acetate EDTA buffer
USGA	United States Golf Course Association
UV	Ultraviolet light
WAS	Week after sprigging

Abstract of Dissertation Presented to the Graduate School
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The fungus-like oomycete *Pythium* causes *Pythium* root rot, one of the most common bermudagrass diseases on Florida golf courses. In a previous nematicide greenhouse experiment, creeping bentgrass growing in pots inoculated with sting nematode easily acquired *Pythium* root rot. Plant-parasitic nematodes, especially root-knot nematode, were observed from most *Pythium* root rot disease samples received by the UF/IFAS Plant Diagnostic Center. Therefore, we hypothesized that plant-parasitic nematodes might be associated with *Pythium* spp. in causing bermudagrass root rot disease.

This research focused on the effects of sting or root-knot nematodes on root infection by three *Pythium* spp. (*P. arrhenomanes*, *P. catenulatum* and *P. middletonii*) on bermudagrass and their mycelia growth. Different nematodes and different *Pythium* spp. interacted with each other in different ways. Sting nematode increased root infection by low virulence *P. catenulatum*, and it might induce plant resistance to high virulence *P. arrhenomanes*. Root-knot nematode reduced infection by either *P. arrhenomanes* or *P. catenulatum*. We also found that bermudagrass roots infested with

plant-parasitic nematodes might have negative effects on growth of *Pythium* mycelia, whether the *Pythium* spp. is virulent or not.

We observed increased levels of benzene sulfonic acid and azelaic acid in nematode infested bermudagrass root exudates, providing a possible explanation of the antagonistic effects observed between nematode infested roots and *Pythium* spp. Benzene sulfonic acid provides an acidic environment which may inhibit *Pythium* mycelia growth. Azelaic acid is involved in the plant immune system and may induce plant resistance to *Pythium* spp.

This study indicated bermudagrass root rot disease identification only based on *Pythium* isolation could be inaccurate. Sometimes plant-parasitic nematodes are the major disease causal agents associated with avirulent or low virulence *Pythium* spp. More efficient bermudagrass root rot disease management strategies can be generated when taking both plant-parasitic nematodes and *Pythium* spp. into consideration.

CHAPTER 1
LITERATURE REVIEW

Why Study Golf Course Turfgrass Management?

Turfgrass is found everywhere in daily life. It beautifies lawns and parks in our cities, provides safe playing surfaces on athletic fields like golf courses and football fields, and plays a role in dust control when planted along highways and airport runways. Turfgrass is also a commercial product when grown on sod farms. Turfgrass not only provides economic value and beauty to our lives (Morris, 2003), but also benefits our physical and mental health (Bauske and Waltz, 2013). Turf also is an important crop based on acreage, Milesi et al. (2005) indicated that in the U.S. turfgrass covers over 40 million acres, which is 2% of the land in the continental U.S. The turf industry is valued $40 billion annually (Morris, 2003). Turf is the top one or two agricultural commodities in states such as Maryland, Pennsylvania, Florida, New Jersey, and North Carolina (Haydu et al., 2006). The turfgrass industry has had a huge contribution to Florida's economy since 1990s (Hodges et al., 1994). In Florida, golf is an essential part in turfgrass industry because of its high economic value. There are 1,103 golf courses and 524 golf communities within the state. Numerous championship golf events are hosted here every year. Florida's golf industry generated approximately $11.0 billion, providing over 132,000 jobs, and $3.6 billion of wage income in 2013 (Anonymous, 2015). Because of the role golf industry plays in the state economy, golf course turfgrass management is very important in Florida.

Bermudagrass (*Cynodon* spp.) is the most widely used turf species on U.S. golf courses (Lyman et al., 2007) due to its green color, medium to fine texture and excellent heat, wear, drought and salt tolerance (Beard, 2002). It is a warm-season grass that

spreads by rhizomes and stolons. Hybrid bermudagrasses (*C. dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy) is widely utilized on golf courses in tropical, subtropical, and temperate climate areas (Burton, 1964; Burton and Elsner, 1965; Reasor et al., 2016).

Research results indicate that turfgrass, including bermudagrass, is affected by many physiological (low cold and shade tolerance), and biological disorders like insects (including chinch bugs, mole cricket, etc.), and numerous pathogenic agents (including fungal pathogens, plant-parasitic nematodes, viruses, bacteria, etc.) (Smiley et al., 2005). Understanding the relationships between turfgrass and these noninfectious and infectious agents provides more efficient strategies for turfgrass management.

The Two Common Bermudagrass Root Problems in Florida

Pythium spp. and plant-parasitic nematodes are two of the most common root problem causal agents on bermudagrass in Florida.

Pythium Root Rot

Pythium diseases have become widespread on turfgrass since 1940s (Abad et al., 1994). *Pythium* root rot is a bermudagrass root disease caused by the soil borne pathogen *Pythium* species (Hodges and Coleman, 1985). The disease occurs in poorly drained conditions and high temperature and humidity environments (Smiley et al., 2005). Symptoms of *Pythium* infected grass roots include reduced growth and small to large yellow or necrotic patches, a tan to brown discoloration may or may not be apparent on turf roots, crowns and stolons (Hodges and Coleman, 1985; Smiley et al., 2005). Based on preliminary data provided by the UF/IFAS Plant Diagnostic Center, *Pythium* root rot is considered one of the major diseases on bermudagrass in Florida since 2012 (Figure 1-1). In the past few decades, about 50 *Pythium* species have been reported on turfgrass in the United States (Nelson and Craft, 1991; Abad et al., 1994;

Feng and Dernoeden, 1999). Species like *P. graminicola*, *P. irregular*, *P. catenulatum*, *P. ultimum*, *P. aphanidermatum*, and *P. torulosum* have been reported from bermudagrass in Florida (Alfieri et al., 1994; Harmon, 2004; Stiles et al., 2007). Research results indicate that some species such as *P. aristosporum* are highly aggressive (causing 61 to 100% disease) to turfgrass; other species like *P. irregulare* are moderately aggressive (causing 21 to 60% disease) (Abad et al., 1994).

Nematodes

Plant-parasitic nematodes are unsegmented roundworms, which are tiny and need to be observed through microscopes (Christie, 1959). They can cause severe economic loss on many crops such as citrus (Shokoohi and Duncan, 2018), strawberry (Deseager and Noling, 2017), turfgrass (Crow, 2001), etc. Based on feeding strategies, plant-parasitic nematodes can be divided into migratory and sedentary endoparasites, and ectoparasites. Ectoparasites, such as *Belonolaimus longicaudatus* (sting nematode), usually cause short and stubby roots; sedentary endoparasites, like *Meloidogyne graminis* (turfgrass root-knot nematode), usually cause galls on roots; and migratory endoparasites, for example *Hoplolaimus galeatus* (lance nematode), usually cause dark and rotten-looking roots.

The golf course putting green root zone mix (a minimum 90% fine sand to fine gravel content) (USGA green section staff, 2018) recommended by the United States Golf Course Association (USGA) provides favorable environment for nematode population development. Plant-parasitic nematodes, especially sting, root-knot, and lance nematodes, cause severe problems on golf courses in Florida, (Crow, 2005). *Belonolaimus longicaudatus* feeds on turfgrass root tips or long turfgrass root sides and causes short and stubby root symptom. *Meloidogyne graminis* penetrates the root with

its entire body and then forms a permanent feeding site. The infection results in minor elongated galls on roots or swelling of the roots (Heald, 1969). *Hoplolaimus galeatus* causes damage to small feeder roots and leads to the death of root tips (Lukens and Miller, 1973). The damage by plant-parasitic nematodes to the root system is responsible for yellow, declining, or dying patches of grass.

Nematodes Associated Disease Complexes

Disease complexes have been studied since 19th century, after the “germ theory” was initiated by Louis Pasteur and Robert Koch. The earliest reports regarding disease complexes were attributed to Pasteur, who observed that synergistic interaction of different microorganisms can led to a disease. Although the study of plant disease causal agents is still mainly focused on individual pathogens, mounting evidence indicates there can be synergistic effects between different pathogens that cause plant diseases (Lamichhane and Venturi, 2015). In the past, research was conducted on plant disease complexes involving several pathogens in same phylum, for example crown and root rot in turfgrass is a disease complex associated with *Pythium* species and *Rhizoctonia* species (Lucas and Mudge, 1997); or belonging to different phyla, such as nematode-fungus disease complexes.

The roles that nematodes play in plant disease complexes have been summarized in several reviews of Powell (1963, 1971a, 1971b), Pitcher (1965, 1978), Bergeson (1972), Taylor (1979), Mai and Abawi (1987), Evans and Haydock (1993), Back et al. (2002), and Williamson and Gleason (2003). Nematode associated plant diseases have three components: nematode, host plants, and other pathogens. Fungi are one of the major plant pathogens known to interact with nematodes on causing plant diseases (Khan, 1993). Nematode-fungus associations have been studied since

the 1800s. Atkinson (1892) first reported *Meloidogyne incognita* increased the severity of *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *vasinfectum.* Nematodes may act as vectors of fungal pathogens, wounding agents, host modifiers, rhizosphere modifiers, or resistance breakers in causing plant diseases (Bergeson, 1972). Nematode fungus associations in disease complexes can be synergistic, antagonistic, and neutral (Back et al., 2002). Synergistic pathogen interactions have been reported more frequently than the other pathogen interactions.

Synergistic disease complexes have been observed on many crops, mainly associate with endoparasitic nematodes and wilt fungi or root rot pathogens. For example, root-knot-*Fusarium* complex on tomato (Jenkins and Coursen, 1957), *Verticillium* wilt–*Pratylenchus* complexes on cotton and pepper (Mountain and McKeen, 1962; Olthof and Reynes, 1969), nematode-*Pythium* complexes on chrysanthemum (Johnson and Littrell, 1969), etc. On turfgrass, zoysiagrass decline caused by *Gaeumannomyces graminis* var*. graminis* was reported to be related with plant-parasitic nematodes in Maryland (Juska, 1972). Morris et al. (2016) reported that *Meloidogyne* spp. interacted with *Pythium aphanidermatum* synergistically on cucumber damping-off. Ahmed and Shahab (2017) indicated that *Meloidogyne incognita* increased root rot symptoms on lentil when inoculated ten days prior to or simultaneously with *Fusarium solani* (Mart.) Sacc.

Some plant-parasitic nematodes modify host plant tissue and make quantitative and qualitative changes in root exudates, making them more favorable to fungal germination, growth and reproduction. *Meloidogyne* spp., *Rotylenchulus reniformis*, and *Hoplolaimus tylenchiformis* delayed the maturation of cotton seedlings, and made them

more susceptible to damping-off by *Pythium debrayanum* and *Rhizoctonia solani*. On cotton, root-knot gall exudates increased the sporangia production of *P. debrayanum* and *R. solani* when compared to healthy cotton root exudates (Brodie and Cooper, 1964). Sikder et al. (2015) observed that *Meloidogyne graminicola* broke resistance to *Pythium arrhenomanes* on the *Pythium*–resistant rice cultivar 'Nipponbare' by suppressing the pathway of salicylic acid biosynthesis. In some cases ectoparasitic nematodes act as wounding agents and can be involved in synergistic disease complexes; for instance, *Belonolaimus longicaudatus* - *Fusarium* wilt on cotton (Holdeman and Graham, 1953; Minton and Minton, 1966).

Antagonistic interactions between nematodes and fungi have also been reported. Infection of *Meloidogyne javanica* on tomato increased the expression of genes associated with plant defense response to other pathogens (Lambert et al., 1999). *Meloidogyne hapla* suppressed alfalfa damping-off caused by *Phytophthora megasperma* f. sp. *medicaginis* (Gray et al., 1990). El-Borai et al. (2002a and 2002b) reported that root infection by *Phytophthora nicotianae* was reduced by *Tylenchulus semipenetrans* on citrus, possibly because *T. semipenetrans* protects its feeding site by producing anti-fungal chemicals. *Pythium* spp. were antagonistic to *Meloidogyne* spp. on rice and sugarcane (Valle-Lamboy and Ayala, 1980; Sikder et al., 2015; Verbeek et al., 2016). Manosalva et al. (2015) found that ascarosides produced by plant-parasitic nematodes induced plants defense responses to many other pathogens including oomycetes.

Hypothesis of My Study

In previous research conducted by Dr. W. T. Crow's lab, *Pythium* root rot was acquired easily when creeping bentgrass was infested with *B. longicaudatus*. In many

diagnoses of bermudagrass suffering from root dysfunction, *B. longicaudatus* and *Pythium* spp. occur concomitantly. This brings forward the question, “do plant-parasitic nematodes predispose bermudagrass to *Pythium* root diseases” The primary hypothesis of this dissertation is that the plant-parasitic nematodes *B. longicaudatus* and *M. graminis* have a synergistic association with *Pythium* spp. in causing root-rot disease on bermudagrass.

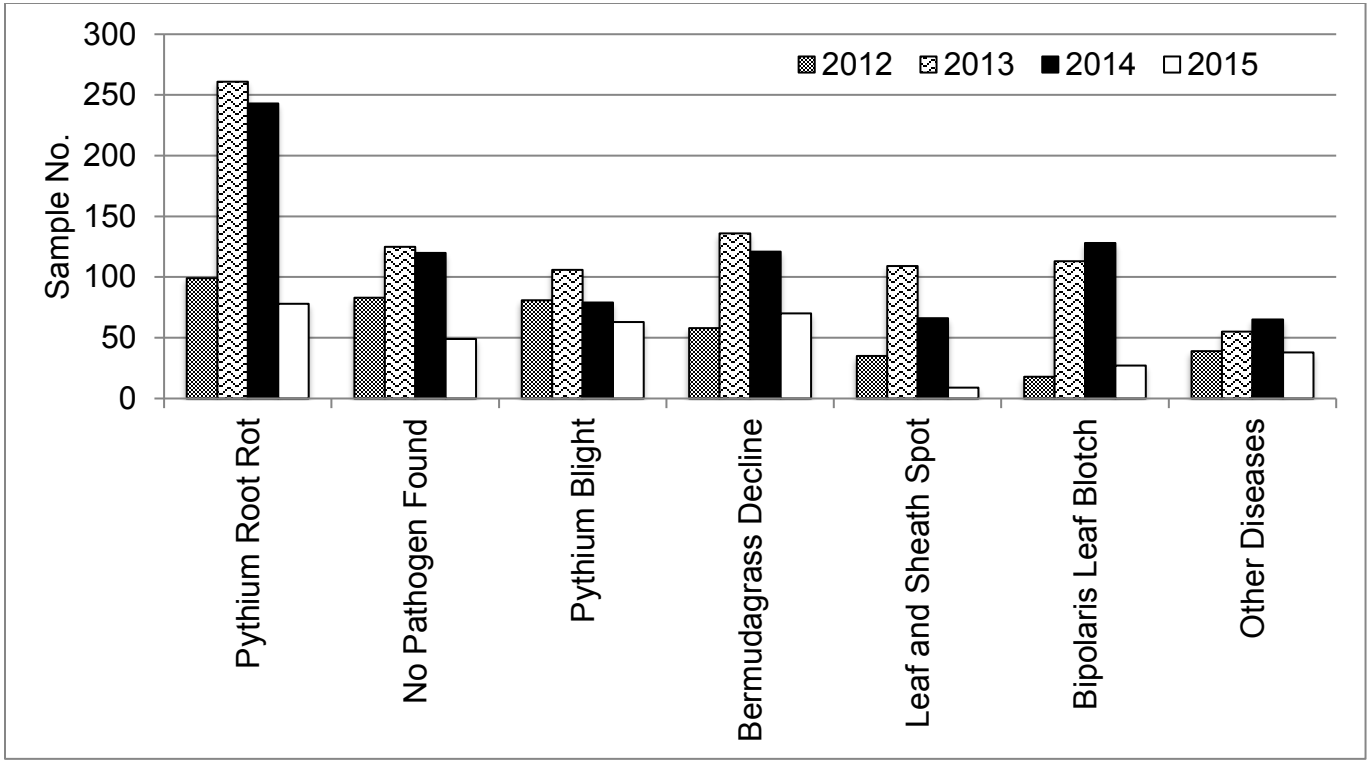


Figure 1-1. The 2012 - 2015 bermudagrass disease sample data from the UF/IFAS Plant Diagnostic Center. X-axis presents the disease observed in all received bermudagrass samples; Y-axis presents the sample number of each disease. The numbers of received *Pythium* root rot samples ranked at the top every year from 2012 to 2015.

CHAPTER 2
PRELIMINARY DATA

Introduction

Bermudagrass root rot disease caused by *Pythium* spp. is considered the one of major diseases on Florida golf greens based on the 2012 - 2015 data (Figure 1-1) provided by the UF/IFAS Plant Diagnostic Center. According to previous greenhouse research experience, bentgrass infested with sting nematode (*Belonolaimus longicaudatus*) easily acquires *Pythium* root rot disease. High plant-parasitic nematode population densities were frequently observed on golf course greens that were diagnosed with *Pythium* root rot disease. (Crow, W.T., pers. comm.).

In the literature review by Powell (1971a), plant-parasitic nematodes associated with *Pythium* spp. led to root disease complexes on many crops, including tomato, tobacco, peach, sugarcane, corn, and chrysanthemum. Bermudagrass root rot disease may be associated with both *Pythium* spp. and plant-parasitic nematodes. Nematode assays of several bermudagrass samples that were positive for *Pythium* spp. infection were conducted to test the hypothesis. Additionally, *Pythium* species were isolated from nematode infested bermudagrass samples received by the Florida Nematode Assay lab. Three of these isolates were retained for use in future studies.

Nematodes Assay of Bermudagrass Root Rot Disease Samples

Materials and Methods

From May 2016 – May 2017, soil from all bermudagrass *Pythium* root rot disease positive samples received by the UF/IFAS Plant Diagnostic Center were collected for nematode assay. Nematodes were extracted from 50 cm^3 of well-mixed sample of soil and roots using a modified mist chamber method (unpublished method developed by

Dr. William T. Crow) for 72 hours. Then, nematodes were identified to genus and counted using an Olympus CK30 inverted microscope at 20× magnification.

Results

A total of 34 bermudagrass root rot disease samples were received. Nematode data of each sample were listed in Table 2-1. Plant-parasitic nematodes were recovered from 30 of these samples (88% of received samples). In 19 of them (56% of received samples) the population density of at least one type nematode presented risk of nematode damage based on the risk thresholds used by the UF Nematode Assay Lab.

Genera of plant-parasitic nematodes recovered from those *Pythium* positive samples included: *Belonolaimus* (sting nematode) (9 samples, 26% of received samples), *Meloidogyne* (root-knot nematode) (23 samples, 67% of received samples), *Hoplolaimus* (lance nematode) (17 samples, 50% of received samples), and *Helicotylenchus* (spiral nematode) (13 samples, 38% of received samples) were observed more frequent than the other plant-parasitic nematodes (Table 2-2, Figure 2-1). Nematode population densities above threshold were observed in 44% of sting nematode, 61% of root-knot nematode, and 29% of lance nematode samples.

Pythium Species Collection from Nematode Samples

Materials and Methods

Five nematode diagnostic samples received by the Florida Nematode Assay lab were processed for preliminary data of nematode-*Pythium* associated with bermudagrass root rot disease. These samples originated from different golf greens on the Key Largo Executive Golf Club in The Villages, FL.

Nematodes were extracted from 100-cm^3 of well-mixed sample soil by centrifugal-flotation technique (Jenkins, 1964) using a 38-µm sieve (Thermo Fisher

Scientific Inc., MA). Plant-parasitic nematode numbers were identified to genus and counted. *Pythium* species were isolated from roots of these samples and identified using morphological and molecular methods. Identified *Pythium* isolates were collected for future studies.

Pythium species were isolated by *Pythium* selective media (PART) developed by the UF/IFAS Plant Diagnostic Center. Corn meal agar (CMA) growth medium (8.5 g in 500 ml of deionized water) was autoclaved at 121 °C for 20 minutes; when the medium was cooled to 55 °C in Fisher Versa-Bath Model 130 (Thermo Fisher Scientific, Waltham, MA), 0.25 g ampicillin (Sigma-Aldrich Corporation, St. Louis, MO), 0.015 g pimaricin (Fisher Scientific International, Inc., Hampton, NH), 0.005 g rifampicin (Sigma-Aldrich Corporation, St. Louis, MO), and 0.0002 g thiamine (Sigma-Aldrich Corporation, St. Louis, MO) were added. Approximately one-cm sections of root tissues exhibiting rot and discoloration symptoms were cut and surface sterilized by soaking in 0.6% sodium hypochlorite for 1 minute. After rinsing in running deionized water for one minute, root sections were blotted dry on sterilized paper tower, plated on PART media, and incubated for 3-5 days at 24 °C to allow pathogen growth.

