ABSTRACT

VAN RYZIN, BENJAMIN JOHN. Etiology and Management of Pythium root rot in North Carolina. (Under direction of James P. Kerns).

Pythium root rot has been an important disease of creeping bentgrass putting greens in the Southeastern United States, however little is known about the etiology, biology and management of the disease. Four *Pythium* spp. were obtained from roots or crowns of creeping bentgrass and other turfgrass species with symptoms of Pythium root rot. Species isolated include *P. torulosum*, *P. vanterpoolii, P. aphanidermatum* and *P. arrhenomanes*. *Pythium torulosum* was the most prevalent species isolated comprising 85% of isolations, followed by *P. vanterpoolii*, comprising 12% of isolates. Pathogenicity of nine *P. torulosum* isolates, seven *P. vanterpoolii* isolates, two *P. arrhenomanes* isolates and one *P. aphanidermatum* isolate was analyzed on post-emergent and mature creeping bentgrass.

Growth chamber experiments were conducted to determine the pathogenicity of *Pythium* spp. on 7-day old and mature 'A-1' creeping bentgrass seedlings. Post-emergent seedlings were grown in cups, inoculated and incubated at 25°C in 100% humidity. After seven days, isolates of *P. arrhenomanes*, *P. vanterpoolii* and *P. aphanidermatum* had caused wilt or necrosis to 100% of inoculated plants. In pathogenicity tests of mature plants, the root zone of 5-week old 'A-1' creeping bentgrass plants was inoculated and plants were incubated at 34°C/28°C. *Pythium aphanidermatum* caused 100% plant death by one week after inoculation, but *P. torulosum*, *P. vanterpoolii* and *P. arrhenomanes* had not caused disease after 5 weeks.

Field experiments were conducted to evaluate preventative and curative control of Pythium root rot. Performance of fungicides for management of the disease was

evaluated in Raleigh, NC on a 'Dominant Plus' creeping bentgrass putting green. Fungicides were preventatively applied when soil temperatures consistently reached 18 to 21^{o}C. Once Pythium root rot symptoms appeared curative fungicide applications began. Cyazofamid provided excellent preventative suppression, as well as curative activity. Mefenoxam, etridiazole, azoxystrobin, pyraclostrobin and fluoxastrobin also provided moderate preventative suppression of symptoms. In vitro assays were conducted to determine the sensitivity of *Pythium* spp. to fungicides. *Pythium* species were highly sensitive to cyazofamid, and fluazinam, and moderately sensitive to etridiazole.

Etiology and Management of Pythium Root Rot in North Carolina

by
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DEDICATION

My work is dedicated to my family. Through my life they have helped influence my interests and supported me in everything I am today. To my late father (1953-2015) whose overall wonderment and creativity helped produce three amazing children. He utilized my interest of the sciences to teach me his vision and understanding of the world. Dad, I hope you are pleased with my work. I will continue to live my life to the fullest in honor of your dedication to our family. To my mother, whose love and nurturing allowed three wonderful children to become phenomenal people. She influenced the direction of my study and pushed me to take the risk. To my sister, whose academic success has been a motivational influence to my further education and influenced my love of biology. Kim, your outspokenness, courage and confidence are something I strive for in my everyday life. To my brother, who has shown me life is not meant to be a comedic-less place full of straight faces and work. Andy, your sense of humor is always quick to break the ice and I will continue to value jokes and humor everyday. Thank you to my Ohana for your unconditional support and love throughout my life. I love you all very much.

BIOGRAPHY

The author of this text, Benjamin J. Van Ryzin, was born February 25, 1989, in Madison, WI to Gary and Pamela Van Ryzin. His interest in the natural sciences began in elementary school where he enrolled in a summer class focusing on entomology and stream ecology. His interest was broadened with physics, biology, anatomy and physiology classes in high school. He graduated from Madison East High School in 2007. The natural progression for Madisonians was to study at the University of Wisconsin-Madison, and because of his earlier interests he was determined to pursue his Bachelor of Science in Biology.