Pythium isolates were cultured on V8 juice agar, CMA, and St. Augustine grass leaf blades for three to five days, and then identified to genus based on the morphological features of hyphae, sporangia and oogonia (Van Der Plaats-Niterink, 1981; Abad et al., 2004). Each isolate was purified by cutting a 0.25-cm^2 mycelia plug from the edge of a colony and subcultured onto CMA. Pure *Pythium* isolates were maintained at 24 °C for 7 days, and three 0.25-cm^2 mycelia plugs were placed in

sterilized capped test tubes containing three sterilized hempseeds submerged in sterilized deionized water for long term storage (Abad et al., 1994).

Pythium isolates were identified to species using a molecular method (Levesque and de Cock, 2004; Klemsdal et al., 2008; Robideau et al., 2011; Binagwa et al., 2016). Prior to DNA extraction, each *Pythium* isolate was grown on CMA for two weeks at 24 °C to allow massive mycelia production. Fresh mycelia tissue was transferred to a 2-ml microcentrifuge tube with 500-µL Genomic Lysis Buffer and broken up by Qsonica Sonicator Q700 (Qsonica L.L.C, Newtown, CT) for one minute. Genomic DNA was extracted using Quick-gDNA™ MiniPrep Kit (Zymo Research, Irvine, CA). Polymerase chain reaction (PCR) amplification of the internal transcribed sequence (ITS) regions was performed using primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') (White et al., 1990). A reaction volume of 25.0-µL contained 8.5-µL nuclease free water, 12.5-µL of 2X One Taq Hot Start MasterMix (BioLabs, New England), 1.25-µL of each primer (10-µM) and 1.5-µL of DNA template. Amplification conditions were achieved in a BIO-RAD MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) programmed for initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for one min. At the end of amplification reaction, a final extension step was accomplished at 72°C for 10 min. PCR products were run at 1.5% agarose gels dissolved in 1× TAE (Tris-Acetate EDTA buffer) concentration as the running solution followed with post staining of ethidium bromide (0.5-µg/ml). Electrophoretic migration was carried out for 25 min electrophoresed at 110 V. The amplified products were visualized and photographed under ultraviolet (UV) light. An

Apex 100 bp ladder (Genesee Scientific Inc., San Diego, CA) was used to estimate the size of PCR products. PCR products with a size of 600 bp and above were sent to the UF Interdisciplinary Center for Biotechnology Research for Sanger sequencing. Consensus ITS sequences of *Pythium* isolates were aligned and trimmed for quality using Geneious software, then compared with ITS sequences of known *Pythium* species available in the GenBank database by performing nucleotide blast search at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/blast.cgi). The MEGA 7 software was used for phylogenetic analysis.

Results

PCR products of the 5 samples with a size of ≥ 600 bp showed banding patterns and were sequenced for phylogenetic classification according to their respective molecular sequences of the ribosomal fragments (Figure 2-3). The trimmed consensus ITS sequences of *Pythium* isolates from bermudagrass samples were listed in Table 2-3. Three different *Pythium* spp. were identified based on nucleotide blast results (Figure 2-4). *Pythium* spp. from sample “1642” is closest to *P. aristosporum* (99% identity) and *P. arrhenomanes* (99% identity); that from sample “1643” is closest to *P. middletonii* (94% identity) and *P. orthogonon* (93% identity); isolate form sample “1651” is closest to *P. catenulatum* (99% identity) and *P. angustatum* (98% identity). Based on literature, *P. aristosporum* and *P. arrhenomanes* cannot be differentiated by molecular methods (Levesque and de Cock, 2004; Robideau et al., 2011). The low percent similarity of sample “1643” ITS sequence is due to few reference sequences that are available for the species.

Morphological features (Van Der Plaats-Niterink, 1981; Abad et al., 2004) were used to confirm *Pythium* species. Isolate “1642” is closer to *P. arrhenomanes* than *P. aristosporum*, because only proliferating and globose sporangia (Figure 2-2A) were observed in the isolate; no oospore presented in any of these medium cultures even in grass-blade cultures (Kerns and Tredway, 2010). The presence of aplerotic oospores, intercalary oogonia, monoclinous antheridia, and globose sporangia (Figure 2-2B) in isolate “1643” makes it closest to *P. middletonii*; the diameter of oospores was in the range 17 to 20 µm and the average was 18 µm. Isolate “1651” is closest to *P. catenulatum* due to the observation of catenulate sporangia (Figure 2-2C).

Samples “1642” and “1651” also had a high sting nematode population density, over 50 sting nematodes per 100-cm^3 of soil (Table 2-4).

Summary

In the bermudagrass *Pythium* root rot disease positive samples diagnosed by the UF/IFAS Plant Diagnostic Center, plant-parasitic nematodes were recovered from 88% of them and nematode numbers above thresholds were observed in 56% of them. Sting nematode, root-knot nematode, and lance nematode appeared more frequent and abundant in these *Pythium* positive samples compared to other plant-parasitic nematodes. These three plant-parasitic nematodes are considered three major nematode problems on bermudagrass golf greens in Florida (Crow, 2005).

In the five nematode samples received by the UF Nematode Assay lab, *Pythium* isolates closely related to *P. arrhenomanes*, *P. middletonii*, and *P. catenulatum* were collected; and two samples, from which *P. arrhenomanes* and *P. catenulatum* were isolated, had high sting nematode population densities. *Pythium arrhenomanes* and *P.*

catenulatum are two common *Pythium* species found on turfgrass (Smiley et al., 2005). *Pythium middletonii* has not been reported on turfgrass.

Based on the high numbers of plant-parasitic nematodes present in bermudagrass *Pythium* root rot disease samples, and the observation of common turfgrass *Pythium* species from bermudagrass samples with high nematode numbers, there is possibility that plant-parasitic nematodes are associated with *Pythium* spp. in causing bermudagrass root rot disease.

Table 2-1. List of plant-parasitic nematode numbers in 50-cm^3 of soil from each sample with a positive diagnosis for bermudagrass *Pythium* root rot disease from May 2016 – May 2017.

Samples	*Belonolaimus* Sting nematode (5*)	*Meloidogyne* Root-knot nematode (40*)	*Hoplolaimus* Lance nematode (20*)	*Helicotylenchus* Spiral nematode (350*)	*Peltamigratus* Spiral nematode (75*)	*Hemicriconemoides* Sheathoid nematode (250*)	*Mesocriconema* Ring nematode (250*)	*Dolichodorus* Awl nematode (5*)
"RT16-246"	0	985	12	0	3	0	0	0
"RT16-247"	0	603	0	3	0	0	0	0
"RT16-251"	15	54	0	2	0	0	0	0
"RT16-252"	0	3	0	0	0	0	0	0
"RT16-255"	12	2	6	46	0	0	0	0
"RT16-270"	0	2	0	4	0	0	0	0
"RT16-298"	0	19	11	30	0	1	0	0
"RT16-304"	0	623	14	0	0	0	13	0
"RT16-314"	0	940	35	0	0	0	0	0
"RT16-324"	1	0	21	0	0	0	0	0
"RT16-353"	1	0	65	0	0	0	0	0
"RT16-361"	1	31	1	0	0	0	0	0
"RT16-362"	0	485	0	0	0	0	0	0
"RT16-363"	5	1	4	0	0	0	1	0
"RT16-366"	0	49	0	0	0	0	0	0
"RT16-425"	0	49	1	0	0	0	0	0
"RT16-471"	26	0	0	2	0	0	0	0
"RT16-472"	0	0	1	0	0	0	0	0
"RT16-482"	0	32	9	3	0	0	0	0
"RT16-488"	3	0	0	0	0	0	0	0
"RT16-554"	1	69	8	7	0	9	0	0
"RT16-564"	0	117	26	3	0	0	1	0
"RT16-567"	0	3	0	1	0	0	0	0
"RT16-590"	0	0	0	0	0	0	0	0
"RT16-591"	0	4	0	2	0	0	0	0
"RT16-628"	0	0	0	0	0	0	0	0
"RT16-641"	0	0	0	0	0	0	0	0
"RT16-642"	0	0	0	0	1	0	0	0
"RT17-62"	0	0	1	0	0	0	0	95
"RT17-76"	0	110	0	2	0	0	0	0
"RT17-111"	0	147	53	0	0	0	0	0
"RT17-118"	0	691	5	0	0	0	0	0
"RT17-182"	0	0	0	0	0	0	0	0
"RT17-190"	0	1,128	0	1,050	0	0	0	0

* Nematode thresholds are based upon number per 50-cm^3 soil. Unpublished data used by the UF Nematode Assay lab.

Table 2-2. Numbers of bermudagrass samples with a positive diagnosis of *Pythium* infection from which different nematode genera were recovered and those with nematode numbers exceeding risk thresholds.

	Belonolaimus Sting nematode (5*)	*Meloidogyne* Root-knot nematode (40*)	*Hoplolaimus* Lance nematode (20*)	*Helicotylenchus* Spiral nematode (350*)	*Peltamigratus* Spiral nematode (75*)	*Hemicriconemoides* Sheathoid nematode (250*)	*Mesocriconema* Ring nematode (250*)	*Dolichodorus* Awl nematode (5*)
Total	9	23	17	13	2	2	2	1
Over threshold	4	14	5	1	0	0	0	1

* Nematode thresholds are based upon number per 50-cm^3 soil. Unpublished data used by the UF Nematode Assay lab.

Table 2-3. The trimmed consensus ITS sequences of three *Pythium* isolates obtained from bermudagrass samples.

Sample	Edited ITS Sequence
“1642”	>GU2_ITS1 TGCGGAAGGATCATTACCACACCAAAAAACTTTCCACGTGAACCGTTGTAATTTTGTTTTG TGCCTTCTTTCGGGAGGGCTAAACGAAGGTTGTCCGCAAGTGTAGTTAATTCTGTACGCG TGGTCTTCCGATGTCTTTTTAAACCCATTACTTAATACTGATCTATACTCCGAGAACGAAAG TTTTTGGTTTTAATCCATAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAA GAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGAATTCAGTGAGTCATCGAAATTT TGAACGCACATTGCACTTTCGGGATATTCCTGGAAGTATGCTTGTATCAGTGTCCGTACAT CAAACTTGCCTTTCTTTTTTTGTGTAGTCAAGGAGAGAGATGGCAGATGTGAGGTGTCTCG CTGACTCCCTCTTCGGAGGAGAAGACGCGAGTCCCTTTAAATGTACGTTCGCTCTTTCTT GTGTCTGAGAGAAGTGTGACCTTCGAATGCGGTGATCTGTTTGGATCGCTTTGCGCGAGT GGGCGACTTCGGTTAGGACGTTAAAGGAAGCAACCATTTTTGGCGGTATGTTAGGCTTCG GCCCGACGTTGCAGCTGAGAGTGTGTGGTTTTCTGTTCTTTCCTTGAGGTGTACCTGTTT GTGTGAGGCAATGGTCTGAGCAAATGGTTATTGTGTGAGAGTGGTTATTGCTCTTGGACG CTCTATTCGTAGAGTAAAGAAGGCAACACCAATTTGGGACTAGTCTGTGGAATGAATGAAT TTTTATTTCGCGGGCGCTTTTCAATTTGGACCTGATATCAAGTAAGACTACCCGCTGAACT TAAGCATATCAATAAGCGGAGGAA
“1643”	>GU3_ITS1 GGATCATTACCACACCTAAAAACTATCCACGTGAACTGTATGATACGATTAGCGCCGTGAC GCGTGCTGCGGGTTTGTAAACGAATCTGTGGTGTGCGGGCTCGGCTGATCGAAGGCTCT TTCATTGCTGCGGGTGTGTGCTTTTCGGAGCGCGTGTTTTGCGGCTTTGTGGGCTGACTT ACTTTTTCAAACCCCTTACTTGAATGACTGATGTATACTGTGAGGACGAAAGTCTTTGCTTT GAACTAGATAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTG CGAACTGCGATACGTAATGCGAATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCAT ATTGCACTTTCGGGTTATGCCTGGAAGTATGTCTGTATCAGTGTCCGTACATCAACCTTGC CTCTCTTTGTCGGTGTAGTCCGGTTTGGAGACGAGCAGACTATGAAGTGTCTTGCGCGTA TTTGCCATTTCGTGTGAACGGCGCGAGTCCTTTTGAAACGACACGATCTCTTCTATTTGCC TTTAGCAACTCGCTTTGGTTTGAACGCATCGGTCTTGGAATCGTTTGCAGTCTCCGGCGA CCTTGGCTTTGGACATTATGGAGGGCACCTCACTTCGCGGTATGTTAAGCTCTTTGTGGC GGAACAATGTTGCGTTTGTGTGTGTGTTTCCGTCTTTGGCTTTGAGGTGTACTGTGAG GTTGTGGGCTTGAGTCCTTGTGCTGTGTGTCAGTAGCTCGGAGGCGGTGTTTTTGTTATT GGATTCTGCGCGTGTATTCGCGTGGGTAGAGAGTATGTATTTGGGAACGATTGTACTGCG CTCTCTTGTGGGGGGCGTGTGTATCTCAATTGGACCTGATATCAGACAAGACTACCCGCT G
“1651”	>GU5_ITS1 GCGGAAGGATCATTACCACACCATAAAAACTTTCCACGTGAACCGTTACAATTATGTTCTG TGCTCTCTCTCGGGAGAGCTGAACGAAGGTAGTGCCGCATGTATGTGCGGCGTCTGCCG ATGTACTTTTAAACCCATTACACTAATACTGAACTATACTCCGAGAACGAAAGTTTTTGGTT TTAATCAATAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTG CGAACTGCGATACGTAATGCGAATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCA CATTGCACTTTCGGGATATTCCTGGAAGTATGCTTGTATCAGTGTCCGTACATCAAACTTG CCTTTCTTTTTTTTGTGTAGTCAAGGAGAGAAATGGCAGAATGTGAGGTGTCTCGCTGACTC CCTCTTCGGAGGAGAAGACGCGAGTCCCTTTAAATGTACGTTCGCTCTTTCTTGTGTCTAA GATGAAGTGTGACTTTCGAACGCAGTGATCTGTTTGGATCGCTTTGCGCGAGTGGGCGAC TTCGGTTAGAACATTAAAGGAAGCAACCTCTATTGGCGGTATGTTAGGCTTCGGCCCGAC TTTGCAGCTGACAGTGTGTTGTTTTCTGTTCTTTCCTTGAGGTGTACCTGTCTTGTGTGAG GCAATGGTCTGGGCAAATGGTTATTGTGTAGTAGAATTTTGCTGCTCTTGGGCGCCCTAC TCGTAGGGTAAAGAAGGCAACACCAATTTGGGACTAGTCTGCGGGGGATGTATTTCTCTT GCGGGCGCATTTTCAATTTGGACCTGATATCAAGTAAGATTACCCGCTGA

Table 2-4. Population density of plant-parasitic nematode genera/100-cm^3 of soil.

Samples	*Belonolaimus* Sting nematode (10*)	*Hoplolaimus* Lance nematode (40*)	*Helicotylenchus* Spiral nematode (700)*	*Caloosia* Sheath nematode (150*)	*Mesocriconema* Ring nematode (500*)
"1641"	17	0	0	0	29
"1642"	59	0	0	0	38
"1643"	26	15	153	9	41
"1645"	27	31	72	0	116
"1651"	58	0	2	0	156

* Nematode thresholds are based upon number per 100-cm^3 soil. Unpublished data used by the UF Nematode Assay lab.

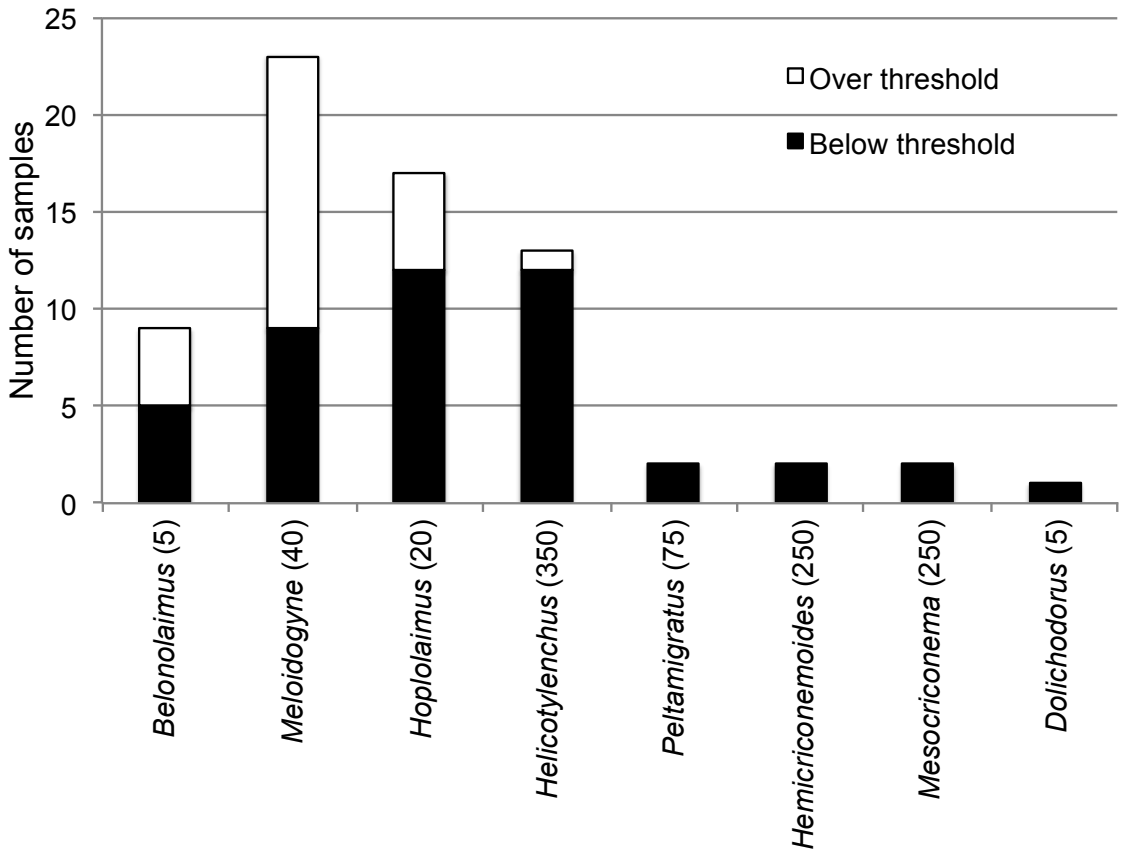
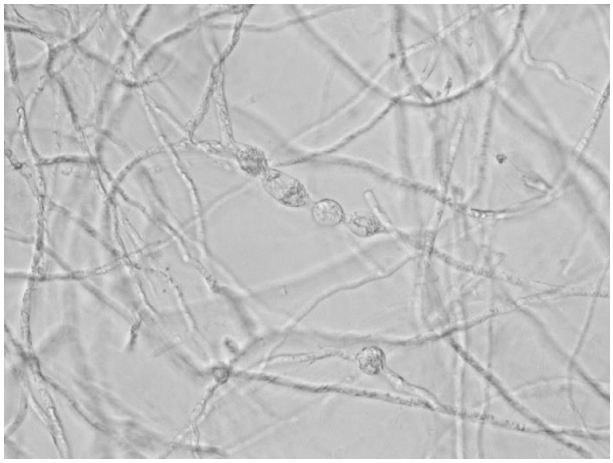
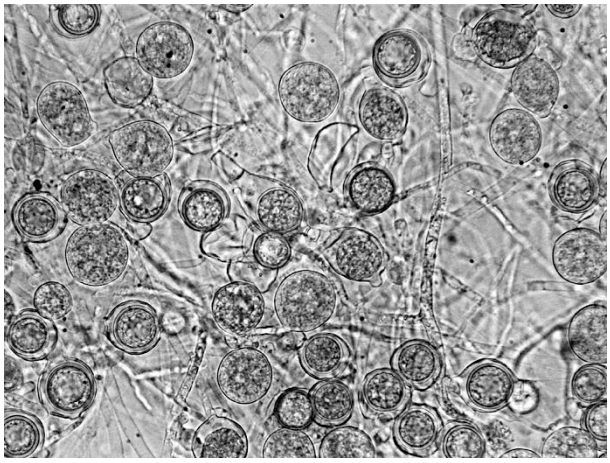


Figure 2-1. Numbers of plant-parasitic nematode genera recovered from bermudagrass *Pythium* root rot disease samples. Bars represent the number of samples where each genus of nematode was detected. White represents the number of samples, in which nematode population density was below threshold; black represents the number of samples, in which the nematode population density was over threshold. Nematode thresholds on bermudagrass are based upon number per 50-cm^3 soil (unpublished data used by the UF Nematode Assay lab).

A



B



C

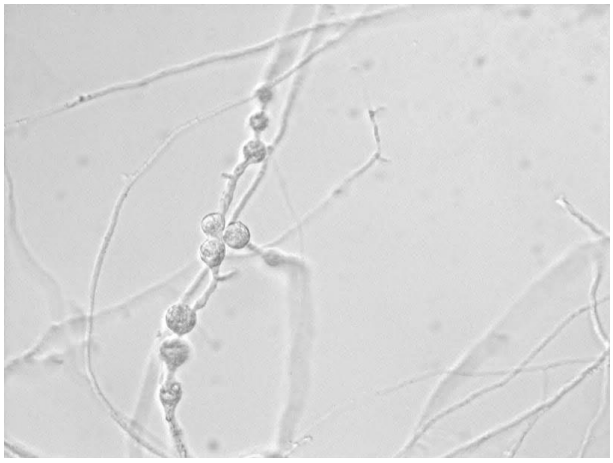


Figure 2-2. Morphological features of *Pythium* spp. isolated from the UF Nematode Assay lab samples. A) Proliferating and globose sporangia observed in isolate “1642”; B) Aplerotic oospores, intercalary oogonia, monoclinous antheridia, and globose sporangia observed in isolate “1643”; C) Catenulate sporangia observed in isolate “1651”.

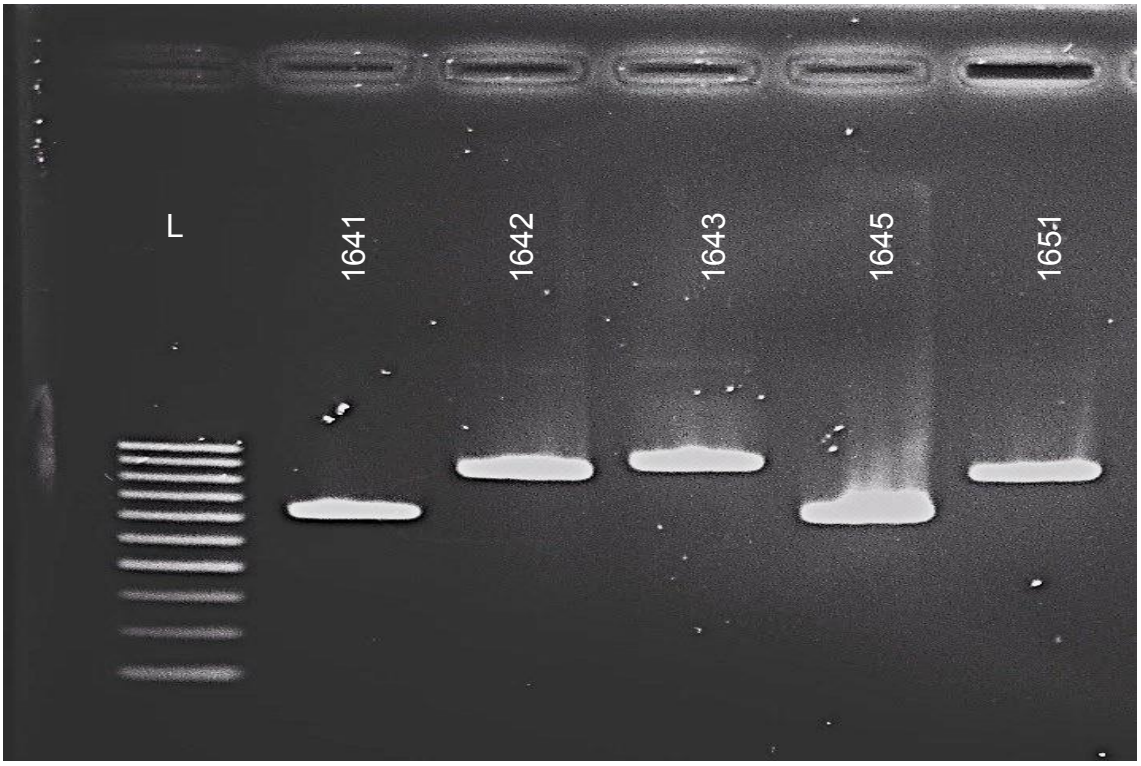


Figure 2-3. Banding patterns for PCR products electrophoresed at 110 V for 25 min. L = Apex 100 bp ladder. Amplification products from massive fungal isolate mycelia obtained using primers ITS1 and ITS4.

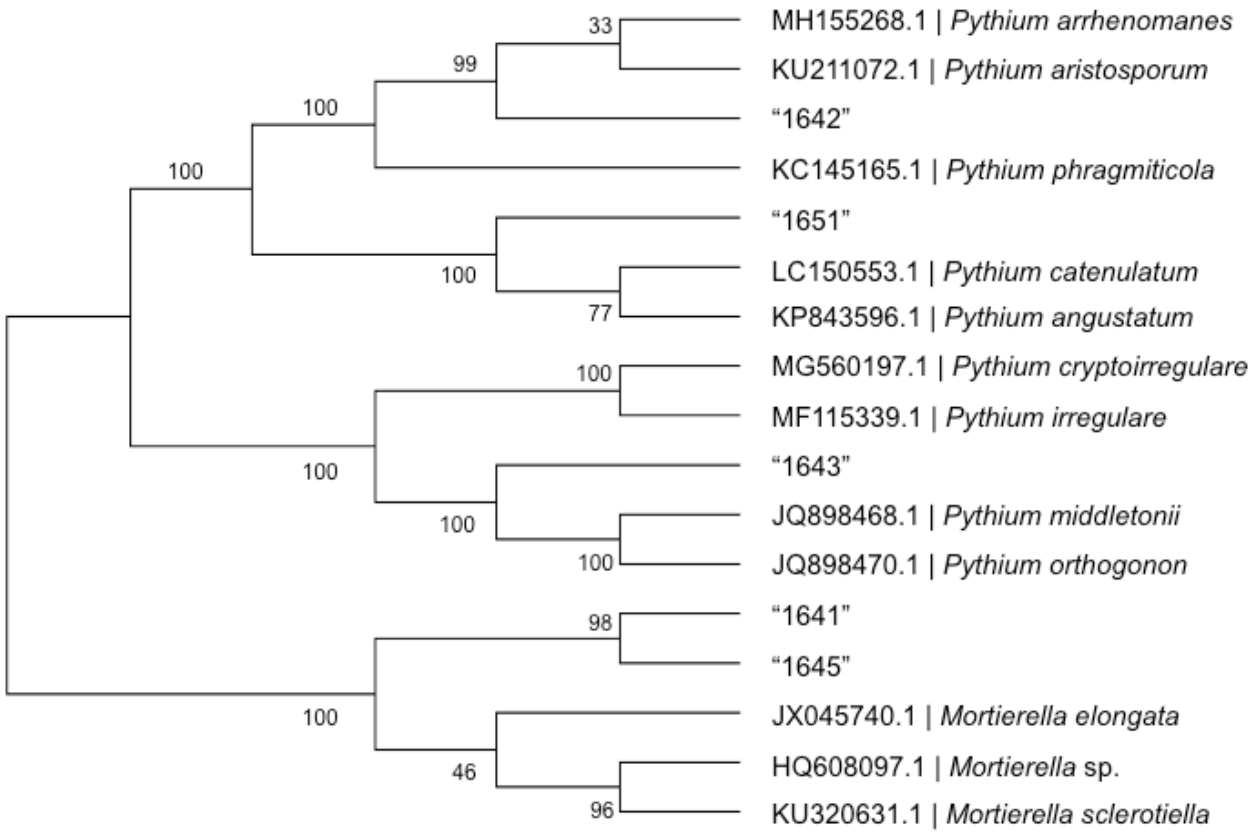


Figure 2-4. Evolutionary phylogenetic relationship among isolated pathogens and already known *Pythium* spp. Generated based on ITS ribosomal DNA sequences aligned by ClustalW and constructed by neighbor-joining tree. The associated taxa were clustered together in the bootstrap test (500 replicates), and the percentage of replicate trees were shown next to the branches. Phylogenetic analyses were conducted in MEGA7.

CHAPTER 3
BERMUDAGRASS ROOT ROT DISEASE COMPLEX TEST

Introduction

The high frequency of plant-parasitic nematode detection in nematode assays of the pre-test bermudagrass root rot disease positive samples indicated *Belonolaimus* spp. (sting nematode), *Meloidogyne* spp. (root-knot nematode), and *Hoplolaimus* spp. (lance nematode), might be associated with *Pythium* species causing bermudagrass root rot disease. Our hypothesis was that plant-parasitic nematodes have synergistic effects on bermudagrass root rot disease caused by *Pythium* spp. Based on literature, different *Pythium* species have varying virulence on different turfgrasses (Abad et al., 1994; Stiles et al., 2007). For example, *P. aphanidermatum* was highly aggressive (causing 61-100% disease) on both *Poa trivialis* and *Lolium perenne*; while *P. irregulare* was moderately aggressive (causing 21-60% disease) on *P. trivialis* and caused low level of disease (causing 1-20% disease) on *L. perenne*.

There were two objectives in this experiment. First, to determine the virulence of the three *Pythium* isolates obtained from the UF Nematode Assay lab samples on “Tifdwarf” bermudagrass (*Cynodon* spp.). Second, to determine if two different plant-parasitic nematodes (*B. longicaudatus* and *M. graminis*) increase the incidence and severity of *Pythium* root rot diseases on bermudagrass.

Material and Methods

Host Plant Preparation

One ‘Tifdwarf’ bermudagrass sprig with two nodes was planted in individual 10-ml pipette tips (Fisher Scientific International, Inc., Hampton, NH) filled with 15-cm^3 autoclaved USGA specification sand (USGA green section staff, 2018) (Figure 3-1A); or

two “Tifdwarf” bermudagrass sprigs with two nodes each were planted in each 5-cm-diameter and 15-cm-height PVC pipe containers filled with 300 cm^3 sterilized USGA specification sand (Figure 3-1B). The turf sprigs were maintained in a greenhouse by irrigating with 1.5-cm of water three times daily and fertilizing with one-ml (for pipette tip) or 50-ml (for PVC pipe) Miracle-Gro Water Soluble All Purpose Plant Food (Scotts Miracle-Gro Products, Inc., Marysville, OH) once biweekly at 5 g fertilizer/L solution.

Experimental Design

Belonolaimus longicaudatus and *M. graminis* were tested in separate experiments for their effects on infection and damage by each of the three *Pythium* species on bermudagrass. Nematode and *Pythium* inoculations occurred on either 4 or 5 weeks after sprigging (WAS). Each experiment had 9 treatments: an uninoculated control and 8 treatments that were inoculated with either the nematode or the *Pythium* spp. individually, or together on the same day, or separately one week apart (Tables 3-1; 3-2). Each of the three *Pythium* species experiments for each nematode were conducted concurrently and shared the uninoculated control and nematode-only treatments. This allowed the relative infectivity and pathogenicity to be compared among the *Pythium* species. The *B. longicaudatus* experiment was conducted twice in pipette tips with ten blocks each time and once in PVC pipe containers with four blocks. The *M. graminis* experiment was conducted three times in PVC pipe containers with four blocks. All experiments used a randomized block design.

Inocula Preparation

Plant-parasitic nematode inocula were extracted using a modified mist chamber method (unpublished method developed by Dr. William T. Crow) for 72 hours. *Belonolaimus longicaudatus* inoculum was cultured on St. Augustinegrass

(*Stenotaphrum secundatum*) and *M. graminis* inoculum was cultured on bermudagrass. Each pipette tip was inoculated with 15 *B. longicaudatus*; and each PVC pipe container was inoculated with either 50 *B. longicaudatus* or 500 *M. graminis*.

Three *Pythium* isolates, *Pythium arrhenomanes*, *P. middletonii*, and *P. catenulatum*, were used as *Pythium* inocula. Each *Pythium* inoculum was prepared by incubating mycelia plugs with sterilized St. Augustinegrass leaves in sterilized deionized water at the rate of three three-mm mycelia plugs for every six 0.5-cm St. Augustinegrass blades. *Pythium* inocula were incubated under continuous fluorescent light at 24°C for three days. Inoculation was performed by burying inoculum (*Pythium*-colonized grass blade) at a 0.5-cm depth. Each pipette tip was inoculated with one *Pythium*-colonized grass blade; each PVC pipe container was inoculated with four *Pythium*-colonized grass blades.

Data Collection and Analysis

Data were collected eight-weeks after grass sprigging. In both *B. longicaudatus* – *Pythium* and *M. graminis* – *Pythium* trials, root length was assessed using WinRHIZO equipment and software (Pang et al., 2011) followed by *Pythium* percent infection measurement. Roots were gently massaged in deionized water to remove sand, and then surface sterilized using the 0.6% sodium hypochlorite method mentioned in Chapter 2. Clean roots of each sample were cut into sections and four root sections were plated on one 100-cm-diameter petri dish (Fisher Scientific International, Inc., Hampton, NH) containing PART medium for *Pythium* isolation. The *Pythium* percent infection was calculated by isolates number / 4 (number of root sections per plate) * 100%.

All statistical analyses were performed using SAS® 9.4 (SAS Institute Inc., Cary, NC). Data from the three repetitions of each experiment were analyzed using the generalized linear model (GLM). If repetition was not significant ($P > 0.1$), the data from the repetitions were combined for analysis; if repetition was significant ($P \leq 0.1$), data in each repetition were analyzed separately. Data was subjected to analysis of variance and treatment means were separated according to Duncan's multiple-range test ($P \leq 0.1$).

Results

Pathogenicity Test of Three *Pythium* Isolates

Treatment Pa4 (*P. arrhenomanes* inoculated 4 WAS) had higher ($P \leq 0.1$) root percent infection than treatments Pc4 (*P. catenulatum* inoculated at 4 WAS) and Pm4 (*P. middletonii* inoculated 4 WAS) in the *B. longicaudatus* – *Pythium* trial (Table 3-3; Figure 3-2A) and Repetitions 2 and 3 of the *M. graminis* – *Pythium* trial (Table 3-4; Figure 3-2B); Treatment Pa5 (*P. arrhenomanes* inoculated 5 WAS) had similar results (Tables 3-3 and 3-4; Figure 3-2) when compared with treatments Pc5 (*P. catenulatum* inoculated 5 WAS) and Pm5 (*P. middletonii* inoculated 5 WAS). *Pythium* percent infection of both *P. catenulatum* (treatments Pc4 and Pc5) and *P. middletonii* (treatments Pm4 and Pm5) were low (less than 20%) in the *B. longicaudatus* – *Pythium* trial (Table 3-3; Figure 3-2A); in the *M. graminis* – *Pythium* trial, *P. catenulatum* had higher root percent infection (61% - 100%) in Repetitions 1 (treatments Pc4 and Pc5) and 3 (treatment Pc4), moderate root percent infection (21% - 60%) in Repetitions 2 (treatment Pc4) and 3 (treatment Pc5), and no infection in treatment Pc5 in Repetition 2 (Table 3-4; Figure 3-2B). *Pythium middletonii* had low root percent infection in both *B.*

longicaudatus – *Pythium* and *M. graminis* – *Pythium* trials (Tables 3-3 and 3-4; Figure 3-2).

Treatment Pa4 reduced bermudagrass root length compared to the untreated control ($P \leq 0.1$) in Repetitions 1 and 2 of both *B. longicaudatus* – *Pythium* (Table 3-3; Figure 3-3A) and *M. graminis* – *Pythium* (Table 3-4; Figure 3-3B) trials. Treatment Pc5 increased ($P \leq 0.1$) bermudagrass root length in Repetitions 2 and 3 of the *B. longicaudatus* – *Pythium* trial (Table 3-3; Figure 3-3A) and in Repetition 1 of the *M. graminis* – *Pythium* trial (Table 3-4; Figure 3-3B). Root effects by *P. middletonii* were not observed in either trial. In both trials, treatment Pa4 caused root damage similar to treatment B4 (*B. longicaudatus* inoculated 4 WAS) or treatment M4 (*M. graminis* inoculated 4 WAS) (Tables 3-3 and 3-4; Figure 3-3).

***Belonolaimus longicaudatus – Pythium* Trial**

Among those treatments inoculated with *P. arrhenomanes*, *Pythium* percent infection of treatment B4Pa5 (*B. longicaudatus* inoculated 1-week before *P. arrhenomanes*) was less ($P \leq 0.1$) than treatment Pa5 (*P. arrhenomanes* inoculated 5 WAS) (Table 3-5; Figure 3-4A); root length of treatment B5Pa4 (*B. longicaudatus* inoculated 1-week after *P. arrhenomanes*) was greater ($P \leq 0.1$) than treatment Pa4 (*P. arrhenomanes* inoculated 4 WAS) in Repetitions 1 and 2 (Table 3-5; Figure 3-4B).

In the *P. catenulatum* inoculated treatments, B5Pc4 (*B. longicaudatus* inoculated 1-week after *P. catenulatum*) had higher ($P \leq 0.1$) *Pythium* percent infection than Pc4 (*P. catenulatum* inoculated 4 WAS) in Repetitions 1 and 2; no *P. catenulatum* root infection was observed in Repetition 3 (Table 3-5; Figure 3-5A). Treatment BPc4 (*B. longicaudatus* and *P. catenulatum* inoculated together 4 WAS) had greater root length

than treatment Pc4 ($P \leq 0.1$) in Repetition 1, and than treatment B4 (*B. longicaudatus* inoculated 4 WAS) in Repetitions 1 and 2 (Table 3-5, Figure 3-5B).

Based on data presented in Table 3-5 and Figure 3-6, effects of *B. longicaudatus* on *Pythium* percent infection and root length of *P. middletonii* inoculated bermudagrass were not consistent in three repetitions.

***Meloidogyne graminis – Pythium* Trial**

In the *M. graminis – P. arrhenomanes* experiment, *Pythium* percent infection from treatment M5Pa4 (*M. graminis* inoculated 1-week after *P. arrhenomanes*) was less ($P \leq 0.1$) than from treatment Pa4 (*P. arrhenomanes* inoculated 4 WAS). Root length results were not consistent among three repetitions (Table 3-6; Figure 3-7).

Among treatments including *P. catenulatum*, treatment Pc4 (*P. catenulatum* inoculated 4 WAS) had greater ($P \leq 0.1$) *Pythium* percent infection than treatment MPc4 (*M. graminis* and *P. catenulatum* inoculated together 4 WAS) in all three repetitions (Table 3-6; Figure 3-8A); the *Pythium* percent infection of treatment M5Pc4 (*M. graminis* inoculated 1-week after *P. catenulatum*) was less ($P \leq 0.1$) than treatment Pc4 in Repetitions 2 and 3 (Table 3-6; Figure 3-8A). When compared with treatment Pc5 (*P. catenulatum* inoculated 5 WAS), treatment MPc5 (*M. graminis* and *P. catenulatum* inoculated together 5 WAS) had greater ($P \leq 0.1$) *Pythium* percent infection in Repetition 3 (Table 3-6; Figure 3-8A). The root length of treatment RC4 was less than that of treatment Pc4 ($P \leq 0.1$) in Repetitions 2 and 3 (Table 3-6, Figure 3-8B).

Based on data presented in Table 3-6 and Figure 3-9, effects of *M. graminis* on *Pythium* percent infection and root length of *P. middletonii* inoculated bermudagrass were not consistent among the three repetitions.

Discussion

Pathogenicity Test of Three *Pythium* Isolates

Results from the *Pythium* pathogenicity test indicate that *P. arrhenomanes* had the greatest bermudagrass root infection and *P. middletonii* had the lowest root infection among the three species tested. Bermudagrass root length was reduced significantly only when *P. arrhenomanes* was inoculated at 4 WAS. Taking *Pythium* infection and root length results together into consideration suggests that *P. arrhenomanes* is highly virulent on bermudagrass, *P. catenulatum* is mildly virulent on bermudagrass, and *P. middletonii* is avirulent on bermudagrass. The results of the pathogenicity test were similar to observations in other studies. Abad et al. (1994) reported that all isolates of *P. arrhenomanes* tested caused >60% disease on bentgrass; *P. catenulatum* is considered a weak bermudagrass pathogen (Smith et al., 1989; Couch, 1995).

Nematode – *Pythium* Complexes

When plant-parasitic nematodes were introduced into *Pythium*-bermudagrass system, instead of increasing disease incidence and severity, each nematode had different effects on the infection by the different *Pythium* spp. and on bermudagrass root health.

Belonolaimus longicaudatus reduced infection by *P. arrhenomanes* when inoculated one-week before *Pythium* inoculation; it increased bermudagrass root length when inoculated one-week after *Pythium* inoculation. Similar to *B. longicaudatus*, *M. graminis* reduced root infection by *P. arrhenomanes* when inoculated one-week after *Pythium* inoculation. Bermudagrass root resistance to *P. arrhenomanes* might also be induced by *M. graminis*, however, no root effect was observed in *M. graminis* - *P. arrhenomanes* complex. These observations indicate that *B. longicaudatus* and *M.*

graminis might have antagonistic effects against highly virulent *P. arrhenomanes* and reduce the root damage caused by the *Pythium* spp. Antagonistic effects may due to indirect (plant resistance induced by plant-parasitic nematodes) or direct (anti-fungal chemicals) pathogen interactions. The small molecules ascarosides produced by plant-parasitic nematodes induced gene expression associated with plant defense response and activated salicylic acid- and jasmonic acid mediated signaling pathways (Manosalva et al., 2015). Nematodes also might inhibit *Pythium* directly, as with eggs of *Tylenchulus semipenetrans* that produce anti-fungal chemicals to inhibit the mycelial growth of *Phytophthora nicotianae* and *Fusarium solani* (El-Borai et al., 2002a).

Infection by *P. catenulatum* on bermudagrass was increased when *B. longicaudatus* was involved, however, infection was reduced when roots were inoculated with *M. graminis* at the same time. *Belonolaimus longicaudatus*, an ectoparasitic that feeds on the root cortex, might wound roots and leave openings for *P. catenulatum* infection (Bergeson, 1972). Similar to *P. arrhenomanes*, the reduction of *P. catenulatum* infection by *M. graminis* might be caused by induced plant-resistance or anti-fungal chemical released by nematodes.

The results of nematode – *P. middletonii* complexes were not consistent. In most repetitions, no *Pythium* infection was observed. The occasional observation of *Pythium* infection might be due to wounding caused by nematodes providing entrance for the *Pythium*.

Summary

Results of the pathogenicity test confirmed pathogenicity of the three *Pythium* isolates. *Pythium arrhenomanes* is highly aggressive on bermudagrass and can cause severe root damage; *P. catenulatum* is a week pathogen on bermudagrass, which may

not lead to root length reduction; *P. middletonii* is not considered a bermudagrass pathogen.

When nematodes and *Pythium* were studied together, different from our hypothesis, no synergistic effects but antagonistic effects were observed when either nematode interacted with high virulent *P. arrhenomanes* on bermudagrass. Therefore, bermudagrass root rot disease identification only based on *Pythium* isolation results without species determination can be inaccurate. In the cases of *P. catenulatum* and *P. middletonii*, sometimes plant parasitic nematodes are the primary causal agent of turf root damage and the associated *Pythium* infection may be from low virulence or avirulent strains.

Accurate disease causal agent identification provides better management strategies for golf course superintendents. Based on our studies, *Pythium* species identification and nematode assay are recommended when a positive *Pythium* infection occurs. Sometimes plant-parasitic nematodes instead of *Pythium* cause the problem. More efficient management strategies can be generated when take both turf pathogens into consideration.

Table 3-1. List of treatments in the *Belonolaimus longicaudatus* – *Pythium* trial.

Treatment	The 1st inoculation (4-week after grass sprigging)	The 2nd inoculation (5-week after grass sprigging)
B4	*B. longicaudatus*	
Pa4	*P. arrhenomanes*	
Pc4	*P. catenulatum*	
Pm4	*P. middletonii*	
BPa4	*B. longicaudatus* + *P. arrhenomanes*	
BPc4	*B. longicaudatus* + *P. catenulatum*	
BPm4	*B. longicaudatus* + *P. middletonii*	
B5		*B. longicaudatus*
Pa5		*P. arrhenomanes*
Pc5		*P. catenulatum*
Pm5		*P. middletonii*
BPa5		*B. longicaudatus* + *P. arrhenomanes*
BPc5		*B. longicaudatus* + *P. catenulatum*
BPm5		*B. longicaudatus* + *P. middletonii*
B4Pa5	*B. longicaudatus*	*P. arrhenomanes*
B4Pc5	*B. longicaudatus*	*P. catenulatum*
B4Pm5	*B. longicaudatus*	*P. middletonii*
B5Pa4	*P. arrhenomanes*	*B. longicaudatus*
B5Pc4	*P. catenulatum*	*B. longicaudatus*
B5Pm4	*P. middletonii*	*B. longicaudatus*
U	Uninoculated control	Uninoculated control

Table 3-2. List of treatments in the *Meloidogyne graminis – Pythium* trial.

Treatment	The 1st inoculation (4-week after grass sprigging)	The 2nd inoculation (5-week after grass sprigging)
M4	*M. graminis*	
Pa4	*P. arrhenomanes*	
Pc4	*P. catenulatum*	
Pm4	*P. middletonii*	
MPa4	*M. graminis* + *P. arrhenomanes*	
MPc4	*M. graminis* + *P. catenulatum*	
MPm4	*M. graminis* + *P. middletonii*	
M5		*M. graminis*
Pa5		*P. arrhenomanes*
Pc5		*P. catenulatum*
Pm5		*P. middletonii*
MPa5		*M. graminis* + *P. arrhenomanes*
MPc5		*M. graminis* + *P. catenulatum*
MPm5		*M. graminis* + *P. middletonii*
M4Pa5	*M. graminis*	*P. arrhenomanes*
M4Pc5	*M. graminis*	*P. catenulatum*
M4Pm5	*M. graminis*	*P. middletonii*
M5Pa4	*P. arrhenomanes*	*M. graminis*
M5Pc4	*P. catenulatum*	*M. graminis*
M5Pm4	*P. middletonii*	*M. graminis*
U	Uninoculated control	Uninoculated control

Table 3-3. An ANOVA table from a GLM procedure for the *Pythium* isolates pathogenicity test in the *Belonolaimus longicaudatus* – *Pythium* trial. Treatments only inoculated with one pathogen and uninoculated control in Table 3-1 were selected for analyses.

		Total		Repetition 1		Repetition 2		Repetition 3	
Dependent Variable	Source	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
PPI*	Repetition	0.37	0.6891						
	Block	0.77	0.6426						
	Treatment	42.35	<.0001						
	Model	18.20	<.0001						
Root length	Repetition	63.41	<.0001						
	Block			0.44	0.9093	0.05	1.0000	4.39	0.0135
	Treatment			2.82	0.0090	7.72	<.0001	5.98	0.0003
	Model	11.43	<.0001	1.56	0.0996	3.81	<.0001	5.55	0.0002

* Dependent variable, *Pythium* percent infection.

Table 3-4. An ANOVA table from a GLM procedure for the *Pythium* isolates pathogenicity test in the *Meloidogyne graminis – Pythium* trial. Treatments only inoculated with one pathogen and uninoculated control in Table 3-2 were selected for analyses.

		Total		Repetition 1		Repetition 2		Repetition 3	
Dependent Variable	Source	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
PPI*	Repetition	2.40	0.0960						
	Block			0.34	0.7961	1.69	0.1953	1.69	0.1953
	Treatment			10.47	<.0001	13.31	<.0001	18.44	<.0001
	Model	16.36	<.0001	7.69	<.0001	10.14	<.0001	13.87	<.0001
Root length	Repetition	21.87	<.0001						
	Block			0.98	0.4199	1.05	0.3876	2.97	0.0522
	Treatment			9.55	<.0001	2.39	0.0469	1.50	0.2091
	Model	6.39	<.0001	7.21	<.0001	2.03	0.0716	1.90	0.0914

* Dependent variable, *Pythium* percent infection.

Table 3-5. An ANOVA Table from a GLM procedure for data in the *Belonolaimus longicaudatus* – *Pythium* trial. Data of three *Pythium* isolates were analyzed separately. Treatments and uninoculated control were listed in Table 3-1.

			Total		Repetition 1		Repetition 2		Repetition 3	
Isolate	Dependent Variable	Source	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
Pythium arrhenomanes	PPI*	Repetition	1.92	0.1496						
		Block	1.54	0.1376						
		Treatment	26.42	<.0001						
		Model	12.02	<.0001						
	Root length	Repetition	61.41	<.0001						
		Block			0.92	0.5145	0.56	0.8233	1.15	0.3479
		Treatment			3.10	0.0046	9.43	<.0001	2.05	0.0828
		Model	9.77	<.0001	1.95	0.0270	4.74	<.0001	1.81	0.1090
Pythium catenulatum	PPI*	Repetition	11.06	<.0001						
		Block			0.81	0.6121	0.68	0.7213	0.00	0.0000
		Treatment			3.93	0.0007	2.97	0.0066	0.00	0.0000
		Model	4.05	<.0001	2.82	0.0084	1.77	0.0516	0.00	0.0000
	Root length	Repetition	66.22	<.0001						
		Block			0.80	0.6206	1.01	0.4398	1.23	0.3206
		Treatment			5.27	<.0001	5.05	<.0001	1.48	0.2178
		Model	11.00	<.0001	2.90	0.0009	3.04	0.0006	1.41	0.2320
Pythium middletonii	PPI*	Repetition	8.41	0.0003						
		Block			0.00	0.0000	0.56	0.8240	0.95	0.4338
		Treatment			0.00	0.0000	2.91	0.0075	0.95	0.4941
		Model	2.11	0.0059	0.00	0.0000	1.69	0.0661	0.95	0.5124
	Root length	Repetition	27.28	<.0001						
		Block			3.07	0.0037	1.18	0.3204	2.16	0.1191
		Treatment			3.32	0.0028	6.69	<.0001	0.89	0.5429
		Model	4.37	<.0001	3.10	0.0004	3.77	<.0001	1.23	0.3192

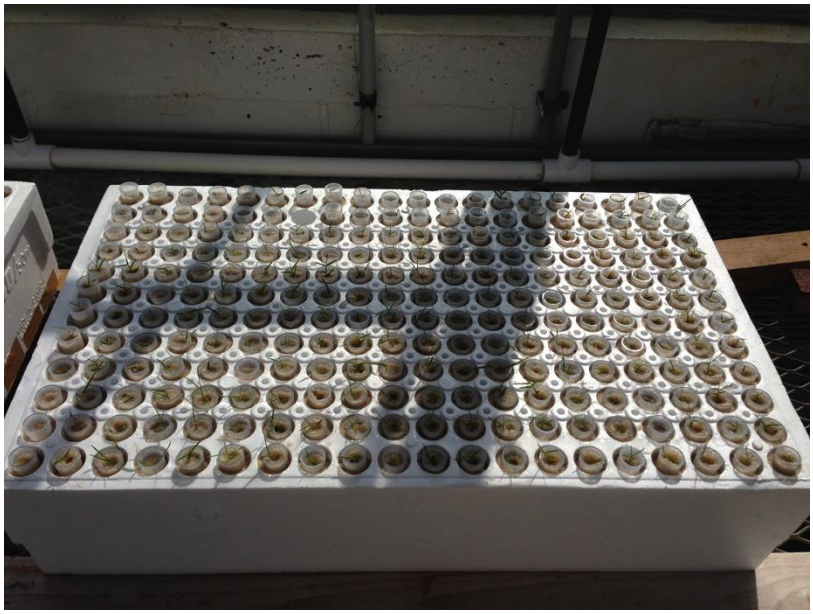
* Dependent variable, *Pythium* percent infection.

Table 3-6. An ANOVA Table from a GLM procedure for data in the *Meloidogyne graminis* – *Pythium* trial. Data of three *Pythium* isolates were analyzed separately. Treatments and uninoculated control were listed in Table 3-2.

			Total		Repetition 1		Repetition 2		Repetition 3	
Isolate	Dependent Variable	Source	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
Pythium arrhenomanes	PPI*	Repetition	1.04	0.3593						
		Block	1.45	0.2325						
		Treatment	24.83	<.0001						
		Model	15.86	<.0001						
	Root length	Repetition	16.26	<.0001						
		Block			1.78	0.1779	0.72	0.5513	5.43	0.0054
		Treatment			4.28	0.0026	0.87	0.5536	1.68	0.1560
		Model	4.21	<.0001	3.60	0.0042	0.83	0.6139	2.70	0.0203
Pythium catenulatum	PPI*	Repetition	21.80	<.0001						
		Block			3.39	0.0353	1.49	0.2418	1.15	0.3483
		Treatment			11.88	<.0001	2.25	0.0592	10.49	<.0001
		Model	8.95	<.0001	9.25	<.0001	2.05	0.0691	7.94	<.0001
	Root length	Repetition	18.53	<.0001						
		Block			0.61	0.6128	0.22	0.8823	3.69	0.0256
		Treatment			3.06	0.0159	3.67	0.0063	4.47	0.0020
		Model	6.82	<.0001	2.39	0.0357	2.73	0.0192	4.26	0.0015
Pythium middletonii	PPI*	Repetition	0.50	0.6096						
		Block	2.02	0.1162						
		Treatment	0.88	0.5326						
		Model	1.09	0.3794						
	Root length	Repetition	22.93	<.0001						
		Block			0.50	0.6882	0.21	0.8883	1.45	0.2525
		Treatment			6.65	0.0001	1.32	0.2815	1.49	0.2125
		Model	5.56	<.0001	4.97	0.0005	1.02	0.4619	1.48	0.2031

* Dependent variable, Pythium percent infection.

A



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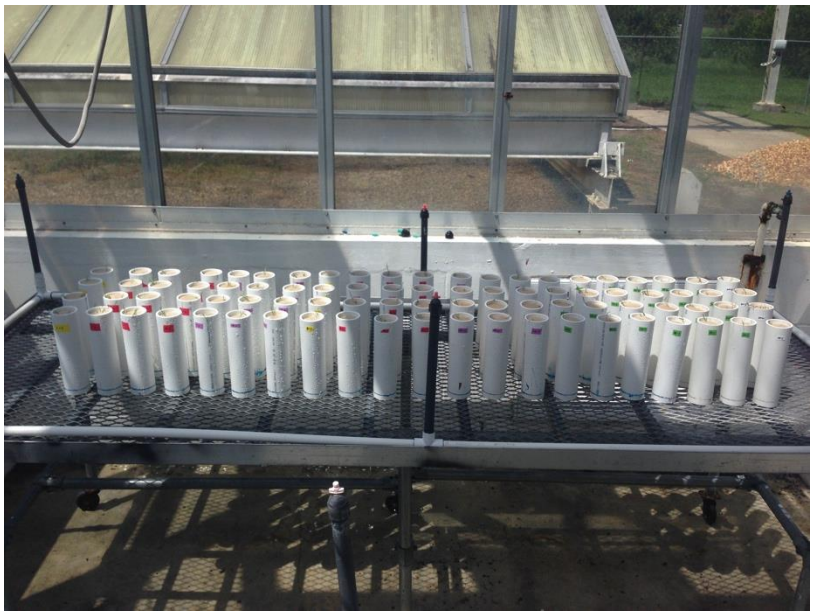
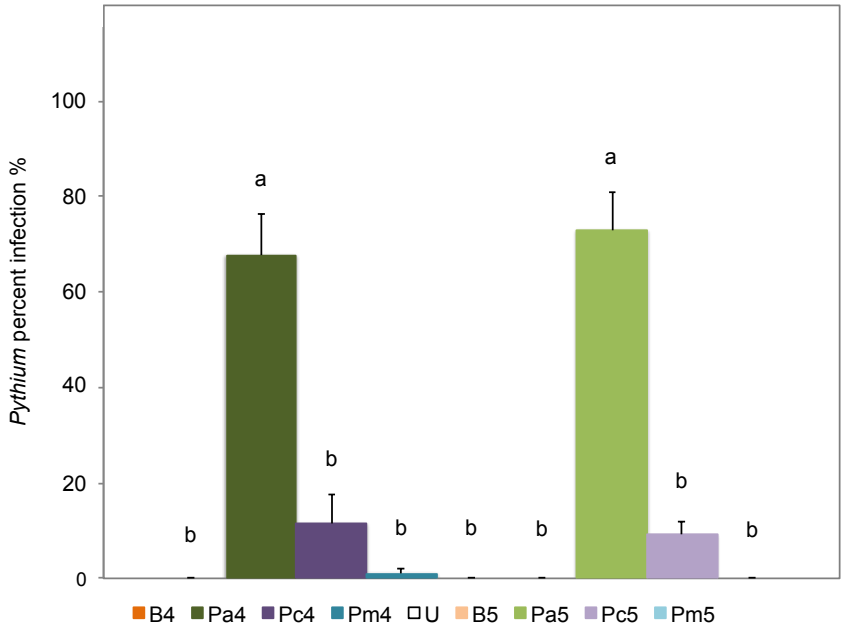


Figure 3-1. Containers in the bermudagrass root rot disease complex test. A) 10-ml pipette tips. B) 5-cm-diameter and 15-cm-height PVC pipes.



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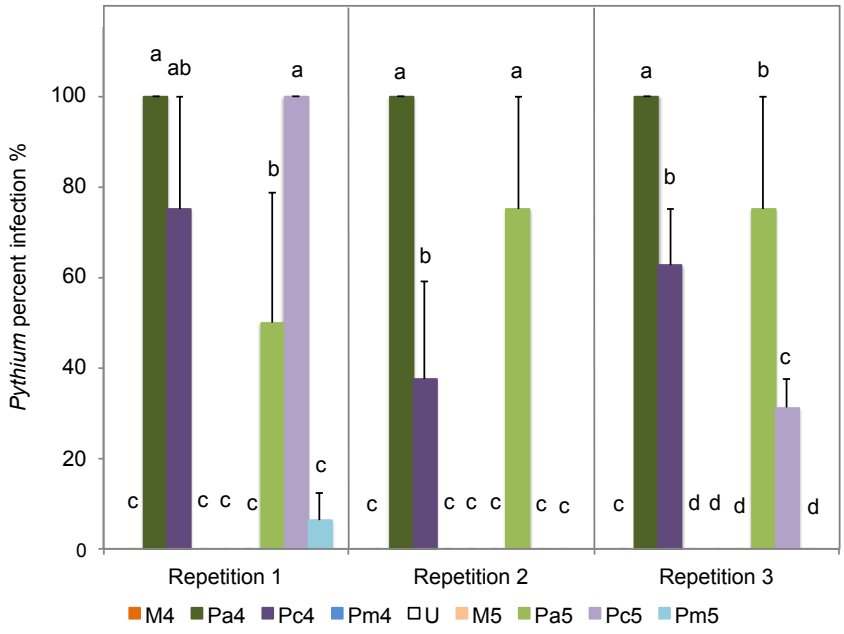
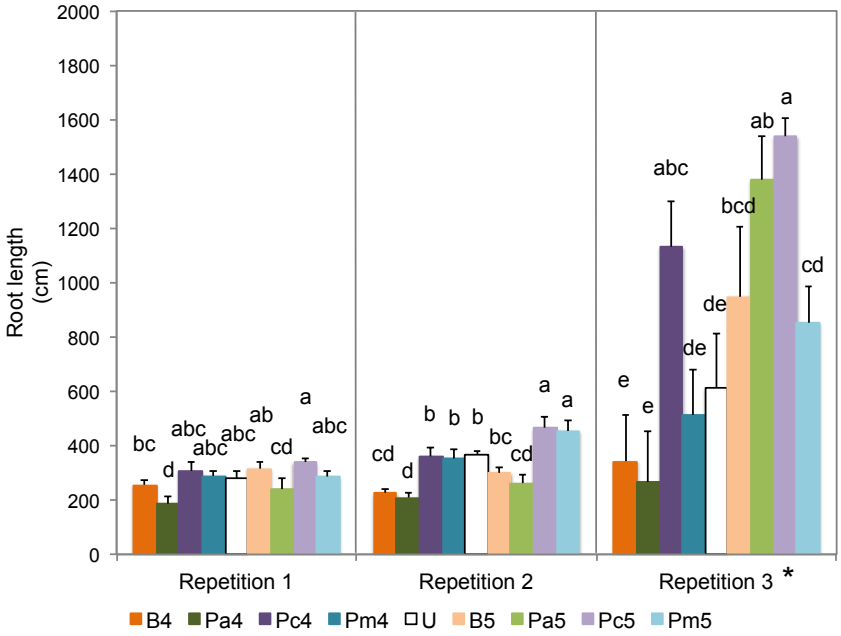
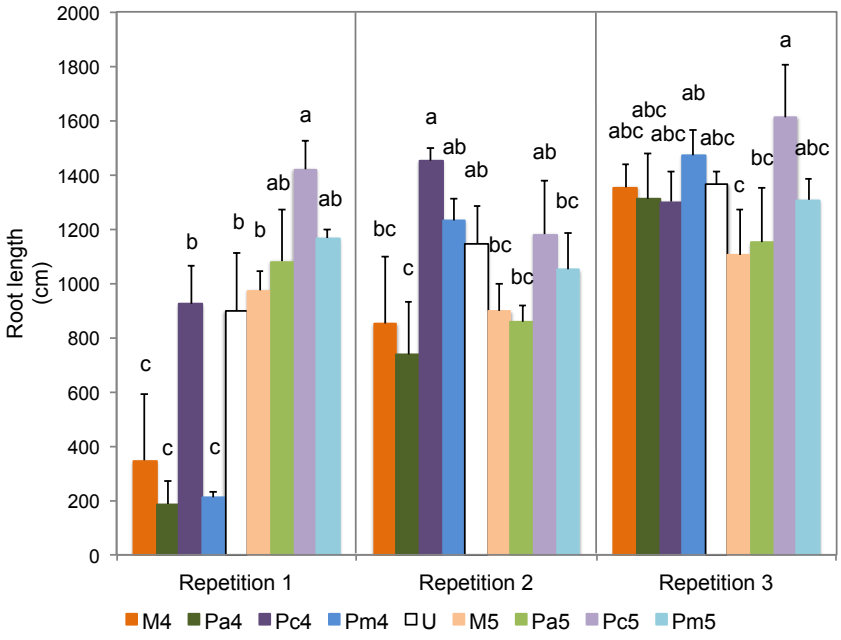


B

Figure 3-2. *Pythium* Percent infection (PPI) of three *Pythium* isolates in the pathogenicity test. Each bar is a treatment treatment mean. Means followed by common letters are not different according to Duncan's multiple-range test ($P \leq 0.1$). Error bar represents standard error of each treatment. A) PPI results of *Belonolaimus longicaudatus* – *Pythium* trial. Treatments are listed in Table 3-1. Data of all three repetitions were combined ($P > 0.05$) (n=24). B) PPI results of *Meloidogyne graminis* – *Pythium* trial. Treatments are listed in Table 3-2. Because data was significant different among repetitions ($P \leq 0.05$), each repetition was analyzed separately (n = 4).

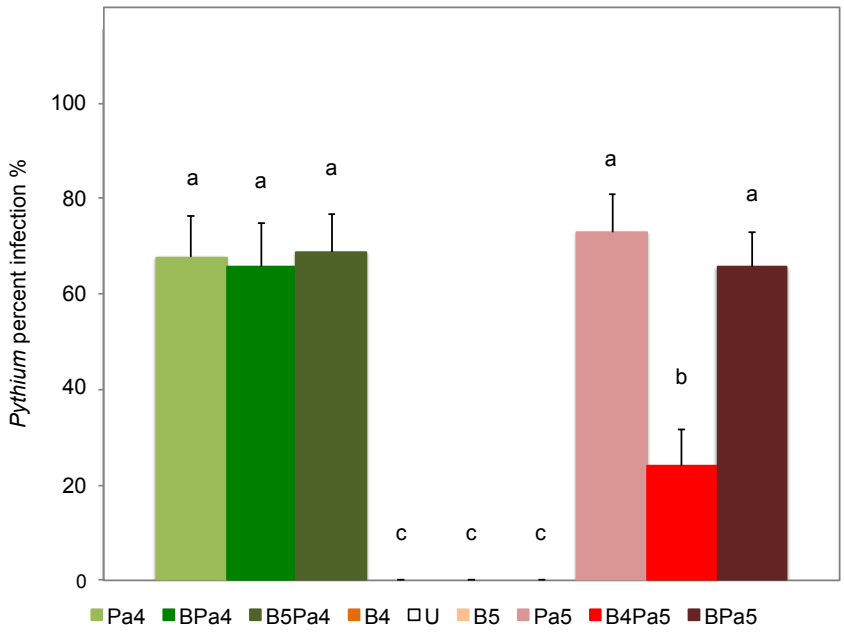


A

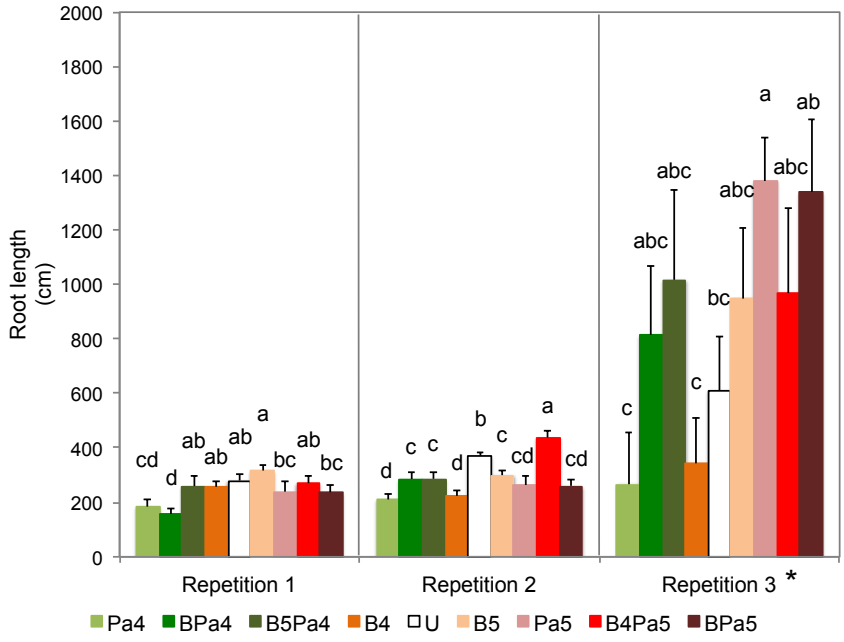


B

Figure 3-3. Bermudagrass root length in the pathogenicity test. Each bar is a treatment mean. Means followed by common letters are not different according to Duncan's multiple-range test ($P \leq 0.1$). Error bar represents standard error of each treatment. A) Root length results of *Belonolaimus longicaudatus* – *Pythium* test. Treatments are listed in Table 3-1 (n = 10 in Repetitions 1 and 2; n = 4 in Repetition 3). * Root length differences among three repetitions were because Repetitions 1 and 2 were conducted in 10-ml pipette tips, while Repetition 3 was conducted in PVC pipes. B) Root length results of *Meloidogyne graminis* – *Pythium* test. Treatments are listed in Table 3-2 (n = 4 in each repetition).

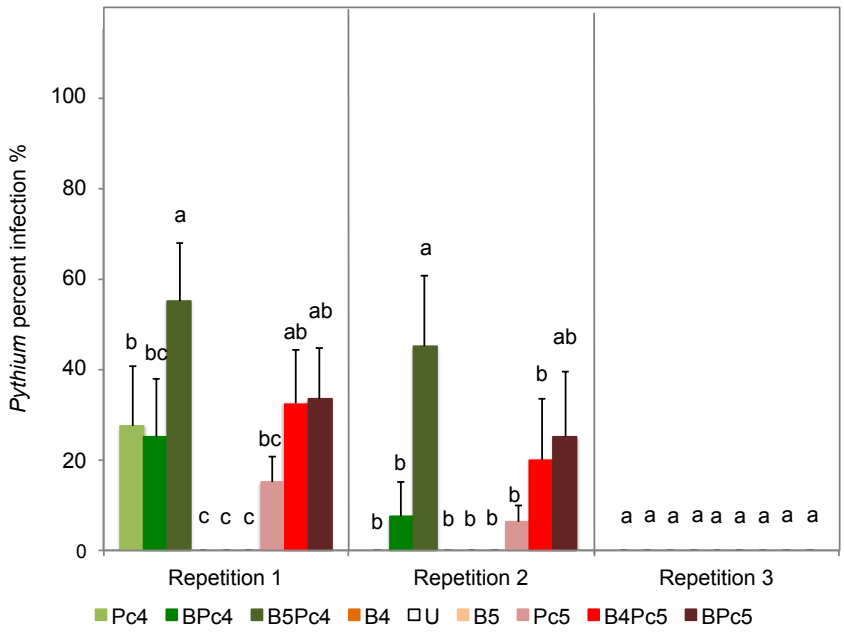


A



B

Figure 3-4. Effects of *Belonolaimus longicaudatus* on *Pythium arrhenomanes* inoculated bermudagrass. Treatments are listed in Table 3-1. Each bar is a treatment mean. Means followed by common letters are not different according to Duncan's multiple-range test ($P \leq 0.1$). Error bar represents standard error of each treatment. A) *Pythium* percent infection results. Data of all three repetitions were combined ($P > 0.05$) (n=24). B) Root length results. Each repetition was analyzed separately ($P \leq 0.05$) (n = 10 in Repetitions 1 and 2, n = 4 in Repetition 3). * Root length differences among three repetitions were because Repetitions 1 and 2 were conducted in 10-ml pipette tips, while Repetition 3 was conducted in PVC pipes.



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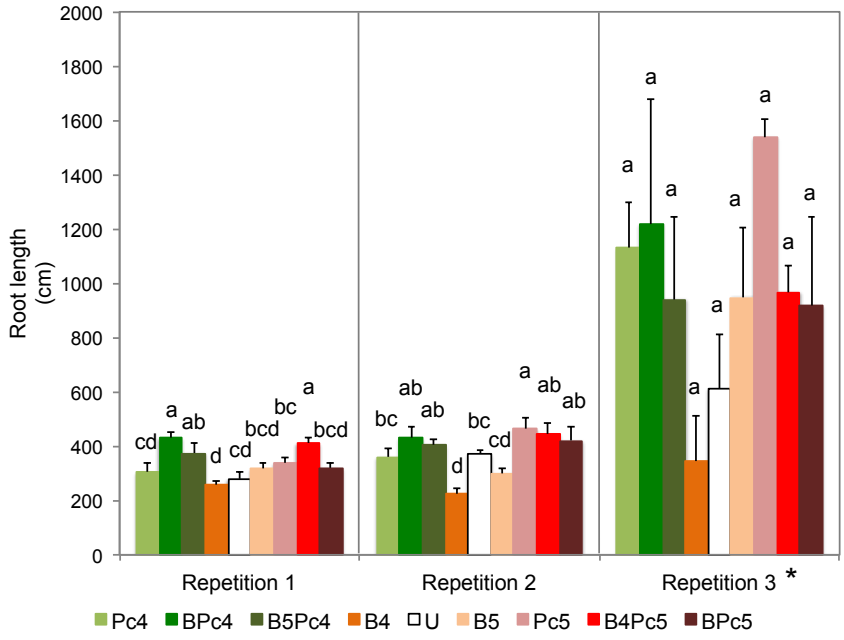


B

Figure 3-5. Effects of *Belonolaimus longicaudatus* on *Pythium catenulatum* inoculated bermudagrass. Treatments are listed in Table 3-1. Each bar is a treatment mean (n = 10 in Repetitions 1 and 2; n = 4 in Repetition 3). Means followed by common letters are not different according to Duncan's multiple-range test ($P \leq 0.1$). Error bar represents standard error of each treatment. A) Effects of *B. longicaudatus* on *Pythium* percent infection. B) Effects of *B. longicaudatus* on root length of bermudagrass inoculated with *P. catenulatum*. * Root length differences among three repetitions were because Repetitions 1 and 2 were conducted in 10-ml pipette tips, while Repetition 3 was conducted in PVC pipes.

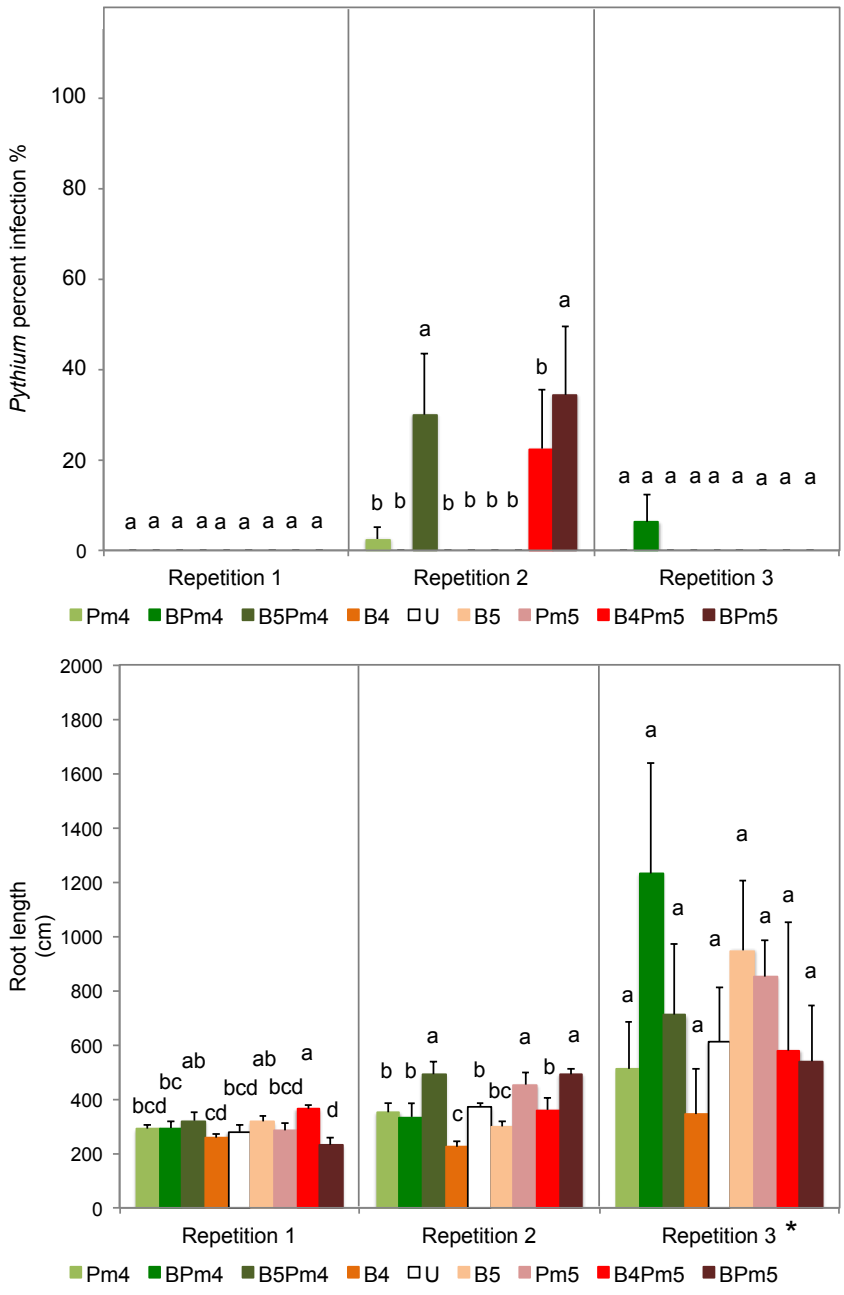


Figure 3-6. Effects of *Belonolaimus longicaudatus* on *Pythium middletonii* inoculated bermudagrass. Treatments are listed in Table 3-1. Each bar is a treatment mean (n = 10 in Repetitions 1 and 2; n = 4 in Repetition 3). Means followed by common letters are not different according to Duncan's multiple-range test ($P \leq 0.1$). Error bar represents standard error of each treatment. A) Effects of *B. longicaudatus* on *Pythium* percent infection. B) Effects of *B. longicaudatus* on root length of bermudagrass inoculated with *P. middletonii*. * Root length differences among three repetitions were because Repetitions 1 and 2 were conducted in 10-ml pipette tips, while Repetition 3 was conducted in PVC pipes.

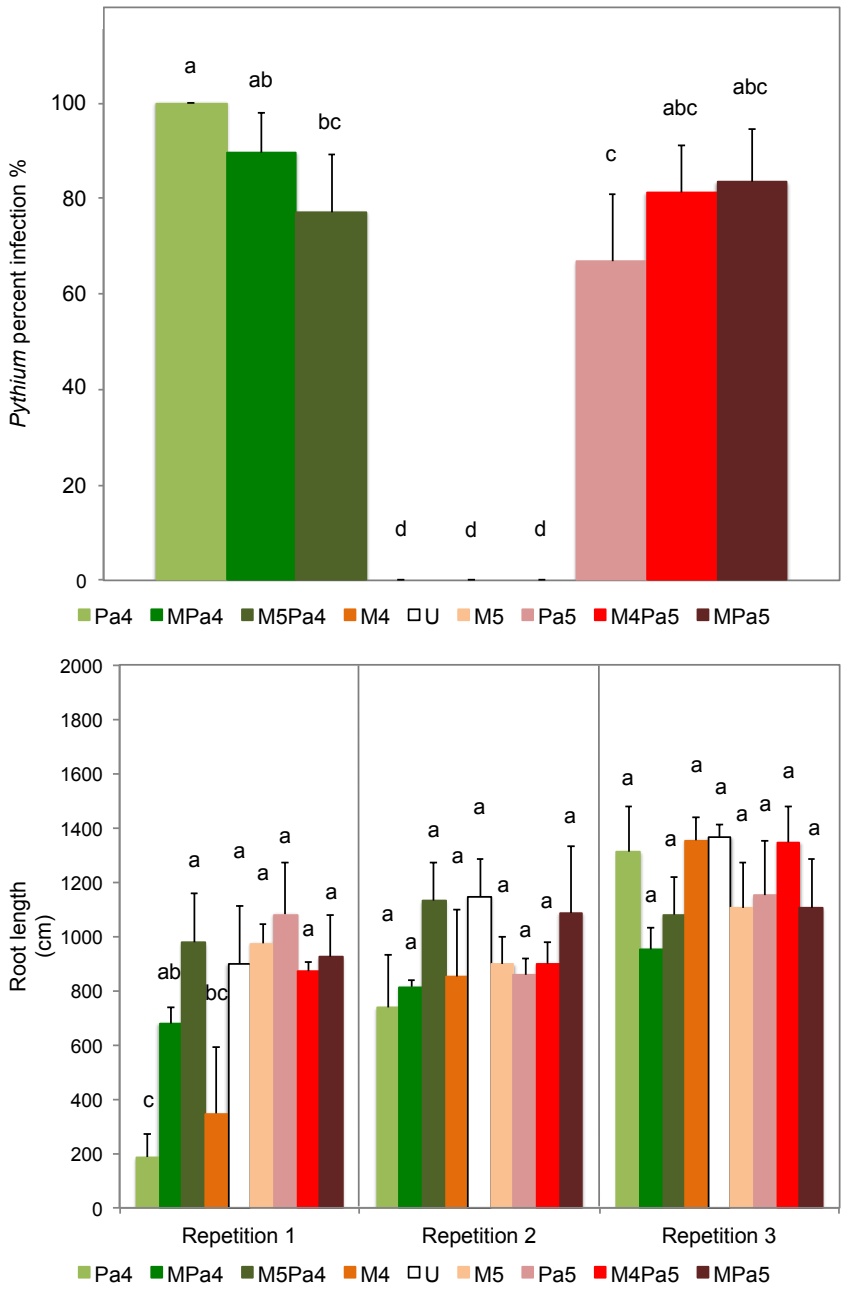
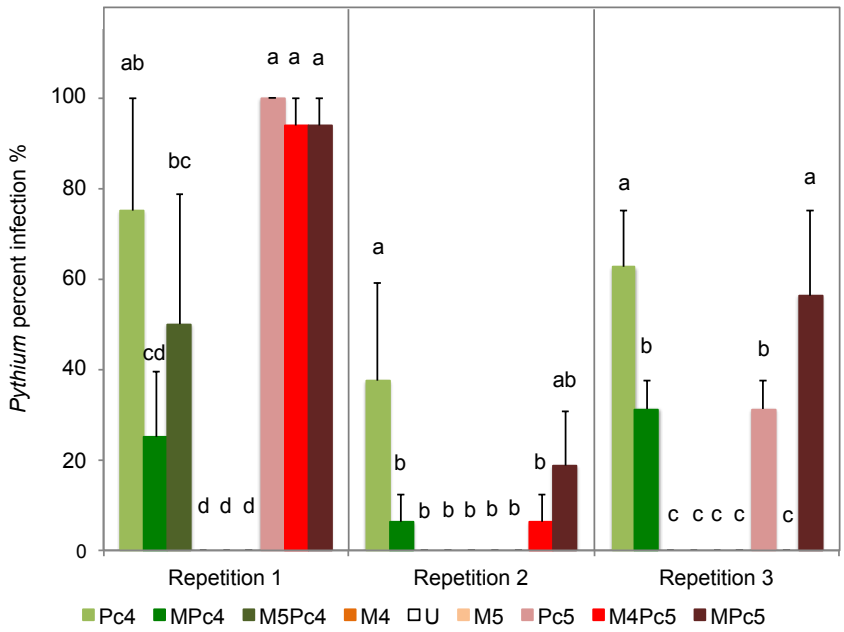
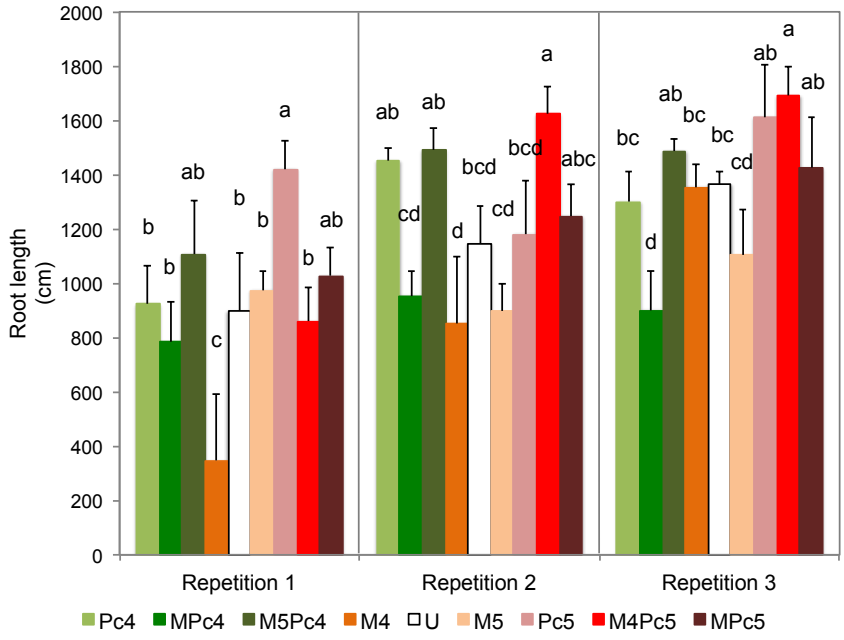


Figure 3-7. Effects of *Meloidogyne graminis* on *Pythium arrhenomanes* inoculated bermudagrass. Treatments are listed in Table 3-2. Each bar is a treatment mean. Means followed by common letters are not different according to Duncan's multiple-range test ($P \leq 0.1$). Error bar represents standard error of each treatment. A) Effects of *M. graminis* on *Pythium* percent infection. Data of all three repetitions were combined ($P > 0.05$) (n=12). B) Effects of *M. graminis* on root length of bermudagrass inoculated with *P. arrhenomanes.* Each repetition was analyzed separately ($P \leq 0.05$) (n = 4).



A



B

Figure 3-8. Effects of *Meloidogyne graminis* on *Pythium catenulatum* inoculated bermudagrass. Treatments are listed in Table 3-2. Each bar is a treatment mean (n=4). Means followed by common letters are not different according to Duncan's multiple-range test ($P \leq 0.1$). Error bar represents standard error of each treatment. A) Effects of *M. graminis* on *Pythium* percent infection. B) Effects of *M. graminis* on root length of bermudagrass inoculated with *P. catenulatum*.

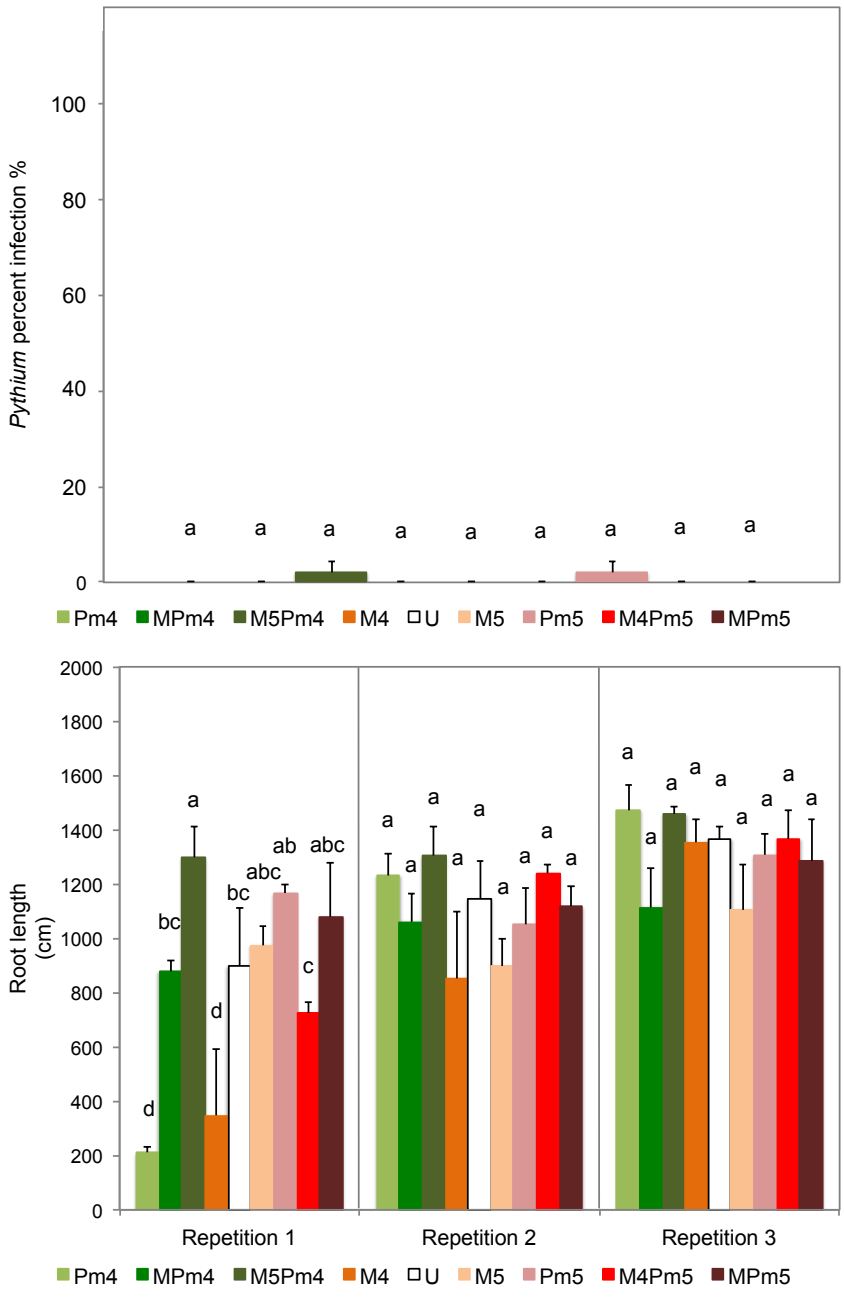


Figure 3-9. Effects of *Meloidogyne graminis* on *Pythium middletonii* inoculated bermudagrass. Treatments are listed in Table 3-2. Each bar is a treatment mean (n=4). Means followed by common letters are not different according to Duncan's multiple-range test ($P \leq 0.1$). Error bar represents standard error of each treatment. A) Effects of *M. graminis* on *Pythium* percent infection. Data of all three repetitions were combined ($P > 0.05$) (n=12). B) Effects of *M. graminis* on root length of bermudagrass inoculated with *P. middletonii*. Each repetition was analyzed separately ($P \leq 0.05$) (n = 4)

CHAPTER 4
PATHOGEN ATTRACTION TEST

Introduction

The development of a plant disease includes three components (host plant, pathogen, and favorable environment) and seven stages (inoculation, penetration, infection, colonization, reproduction, dissemination, and survival). Agrios (2005) indicated that inoculation (introducing pathogens to host plants) was very important to the occurrence of plant disease. Most pathogens are carried to host plants by wind, water and vectors. Plant-parasitic nematodes and zoospores may be attracted to host plants by the substances released from plant roots. *Meloidogyne hapla* and *M. javanica* juveniles were attracted to tomato root tips, and the number of attracted nematodes increased over time (Wang et al., 2009; Čepulytė et al, 2018). Zoospores of *Pythium aphanidermatum* were attracted to roots of many crops including alfalfa, bean, tomato, etc. (Royle and Hickman, 1964); zoospores of *P. dissotocum* were attracted to cotton root cap cells (Goldberg et al., 1989).

In a disease complex, co-occurring pathogens may affect each other antagonistically and/or synergistically. In the *Ascochyta* blight complex on pea, the co-occurring pathogens *Mycosphaerella pinodes* and *Phoma medicaginis* var*. pinodella* showed antagonistic effects on disease development when presented together on host plants; synergistic effects on damage were observed when one pathogen was inoculated ahead of the other pathogen (Le May et al., 2009). In a previous bermudagrass root rot complex disease study, both *Belonolaimus longicaudatus* and *M. graminis* had antagonistic effects on infection by *Pythium arrhenomanes*; and

bermudagrass root infection by *P. catenulatum* was increased by *B. longicaudatus* but reduced by *M. graminis*.

Greenhouse and lab tests were conducted to study the effects of nematode or *Pythium* spp. infected roots on the movement and infection of the other pathogen. Our hypotheses were that: a) bermudagrass roots inoculated with *M. graminis* or one of the three *Pythium* isolates (*P. arrhenomanes, P. catenulatum* and *P. middletonii*) would be less attractive to the other pathogen, b) *B. longicaudatus* or *P. arrhenomanes* inoculated roots would repel or reduced infection by the other pathogen, and c) that *B. longicaudatus*, or one of the two *Pythium* isolates (*P. catenulatum* and *P. middletonii*) inoculated roots would attract or increase the infection by the other pathogen.

Materials and Methods

Inocula Preparation

Three *Pythium* isolates (*Pythium arrhenomanes*, *P. catenulatum*, and *P. middletonii*) were used as *Pythium* inocula. Each *Pythium* inoculum was prepared as described in Chapter 3. Mycelia plugs were incubated with sterilized 0.5-cm St. Augustinegrass (*Stenotaphrum secundatum*) leave blades in sterilized deionized water under continuous fluorescent light at 24°C for three days. Inoculation was performed by burying inoculum (*Pythium*-colonized grass blades) at a 0.5-cm depth.

Nematode inocula, *B. longicaudatus* and *M. graminis*, were extracted using a modified mist chamber method (unpublished method developed by Dr. William T. Crow) for 72 hours. *Belonolaimus longicaudatus* were cultured on St. Augustinegrass and *M. graminis* were cultured on bermudagrass.

Greenhouse Attraction Test

U-shape PVC containers assembled by connecting three 4-cm-diameter 15-cm-length PVC pipes through two 90-degree and 5-cm-diameter PVC elbows were used in this greenhouse trial. A hole was made in the middle of each container bridge for inoculation (Figure 4-1). 'Tifdwarf' bermudagrass sprigs with four-node were planted in seed starting trays filled with autoclaved USGA specification sand (USGA green section staff, 2018) for two weeks, then transplanted into the U-shape PVC containers filled with 1000-cm^3 sterilized USGA specification sand. Two bermudagrass sprigs with similar root length and amount were planted in each 15-cm-height PVC arm of each container. Grass sprigs were maintained in greenhouse by irrigating with 1.5-cm of water three times daily and fertilizing with one-ml (for pipette tip) or 50-ml (for PVC pipe) Miracle-Gro Water Soluble All Purpose Plant Food (Scotts Miracle-Gro Products, Inc., Marysville, OH) once biweekly at 5 g fertilizer/L solution.

All containers were separated into four groups, each with 15 containers. In the first group, two weeks after transplanting, one PVC arm in each container was inoculated with *Pythium* by burying four *Pythium*-colonized grass blades of one of the three *Pythium* spp. at a 0.5-cm depth. *Belonolaimus longicaudatus* were inoculated one week later into the container bridge by pipetting 2-ml of nematode suspension containing 25 nematodes into the hole in the bridge center. For the second group, 2-ml of *B. longicaudatus* suspension containing 25 nematodes were pipetted into the top portion of one PVC arm and four *Pythium*-colonized grass blades were buried into the hole in the middle bridge at 0.5-cm depth one week later. In the third group, two weeks after transplanting, one PVC arm in each container was inoculated with one of the three *Pythium* spp. as described for group one and 2-ml of *M. graminis* suspension containing

500 J2 were pipetted into the hole in the bridge center one week later. For the forth group, 2-ml of *M. graminis* suspension containing 500 J2 were pipetted into the top portion of one PVC arm and *Pythium* was inoculated into the bridge as described for group two one week later. In each container, the PVC arm inoculated with neither *Pythium* inoculum nor nematode was the control. This test was repeated twice in greenhouse. All containers were arranged in a completely randomized design with 5 replications in each repetition of the *B. longicaudatus* trial, and either 5 replications or 10 replications for the two repetitions of the *M. graminis* trial.

Six weeks after bermudagrass sprigging, U-shape PVC containers were disassembled into three parts including two arms each contained one 4-cm-diameter 15-cm-length PVC pipe and one five-cm diameter 90-degree PVC elbow, and one bridge which was 4-cm-diameter 15-cm-length PVC pipe. Results were collected from the PVC arms. In groups one and two, *B. longicaudatus* were extracted from soil in each container arm using a centrifugal-flotation technique (Jenkins, 1964) using a 38-µm sieve (Thermo Fisher Scientific Inc., MA), and nematodes were identified into genus and counted using an Olympus CK30 inverted microscope at 20× magnification. The bermudagrass roots in each arm were washed and *Pythium* percent infection was recorded as described in Chapter 3. In groups three and four, *M. graminis* were extracted from soil in each container arm using the same method as *B. longicaudatus*. In Repetition 1, half of the roots in each arm were processed using a modification of an acid fuchsine staining method (Byrd et al., 1983) followed by counting nematodes and number of *Pythium* zoospores in roots. Roots with no zoospore attachment were marked as “0” and those with zoospore attachment were recorded as “1” (Figure 4-3);

the other half roots were processed to measure *Pythium* percent infection. In Repetition 2, the modified acid fuchsine staining method was applied to entire root systems, and only nematode numbers and zoospore observation results were recorded.

Lab Attraction Test

One 'Tifdwarf' bermudagrass sprig with two nodes was planted into each 5-ml pipette tip (Fisher Scientific International, Inc., Hampton, NH) filled with 6-cm^3 autoclaved USGA specification sand (USGA green section staff, 2018) (Figure 4-2). The bermudagrass sprigs were maintained in greenhouse by irrigating with 1.5-cm of water three times daily and fertilizing with 1-ml (for pipette tip) or 50-ml (for PVC pipe) Miracle-Gro Water Soluble All Purpose Plant Food (Scotts Miracle-Gro Products, Inc., Marysville, OH) once biweekly at 5 g fertilizer/L solution. Four weeks after bermudagrass sprigging, 15 *B. longicaudatus*, 30 *M. graminis* or one of the three *Pythium* species inocula (two *Pythium*-colonized grass blades for each species) were inoculated separately into the pipette tips planted with grass. Each treatment pipette tip had an uninoculated pipette tip to compare with. This test had 10 replications of each treatment and was repeated three times. Bermudagrass roots in each pipette tip were collected two-week after inoculation and surface sterilized by 0.6% sodium hypochlorite method mentioned in Chapter 2.

Pythium selective PART media were used to study the effects of plant-parasitic nematode infested bermudagrass roots on the mycelia growth of each *Pythium* spp. Surface sterilized *B. longicaudatus* or *M. graminis* infested roots and uninoculated roots were placed on two ends of each 100-diameter petri dish (Fisher Scientific International, Inc., Hampton, NH) contained PART media; one 3 x 3 mm mycelia plug of each *Pythium* spp. was placed in the middle of each petri dish. All petri dishes were

incubated at 24 °C for three to five days. Mycelia growth results of each *Pythium* spp. on each nematode treated and untreated roots were recorded. Roots that stopped mycelia growth were marked as “0” and those did not stop the growth of mycelia were recorded as “1” (Figure 4-4).

Pluronic F-127 gel was used to study the effects of each *Pythium* spp. infested bermudagrass roots on the movement of *B. longicaudatus* or *M. graminis*. To make 23% 100-ml gel, 23 g Pluronic F-127 powder (Sigma-Aldrich Corporation, St. Louis, MO) was added to 60-ml cold, deionized water, and stirred to dissolve at 6 °C for 24 hours; then cold deionized water was added to make the total solution 100 ml and continuously stirred at 6 °C for another hour to make solution was well mixed (Wang et al., 2009). The dissolved gel was stored at 10 °C. Five-ml of 23% Pluronic gel containing 20 *B. longicaudatus* or 50 *M. graminis* J2*,* fresh extracted by a modified mist chamber method (unpublished method developed by Dr. William T. Crow), was poured into 5-cm-diameter petri dishes (Fisher Scientific International, Inc., Hampton, NH) at 10 °C. Surface sterilized *Pythium* inoculated and non-inoculated roots were placed on two ends of petri dish, then the petri dish was transferred to 24 °C for the gel to solidify. The number of plant-parasitic nematodes around the root surface (0.5-cm) was counted 24 hours later using an Olympus CK30 inverted microscope.

Data Analyses

All statistical analyses were performed using SAS® 9.4 (SAS Institute Inc., Cary, NC). T-tests were conducted to compare each inoculated treatment with the uninoculated control.

Results

Pythium Inoculated Roots - Nematode

In the greenhouse attraction test, only *P. middletonii* inoculated roots had more (P = 0.07) *B. longicaudatus* recovered than uninoculated roots in Repetition 1 (Table 4-1). In the lab attraction test, the number of *B. longicaudatus* was higher around *P. middletonii* (P = 0.002 in Repetition 1) or *P. arrhenomanes* (P = 0.04 in Repetition 2 and P = 0.03 in Repetition 3) infected bermudagrass roots than from around roots of the uninoculated (Table 4-2); roots inoculated with *P. catenulatum* (P < 0.0001 in Repetition1 and P = 0.01 in Repetition 2) or *P. arrhenomanes* (P = 0.09 in Repetition 1) were less attractive to *B. longicaudatus* than uninoculated roots in Repetitions 1 (P = 0.001) and 2 (P = 0.01) of the lab attraction test (Table 4-2).

Meloidogyne graminis was more attracted (P = 0.08) by bermudagrass roots inoculated with *P. arrhenomanes* in Repetition 1 in the greenhouse attraction test (Table 4-3). In the lab attraction test, bermudagrass roots inoculated with *P. middletonii* or *P. arrhenomanes* were less attractive to *M. graminis* compared with inoculated roots, Repetition 2 for *P. middletonii* (P = 0.08) and Repetition 3 for *P. arrhenomanes* (P = 0.002) (Table 4-4). *Meloidogyne graminis* had no preference for roots inoculated with *P. catenulatum* in any of the tests (Tables 4-3, 4-4).

Nematode Infested Roots - *Pythium*

In the greenhouse attraction test, no *Pythium* infection was observed on *B. longicaudatus* infested or non-infested bermudagrass roots (Table 4-5); there was no *Pythium* percent infection difference between *M. graminis* inoculated and uninoculated bermudagrass roots (Table 4-8). More *P. catenulatum* zoospores were observed on *M.*

graminis infested roots in Repetition 1 (P = 0.07) than around uninoculated roots (Table 4-7).

In the lab attraction test, mycelia contact from *P. arrhenomanes* (P = 0.04) was less for roots inoculated with *B. longicaudatus* than for uninoculated roots (Table 4-6). *Meloidogyne graminis* infested roots had less (P = 0.04) contact from *P. catenulatum* than uninoculated roots in Repetition 2 (Table 4-9).

Discussion

For the greenhouse tests, the number of nematodes recovered, percent *Pythium* infection, and the zoospore attachments were all very low and, therefore, the results from those trials were inconclusive. Similarly, in the lab attraction test the numbers of nematodes interacting with roots were low and the results were highly variable among repetitions. Therefore, while some significant differences occurred it is difficult to draw any conclusions from that data.

Bermudagrass roots infested with either *B. longicaudatus* or *M. graminis* reduced mycelia growth of three *Pythium* spp., although inconsistently. The suppression on mycelia growth might be caused by nematodes inducing bermudagrass roots to release defence chemicals. Anti-fungal chemicals were produced by *Tylenchulus semipenetrans* to inhibit the mycelial growth of *Phytophthora nicotianae* and *Fusarium solani* and protect their feeding sites in citrus roots (El-Borai et al., 2002a). Bais et al. (2002) observed rosmarinic acid (RA) in sweet basil root exudate when plant roots were elicited using *Phytophthora cinnamon* and *Pythium ultimum*. RA is a caffeic acid ester demonstrated has potential antimicrobial ability. Naphthoquinones was present in the root exudates of gromwell, which had biological activity against soil-borne fungi and bacteria (Brigham et al, 1999). In order to confirm the presence of defence chemicals in

the root exudates, a metabolomics tests on pathogen infested bermudagrass root exudates should be conducted.

Based on these results, the effects of nematode or *Pythium* inoculated roots on the movement and infection of the other pathogen were not fully answered. More replications and larger nematode population density should be involved if experiments will be conducted again.

Table 4-1. Results from a greenhouse attraction test evaluating the number of *Belonolaimus longicaudatus* around *Pythium* inoculated (I) and uninoculated (U) bermudagrass roots.

Treatment	Repetition 1		Repetition 2	
	I	U	I	U
P. middletonii	0.6 ± 0.5 *	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. catenulatum	0.0 ± 0.0	0.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
P. arrhenomanes	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Mean with * is different from uninoculated control according to t-test, P ≤ 0.1.

Table 4-2. Results from a lab attraction test evaluating the number of *Belonolaimus longicaudatus* around *Pythium* inoculated (I) and uninoculated (U) bermudagrass roots.

Treatment	Repetition 1		Repetition 2		Repetition 3	
	I	U	I	U	I	U
P. middletonii	1.0 ± 0.7 ***	0.1 ± 0.3	0.9 ± 0.7	0.4 ± 0.7	2.2 ± 1.5	2.0 ± 1.3
P. catenulatum	0.1 ± 0.3 ***	1.1 ± 0.6	0.2 ± 0.4 **	1.3 ± 1.1	3.0 ± 1.9	3.5 ± 2.7
P. arrhenomanes	0.0 ± 0.0 *	1.1 ± 1.9	0.9 ± 0.9 **	0.2 ± 0.4	3.5 ± 2.3 **	1.5 ± 1.4

Means with *, **, *** are different from uninoculated control according to t-test, P ≤ 0.1, 0.05, 0.01, respectively.

Table 4-3. Results from a greenhouse attraction test evaluating the number of *Meloidogyne graminis* around *Pythium* inoculated (I) and uninoculated (U) bermudagrass roots.

Treatment	Repetition 1		Repetition 2	
	I	U	I	U
P. middletonii	1.4 ± 1.7	0.4 ± 0.9	1.4 ± 1.7	0.3 ± 0.6
P. catenulatum	2.8 ± 4.1	0.8 ± 1.8	2.0 ± 2.9	3.3 ± 3.8
P. arrhenomanes	3.6 ± 2.6 *	0.8 ± 1.8	0.1 ± 0.3	0.3 ± 0.7

Mean with * is different from uninoculated control according to t-test, $P \leq 0.1$.

Table 4-4. Results from a lab attraction test evaluating the number of *Meloidogyne graminis* around *Pythium* inoculated (I) and uninoculated (U) bermudagrass roots.

Treatment	Repetition 1		Repetition 2		Repetition 3	
	I	U	I	U	I	U
P. middletonii	5.4 ± 6.6	5.2 ± 2.4	0.5 ± 1.0 *	1.3 ± 0.9	1.3 ± 0.9	2.1 ± 1.7
P. catenulatum	4.0 ± 2.9	3.8 ± 3.5	0.1 ± 0.3	0.5 ± 0.7	2.0 ± 2.1	2.3 ± 2.2
P. arrhenomanes	4.4 ± 5.3	6.3 ± 7.3	0.3 ± 0.5	0.2 ± 0.6	1.2 ± 0.9 ***	3.3 ± 1.6

Means with *, *** are different from uninoculated control according to t-test, $P \leq 0.1$, 0.01, respectively.

Table 4-5. Results from a greenhouse attraction test evaluating the *Pythium* percent infection (%) on *Belonolaimus longicaudatus* inoculated (I) and uninoculated (U) bermudagrass roots.

Treatment	Repetition 1		Repetition 2	
	I	U	I	U
P. middletonii	0 ± 0	0 ± 0	0 ± 0	0 ± 0
P. catenulatum	0 ± 0	0 ± 0	0 ± 0	0 ± 0
P. arrhenomanes	0 ± 0	0 ± 0	0 ± 0	0 ± 0

No significant differences between inoculated treatment and uninoculated control was observed ($P > 0.1$) according to t-test.

Table 4-6. Results from a lab attraction test evaluating *Pythium* mycelia growth on *Belonolaimus longicaudatus* inoculated (I) and uninoculated (U) bermudagrass roots.

Treatment	Repetition 1		Repetition 2	
	I	U	I	U
P. middletonii	0.5 ± 0.5	0.8 ± 0.4	1.0 ± 0.0	1.0 ± 0.0
P. catenulatum	0.8 ± 0.4	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
P. arrhenomanes	0.6 ± 0.5 **	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0

Mean with ** is different from uninoculated control according to t-test, $P \leq 0.05$.

Table 4-7. Results from a greenhouse attraction test evaluating *Pythium* zoospore observation on *Meloidogyne graminis* inoculated (I) and uninoculated (U) bermudagrass roots.

Treatment	Repetition 1		Repetition 2	
	I	U	I	U
P. middletonii	0.8 ± 0.4	0.4 ± 0.5	0.8 ± 0.4	1.0 ± 0.0
P. catenulatum	0.8 ± 0.4 *	0.2 ± 0.4	1.0 ± 0.0	0.9 ± 0.3
P. arrhenomanes	0.8 ± 0.4	0.4 ± 0.5	0.8 ± 0.4	0.8 ± 0.4

Mean with * is different from uninoculated control according to t-test, P ≤ 0.1.

Table 4-8. Results from a greenhouse attraction test evaluating the *Pythium* percent infection (%) on *Meloidogyne graminis* inoculated (I) and uninoculated (U) bermudagrass roots.

Treatment	Repetition 1	
	I	U
P. middletonii	20 ± 27	20 ± 27
P. catenulatum	50 ± 40	15 ± 14
P. arrhenomanes	55 ± 37	75 ± 35

No significant differences between inoculated treatment and uninoculated control was observed (*P* > 0.1) according to t-test.

Table 4-9. Results from a lab attraction test evaluating *Pythium* mycelia growth on *Meloidogyne graminis* inoculated (I) and uninoculated (U) bermudagrass roots.

Treatment	Repetition 1		Repetition 2	
	I	U	I	U
P. middletonii	0.7 ± 0.5	0.8 ± 0.4	0.5 ± 0.5	0.7 ± 0.5
P. catenulatum	1.0 ± 0.0	1.0 ± 0.0	0.6 ± 0.5 **	1.0 ± 0.0
P. arrhenomanes	1.0 ± 0.0	1.0 ± 0.0	0.8 ± 0.4	1.0 ± 0.0

Mean with ** is different from uninoculated control according to LSMeans, P ≤ 0.05.

Figure 4-1. U-shape PVC containers applied in the greenhouse attraction test. Each container was assembled by connecting three four-cm diameter 15-cm length PVC pipes through two 90-degree five-cm diameter PVC elbows. The left elbow was inoculated with one pathogen (nematode or *Pythium* spp.) as treatment; the right elbow was inoculated with nothing as control. One-week after treatment inoculation, the other pathogen (*Pythium* spp. or nematode) was inoculated to the hole in the middle of the container bridge.

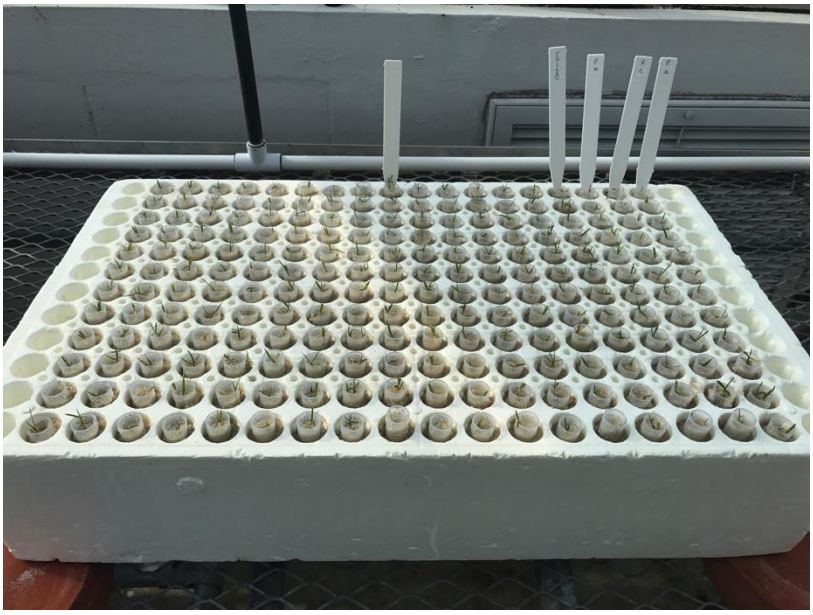
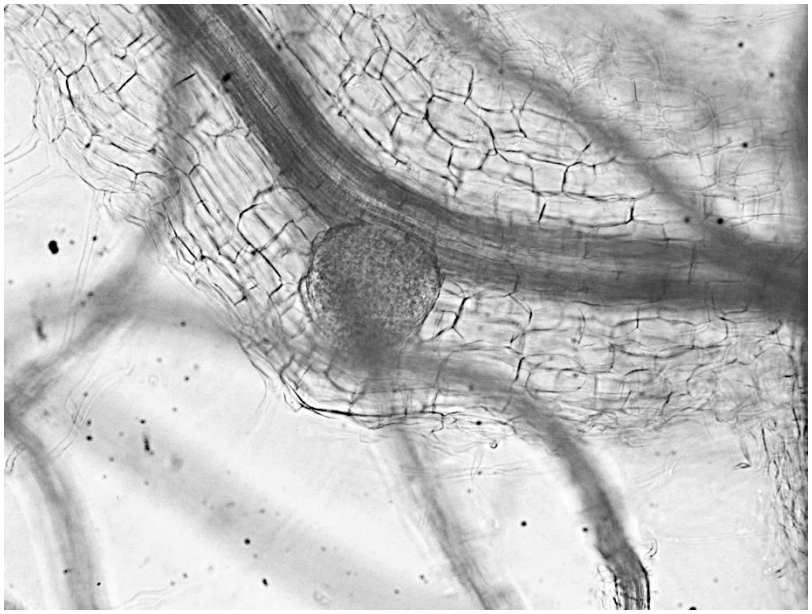


Figure 4-2. Bemudagrass sprigs planted in five-ml pipette tips in the lab attraction test.

A



B

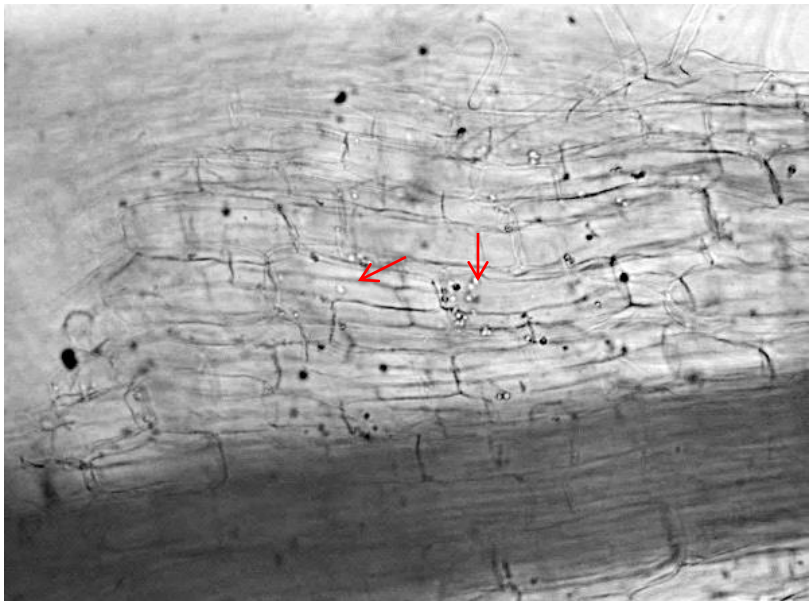


Figure 4-3. One example of bermudagrass roots with and without *Pythium* zoospore attachment. Zoospores indicated by red arrows. A) Roots with no *Pythium* zoospore attachment were marked as “0”. B) Roots had *Pythium* zoospore attached were recorded as “1”.

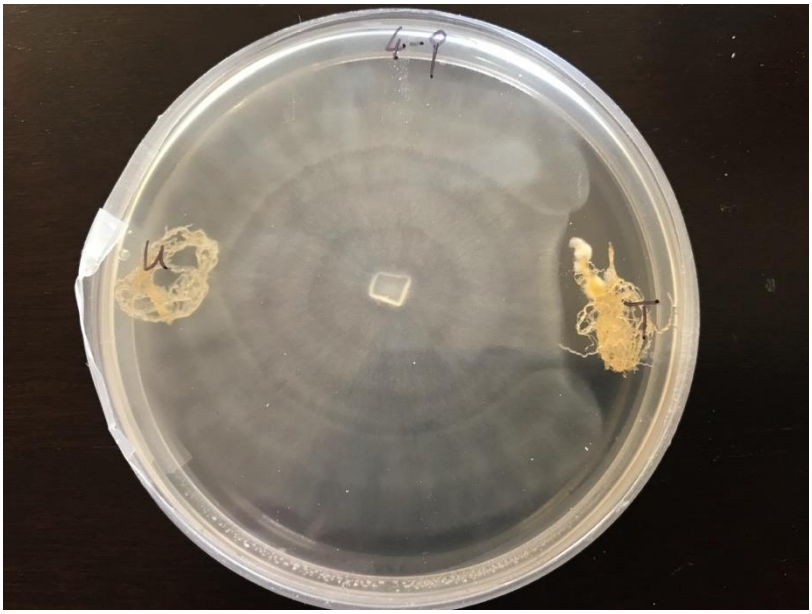


Figure 4-4. One example of nematode-infected bermudagrass roots stopping *Pythium* mycelia growth. Roots stopped mycelia growth (on the right side of PART medium) were marked as “0” and those did not stop the growth of mycelia (on the left side of PART medium) were recorded as “1”. In this example, “T ” is bermudagrass roots inoculated with *Belonolaimus longicaudatus*, “U” is uninoculated roots, and *P. middletonii* mycelia plug is placed in the middle.

CHAPTER 5
ROOT EXUDATE METABOLOMIC TEST

Introduction

Plant roots diffuse many compounds that impact interactions in rhizosphere, including root-root, root-insect, and root-microbe interactions. These interactions can be classified as positive, negative, or neutral associations (Bais et al., 2006). Hiltner first used “rhizosphere effect” in 1904 to describe the interaction between plant roots and organisms in soil. Plant roots diffuse nutrients in exudates that attract many organisms in soil including soil-borne pathogens, nematodes, insects, etc. The chemical attraction of soil organisms to roots is also called “chemotaxis” (Bais et al., 2004). Chemotaxis studies have been conducted on many organisms including oomycete zoospores and plant-parasitic nematodes (Royle and Hickman, 1963; Goldberg et al., 1989; Zhou and Paulitz, 1993; Nicol et al., 2003; Wang et al., 2009; Čepulytė et al., 2018). Plant root exudates can also contain compounds such as rosmarinic acid, salicylic acid, etc. that have antagonistic effects on soil organisms (Bais et al., 2002; Branch et al., 2004).

Our specific research interest was nematode-*Pythium*-root interactions. Root exudates provide carbon sources that attract plant-parasitic nematodes and *Pythium* spp. to the rhizosphere. However, they may also contain adverse factors to *Pythium* spp. and plant-parasitic nematodes. Antagonistic effects were observed in our previous experiments.

In this experiment, compounds in root exudates of bermudagrass inoculated with and without different pathogens were identified and compared. My hypothesis was that fungicidal or nematicidal compounds would be detected in nematode or *Pythium* inoculated bermudagrass root exudates.

Materials and Methods

Root Exudates Collection

Two 'Tifdwarf' bermudagrass sprigs with four nodes each were planted into 4-cm-diameter ConeTainers™ (Stuewe & Sons., Inc., Tangent, OR) filled with 125-cm^3 autoclaved USGA specification sand (USGA green section staff, 2018). Two weeks after grass sprigging all ConeTainers were divided into six groups with twelve ConeTainers in each group. Five groups were inoculated with 50 *Belonolaimus longicaudatus*, 130 *Meloidogyne graminis*, four *Pythium arrhenomanes*-colonized St. Augustinegrass blades, four *P. catenulatum*-colonized St. Augustinegrass blades, or four *P. middletonii*-colonized St. Augustinegrass blades, separately. The last group was an uninoculated control. Plant-parasitic nematode and *Pythium* inocula were prepared with the methods described in Chapter 3. Bermudagrass sprigs were maintained in greenhouse by irrigating with 1.5-cm of water three times daily and fertilizing with one-ml (for pipette tip) or 50-ml (for PVC pipe) Miracle-Gro Water Soluble All Purpose Plant Food (Scotts Miracle-Gro Products, Inc., Marysville, OH) once every two weeks at 5 g fertilizer/L solution. Six-week after sprigging, all ConeTainers were taken down for root exudates collection.

Containers of each treatment were subdivided into four sets with three ConeTainers in each set. All bermudagrass roots in each set were washed in running deionized water followed by rinsing with sterilized Milli-Q water for 30 sec, then transferred to 120-ml sterilized glass flasks containing 100-ml of sterilized Milli-Q water for 24 hours at 24 °C. Milli-Q water containing exudates was immediately filter sterilized using 0.22-µm syringe filters (Fisher Scientific International, Inc., Hampton, NH). Thirty-five-ml of root exudates from each treatment were immediately frozen using liquid

nitrogen, then lyophilized using a Labconco FreeZone Bulk Tray Dryer (Labconco Corporation, Kansas City, MO) and stored in −80 °C.

Root Exudates Analysis

Six root exudates samples (one in each treatment and the control) were sent to the UF/SECIM Cores for a LC-MS Global metabolomics test on an UPLC-HRMS (Model: Thermo Ultimate 3000 UPLC and Thermo Q Exactive mass spectrometer) (Khan et al., 2018).

MZmine 2.34 software was used to analyze the raw data collected from the UPLC-HRMS. After removing additives and complexes from the data set, data from positive and negative ion modes were blast through mzCloud™ (an advanced mass spectral database).

Results

The metabolite compound blast results of the six root-exudate samples are listed in Table 5-1. There were 143 targeted metabolites observed in all six samples; 53 metabolites were found through negative ion mode, and 90 metabolites were obtained through positive ion mode.

Among all compounds analyzed, isoleukotoxin, benzoic acid, isorhamnetin, acetamide, trans-cinnamic acid, adenine, D-alanine methyl ester, L-alanine methyl ester, L(+)-2-aminobutyric acid, melamine, methionine, methyl picolinate, spermidine, and gamma-aminobutyric acid (GABA) were only present in the pathogen non-inoculated bermudagrass (control U).

Metabolites 1,5-anhydro-D-glucitol, D-(+)-fucose, L(-)-fucose, benzene sulfonic acid, and 6,7-dihydro-1H-[1,4]dioxino[2',3' 4,5]benzo[d]imidazole-2-thiol were only observed in bermudagrass inoculated with *B. longicaudatus* (treatment S); metabolite

2,5-di-*tert*-butylhydroquinone was only found in bermudagrass inoculated with either nematode, (treatments B and M). Azelaic acid was presented in treatments B and M, and also in bermudagrass inoculated with *P. middletonii* (Pm).

The metabolite benzamide was only observed in bermudagrass inoculated with *P. arrhenomanes* (treatment Pa). Acetic acid was only found in treatments Pa and Pm.

Citrinin was present in treatments Pa, M, B, and *P. catenulatum* inoculated bermudagrass (treatment Pc). Sulcatol was found in all test treatments, but not in the uninoculated control. The metabolites benzeneoctanoic acid, citric acid, isocitric acid, 4-Methyl-N,N-dimethylcathinone, and DEET were observed in treatments Pc, Pm and control U.

Discussion

Benzene sulfonic acid was only present in *B. longicaudatus* infested bermudagrass. Benzoic acid, a plant phenolic compound generated through the phenylpropanoid pathway, is known to inhibit fungal growth. Acidic conditions are more favor to the inhibitory effects of benzoic acid on fungal growth (Matsuzaki et al., 2008). Propolis has an inhibitory effect on the hyphal growth of *Pythium insidiusum*, and benzoic acid is one of the main compounds in propolis (Araújo et al., 2016). Because benzene sulfonic acid is more acidic than benzoic acid, it may inhibit *Pythium* hyphal growth. This may be one reason for observations in Chapter 3 that *P. arrhenomanes* infection on bermudagrass roots was reduced when *B. longicaudatus* was inoculated one-week ahead of *P. arrhenomanes*, and Chapter 4 that *B. longicaudatus* infested bermudagrass roots reduced mycelia growth of all three *Pythium* spp. to some degree.

Azelaic acid was found in exudates from bermudagrass inoculated with *B. longicaudatus*, *M. graminis*, and *P. middletonii*. Azelaic acid together with azelaic acid

induced 1 (AZI1) gene are involved in plant systemic immunity (Jung et al., 2009). AZI1 gene is induced by azelaic acid when the plant is under biotic and abiotic stress. For example, either infection by the nematode *Heterodera schachtii* or drought results in the accumulation of salicylic acid, a defense signal upon infection (Atkinson et al., 2013). Azelaic acid may be another reason for the decrease of *P. arrhenomanes* percent infection on *B. longicaudatus* infested bermudagrass roots and *Pythium* mycelia growth reduction by *B. longicaudatus* or *M. graminis* infested bermudagrass roots.

The presence of benzene sulfonic acid in *B. longicaudatus* infested bermudagrass root exudates and azelaic acid in the root exudates of bermudagrass infested with *B. longicaudatus* and *M. graminis* might account for the antagonistic effects on *Pythium* infection and mycelial growth.

Benzamide was only observed in *P. arrhenomanes* inoculated bermudagrass inoculated with *P. arrhenomanes*. Benzamide has been reported have nematicidal and egg-hatch inhibiting effects on *M. incognita* (Hackney and Dickerson, 1975; Goswami and Vijayalakshmi, 1986; Adegbite and Adesiyan, 2005; Asif et al., 2014).

The metabolite 2-({2-[(1-benzylpiperidin-4-yl)amino]-2-oxoethyl}thio) acetic acid was present only in exudates from bermudagrass inoculated with *P. arrhenomanes* or *P. middletonii*. Reports in literature indicate that acetic acid produced by *Purporeum lilacinum* (a nematophagous fungus) and *Trichoderma longibrachiatum* had nematotoxic effects on juveniles of *Meloidogyne* spp. Nematode juveniles were paralyzed in acetic acid with concentrations up to dilution 1/800 w/v; and nematode juvenile immobilization required time was from five minutes in 1 M/l concentration to 24 hours in dilution 1/800 w/v (Djian-Caporalino et al., 1991). Seo and Kim (2014) observed complete mortality of

M. incognita juveniles when exposed to acetic acid concentrations of 1.0%, 0.5%, 0.2% and 0.1%. As a deviant of acetic acid, 2-({2-[(1-benzylpiperidin-4-yl) amino]-2-oxoethyl}thio) acetic acid may also have nematicidal effects on *M. graminis* juveniles. The compound 2-({2-[(1-benzylpiperidin-4-yl) amino]-2-oxoethyl}thio) acetic acid may be the reason for fewer *M. graminis* observations around bermudagrass roots inoculated with *P. arrhenomanes* or *P. middletonii* when compared to pathogen non-inoculated control.

The hypothesis of this experiment was not fully tested. To make results solid, more replications of treatment and control should be involved in future studies. The fungicidal or nematicide effects of compound observed in root exudates are reported in literature. To confirm the pesticidal effects, compounds will be tested directly on reproduction, mobility and mortality of *B. longicaudatus* and *M. graminis*, and mycelial growth and infection ability of the three *Pythium* isolates.

Table 5-1. Metabolites observed in root exudates from six bermudagrasses samples. Samples are: bermudagrass inoculated with *Pythium arrhenomanes* (Pa), *P. catenulatum* (Pc), *P. middletonii* (Pm), *Belonolaimus longicaudatus* (B), or *Meloidogyne gramnins* (M), and pathogen non-inoculated (U) bermudagrass. + Means metabolite presented in sample; - means metabolite was absent in sample.

#	Polarity	Metabolite name	Pa	Pc	Pm	M	B	U
1	Negative	Isoleukotoxin	-	-	-	-	-	+
2	Negative	Benzoic acid	-	-	-	-	-	+
3	Negative	Isorhamnetin	-	-	-	-	-	+
4	Negative	Acetamide	-	-	-	-	-	+
5	Negative	trans-Cinnamic acid	-	-	-	-	-	+
6	Negative	1,5-Anhydro-D-glucitol	-	-	-	-	+	-
7	Negative	D-(+)-Fucose	-	-	-	-	+	-
8	Negative	L(-)-Fucose	-	-	-	-	+	-
9	Negative	Benzene sulfonic acid	-	-	-	-	+	-
10	Negative	2,5-di-tert-Butylhydroquinone	-	-	-	+	+	-
11	Negative	2-Butoxyacetic acid	-	-	+	-	-	-
12	Negative	2-Hydroxy-2-methylbutyric acid	-	-	+	-	-	-
13	Negative	2-Hydroxycaproic acid	-	-	+	-	-	-
14	Negative	2-Hydroxyisovaleric acid	-	-	+	-	-	-
15	Negative	2-Hydroxyphenylalanine	-	-	+	-	-	-
16	Negative	2-Hydroxyvaleric acid	-	-	+	-	-	-
17	Negative	2-Methyl-3-hydroxybutyric acid	-	-	+	-	-	-
18	Negative	2,3-Dihydroxybenzoic acid	-	-	+	-	-	-
19	Negative	2,4-Dihydroxybenzoic acid	-	-	+	-	-	-
20	Negative	3-(4-Hydroxyphenyl) propionic acid	-	-	+	-	-	-
21	Negative	3-Amino-3-(4-hydroxyphenyl) propanoic acid	-	-	+	-	-	-
22	Negative	3-Hydroxy-3-methylbutanoic acid	-	-	+	-	-	-
23	Negative	3-Hydroxyvaleric acid	-	-	+	-	-	-
24	Negative	3-Methyladipic acid	-	-	+	-	-	-
25	Negative	3-Phenyllactic acid	-	-	+	-	-	-
26	Negative	3,5-Dihydroxybenzoic acid	-	-	+	-	-	-

Table 5-1. Continued.

#	Polarity	Metabolite name	Pa	Pc	Pm	M	B	U
27	Negative	6-Hydroxycaproic acid	-	-	+	-	-	-
28	Negative	D(+)-Phenyllactic acid	-	-	+	-	-	-
29	Negative	Gentisic acid	-	-	+	-	-	-
30	Negative	L-(-)-3-Phenyllactic acid	-	-	+	-	-	-
31	Negative	L-Tyrosine	-	-	+	-	-	-
32	Negative	Pimelic acid	-	-	+	-	-	-
33	Negative	Protocatechuic acid	-	-	+	-	-	-
34	Negative	Quercetin	-	-	+	-	-	-
35	Negative	Suberic acid	-	-	+	-	-	-
36	Negative	UDP-N-acetylglucosamine	-	-	+	-	-	-
37	Negative	Uracil	-	-	+	-	-	-
38	Negative	Uric acid	-	-	+	-	-	-
39	Negative	15-octadecenoic acid	-	-	+	-	-	+
40	Negative	2-Methylglutaric acid	-	-	+	-	-	+
41	Negative	2,2-Dimethylsuccinic acid	-	-	+	-	-	+
42	Negative	3-Methylglutaric acid	-	-	+	-	-	+
43	Negative	4-Oxoproline	-	-	+	-	-	+
44	Negative	Adipic acid	-	-	+	-	-	+
45	Negative	L-Threonic acid-1,4-lactone	-	-	+	-	-	+
46	Negative	Methylmalonic acid	-	-	+	-	-	+
47	Negative	Succinic acid	-	-	+	-	-	+
48	Negative	Xanthine	-	-	+	-	-	+
49	Negative	Azelaic acid	-	-	+	+	+	-
50	Negative	Benzeneoctanoic acid	-	+	+	-	-	+
51	Negative	Citric acid	-	+	+	-	-	+
52	Negative	Isocitric acid	-	+	+	-	-	+
53	Negative	Naringenin	+	+	+	-	-	+
54	Positive	Adenine	-	-	-	-	-	+
55	Positive	D-Alanine methyl ester	-	-	-	-	-	+

Table 5-1. Continued.

#	Polarity	Metabolite name	Pa	Pc	Pm	M	B	U
56	Positive	L-Alanine methyl ester	-	-	-	-	-	+
57	Positive	L(+)-2-Aminobutyric acid	-	-	-	-	-	+
58	Positive	Melamine	-	-	-	-	-	+
59	Positive	Methionine	-	-	-	-	-	+
60	Positive	Methyl picolinate	-	-	-	-	-	+
61	Positive	Spermidine	-	-	-	-	-	+
62	Positive	Y-Aminobutyric acid (GABA)	-	-	-	-	-	+
63	Positive	6,7-Dihydro-1H-[1,4]dioxino[2',3' 4,5]benzo[d]imidazole-2-thiol	-	-	-	-	+	-
64	Positive	1H-Imidazole-4-carboxylic acid	-	-	+	-	-	-
65	Positive	2-(Methylamino) isobutyric acid	-	-	+	-	-	-
66	Positive	2-Aminoadipic acid	-	-	+	-	-	-
67	Positive	2-Aminonicotinic acid	-	-	+	-	-	-
68	Positive	4-Aminonicotinic acid	-	-	+	-	-	-
69	Positive	5-Aminonicotinic acid	-	-	+	-	-	-
70	Positive	6-Aminonicotinic acid	-	-	+	-	-	-
71	Positive	7-Oxobenz[de]anthracene	-	-	+	-	-	-
72	Positive	Acetanilide	-	-	+	-	-	-
73	Positive	D-(+)-Pyroglutamic Acid	-	-	+	-	-	-
74	Positive	Guanine	-	-	+	-	-	-
75	Positive	Kanosamine	-	-	+	-	-	-
76	Positive	L-2-Aminoadipic acid	-	-	+	-	-	-
77	Positive	L-Pyroglutamic acid	-	-	+	-	-	-
78	Positive	L-Valine	-	-	+	-	-	-
79	Positive	Maleic hydrazide	-	-	+	-	-	-
80	Positive	Methionine	-	-	+	-	-	-
81	Positive	Nicotinamide 1-oxide	-	-	+	-	-	-
82	Positive	Norepinephrine	-	-	+	-	-	-
83	Positive	Pyridoxine	-	-	+	-	-	-
84	Positive	Uracil	-	-	+	-	-	-

Table 5-1. Continued.

#	Polarity	Metabolite name	Pa	Pc	Pm	M	B	U
85	Positive	Urocanic acid	-	-	+	-	-	-
86	Positive	2-Hydroxyphenylalanine	-	-	+	-	-	+
87	Positive	2-Pyridylacetic acid	-	-	+	-	-	+
88	Positive	2,2,6,6-Tetramethyl-1-piperidinol (TEMPO)	-	-	+	-	-	+
89	Positive	2,2,6,6-Tetramethyl-4-piperidinol	-	-	+	-	-	+
90	Positive	3-Pyridylacetic acid	-	-	+	-	-	+
91	Positive	4-Aminobenzoic acid	-	-	+	-	-	+
92	Positive	4-Pyridineacetic acid	-	-	+	-	-	+
93	Positive	6-Aminocaproic acid	-	-	+	-	-	+
94	Positive	Allopurinol	-	-	+	-	-	+
95	Positive	Anthranilic acid	-	-	+	-	-	+
96	Positive	D-Carnitine	-	-	+	-	-	+
97	Positive	DL-Carnitine	-	-	+	-	-	+
98	Positive	Hexadecanamide	-	-	+	-	-	+
99	Positive	Hypoxanthine	-	-	+	-	-	+
100	Positive	Isoleucine	-	-	+	-	-	+
101	Positive	L-(-)-Methionine	-	-	+	-	-	+
102	Positive	L-Glutamic acid	-	-	+	-	-	+
103	Positive	L-Isoleucine	-	-	+	-	-	+
104	Positive	L-Norleucine	-	-	+	-	-	+
105	Positive	L-Tyrosine	-	-	+	-	-	+
106	Positive	L(-)-Carnitine	-	-	+	-	-	+
107	Positive	Leucine	-	-	+	-	-	+
108	Positive	Methyl isonicotinate	-	-	+	-	-	+
109	Positive	N-Methyl-D-aspartic acid (NMDA)	-	-	+	-	-	+
110	Positive	Salicylamide	-	-	+	-	-	+
111	Positive	Trigonelline	-	-	+	-	-	+
112	Positive	Metolachlor OXA	-	-	+	-	+	-
113	Positive	PEG n5	-	-	+	-	+	-

Table 5-1. Continued.

#	Polarity	Metabolite name	Pa	Pc	Pm	M	B	U
114	Positive	5-Aminovaleric acid	-	-	+	+	-	+
115	Positive	Betaine	-	-	+	+	-	+
116	Positive	Choline	-	-	+	+	-	+
117	Positive	Triisopropanolamine	-	-	+	+	-	+
118	Positive	Valine	-	-	+	+	-	+
119	Positive	Oleamide	-	-	+	+	+	+
120	Positive	4-tert-Butylcyclohexyl acetate	-	+	-	-	-	+
121	Positive	N,N-Diisopropylethylamine (DIPEA)	-	+	-	-	-	+
122	Positive	Oxybenzone	-	+	-	-	+	+
123	Positive	Resveratrol	-	+	-	-	+	+
124	Positive	Trioxsalen	-	+	-	-	+	+
125	Positive	N,N-Diethylethanolamine	-	+	-	+	-	-
126	Positive	4-Methyl-N,N-dimethylcathinone	-	+	+	-	-	+
127	Positive	DEET	-	+	+	-	-	+
128	Positive	Metalaxyl	-	+	+	-	+	+
129	Positive	Benzamide	+	-	-	-	-	-
130	Positive	2-({2-[(1-Benzylpiperidin-4-yl) amino]-2-oxoethyl}thio) acetic acid	+	-	+	-	-	-
131	Positive	1-Aminocyclohexanecarboxylic acid	+	-	+	-	-	+
132	Positive	DL-Stachydrine	+	-	+	-	-	+
133	Positive	L-Phenylalanine	+	-	+	+	-	+
134	Positive	Citrinin	+	+	-	+	+	-
135	Positive	Benzotriazole	+	+	+	-	+	-
136	Positive	Sulcatol	+	+	+	+	+	-
137	Positive	2-Hydroxycinnamic acid	+	+	+	+	+	+
138	Positive	3,4-Dihydroxyphenylpropionic acid	+	+	+	+	+	+
139	Positive	4-Coumaric acid	+	+	+	+	+	+
140	Positive	Cytosine	+	+	+	+	+	+
141	Positive	Ethylenediaminetetraacetic acid (EDTA)	+	+	+	+	+	+
142	Positive	Isocytosine	+	+	+	+	+	+

Table 5-1. Continued.

#	Polarity	Metabolite name	Pa	Pc	Pm	M	B	U
143	Positive	N-Benzylformamide	+	+	+	+	+	+

CHAPTER 6
CONCLUSION

The results of this Ph.D. project indicate that plant-parasitic nematode – *Pythium* association on bermudagrass is complicated and species determined. This study emphasized the importance of accurate disease causal agent identification. A positive *Pythium* sample does not always mean *Pythium* is the primary problem on a golf green. Sometimes plant-parasitic nematodes instead of *Pythium* are the primary problem. Both *Pythium* species identification and nematode assay are recommended when a positive *Pythium* sample is received. It is better to treat nematodes and *Pythium* together when both of them present in diseased bermudagrass samples.

In the greenhouse disease complex test, different plant-parasitic nematodes had different effects on different *Pythium* species infection on bermudagrass. *Belonolaimus longicaudatus* reduced the bermudagrass infection by *Pythium arrhenomanes* when nematodes were inoculated ahead of *Pythium*; however, it increased the infection by *P. catenulatum* or *P. middletonii*. *Meloidogyne graminis* reduced the bermudagrass infection by *P. arrhenomanes* or *P. catenulatum*, and had no effect on the infection by *P. middletonii*. In the lab attraction test, bermudagrass roots infested with either *B. longicaudatus* or *M. graminis* reduced the mycelia growth of the three *Pythium* spp. to some degree. According to those results, plant-parasitic nematodes may induce plant resistance to *Pythium* spp.

The antagonistic effects of plant-parasitic nematode inoculated bermudagrass roots on infection and mycelial growth of *Pythium* isolates in the greenhouse and lab tests might be associated with metabolites benzene sulfonic acid and azelaic acid, which presented in nematode inoculated root exudates. Acidic conditions provided by

benzene sulfonic acid may inhibit *Pythium* mycelia growth. Azelaic acid induces the 1 (AZI1) gene and activates the salicylic acid pathway, which is involved in plant defense responses to pathogen infection. To confirm this hypothesis, additional trials need to be conducted on the nematode effects on the expression of AZI1 gene in bermudagrass, and the effects of salicylic acid and benzene sulfonic acid on *Pythium* infection and mycelia growth.

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BIOGRAPHICAL SKETCH

Mengyi Gu graduated from Northeast Agricultural University with a Bachelor of Science degree in Landscape in 2012. In the same year, she also graduated from the Michigan State University - China Turf Program with a Bachelor of Science degree in Turfgrass Management. In fall 2011, she did her internship at the Jimmie Austin University of Oklahoma Golf Club, Norman, OK, where a love of turfgrass management was born. She relocated to Gainesville, FL, to join Dr. William T. Crow's lab at the University of Florida and to pursue the Master of Science and Doctor of Philosophy degrees in the Entomology and Nematology Department in 2012. Mengyi accepted her Master in the field of nematology with thesis titled "The efficacy of potential nematicides for golf courses in Florida" in August 2014 and kept on working with her PhD in the same lab. In May 2019, she received her PhD with the dissertation "Bermudagrass root rot complexes associated with plant-parasitic nematodes and *Pythium* species on golf courses in Florida".