As an undergraduate, Ben worked for Dr. James Kerns as an hourly employee. He helped in laboratory procedures as well as field studies throughout the state of Wisconsin. Ben used the lab as an opportunity to learn what scientific research involved through student research credits, where he determined whether turfgrass seed was a potential source of inoculum for the dollar spot disease. Through this introductory research project, Ben learned plant pathology was an area of study he would like to delve into more.

He decided to pursue a Masters of Science in Plant Pathology with Dr. Kerns. During his time at North Carolina State University, Ben fell in love with researching plant pathogens and decided he would continue researching the biology and management of plant pests. His degree requirements were fulfilled March of 2016 with the approval and acceptance of this thesis. He has accepted a position at the University of Minnesota-Twin Cities as a Research Fellow.

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Thank you to Dr. James Kerns, who took the chance on me, acquired funding and helped direct this MS thesis. Jim, you gave me the space to learn and resources to become a member of the scientific community, it is something I will always be grateful you did. You were quick to tell me my faults, but you also gave me great advice and listened to my opinion when issues arose. The six years of working for you have been awesome, I have seen and done more than I thought I would, and I always had your support helping me through it all. Here is to the continued success and laughter coming from the turfpathology lab!

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INTRODUCTION

North Carolina's golf industry plays a significant role to the state's economy. In a recent survey in 2011, the golf industry in the state of North Carolina supported $4.2 billion of total economic activity, along with 53,000 jobs and $1.3 billion in wage income (55). Nearly 600 golf courses are found throughout the state, ranging from small public courses to private country clubs to golf clubs that host United States Golf Association (USGA) and Professional Golf Association tour championships. The North Carolina golf industry also enables real estate property and tourism industries in the state to profit (55).

Putting greens are the most important management areas on a golf course. These areas incur the most stress such as heavy traffic from patrons, intensive maintenance, seasonal environmental stress, and a soil substrate with limited water and nutrient holding capacity. Turfgrass species used for putting greens must withstand stresses and remain uniform, dense and aesthetically pleasing, but turfgrass managers have limited options when choosing a surface for their putting greens. One of the most popular grasses used is *Agrostis stolonifera*, commonly known as creeping bentgrass. Its ability to grow in a wide range of climatic conditions and to self-repair, via stolon growth, has led to plantings on golf greens, fairways and tees around the world. It is adapted to temperatures between 25-30°C but is susceptible to heat stress when temperatures exceed 32°. This stress predisposes the turf to disease. Turfgrass species that are grown for putting greens must be maintained according to standards published by the USGA on stand height. USGA also recommends a relatively high percentage of sand and low percentage of

organic matter that retains water and nutrients in the putting green substrate (56). These standards were put in place so that water freely drains from the rootzone to allow for playability in the event of extreme rainfall, as well as to reduce soil compaction. Creeping bentgrass putting greens must be highly maintained in order to reduce the seasonal stresses. Aerification is the process used to allow air, water and nutrients to reach the root zone, which also alleviates soil compaction, and improves soil drainage and drying. Aerification is followed by topdressing the golf green with a layering of sand to fill aerification holes. Topdressing can be a regular application during the growing season that modifies the topsoil and reduces thatch buildup (12). Neglecting aerification and topdressing maintenance often causes creeping bentgrass to succumb to disease and abiotic stresses.

First reports of Pythium root rot on turfgrasses were published in the 1940's. *Pythium* species have a broad host range within turfgrass species; foliar and root infections are common. Pythium root rot is common in creeping bentgrass golf greens and is destructive enough to result in plant death. Symptoms begin as small necrotic patches that appear to be thinning the grasses' uniform appearance. Under favorable environmental conditions patches may coalesce. Symptoms are often seen following paths of water movement. Stressful summer temperatures cause affected areas to become less dense and slow growing, which persists until conditions become favorable to the growth of the plant. Roots, stolons and crowns often have dark, discolored lesions, while primary and secondary roots appear stunted and often lack root hairs. It is still uncertain which *Pythium* spp. are the causal agent of the disease, or whether multiple species of *Pythium* infect and reduce plant root growth throughout the year. However, we

hypothesize that *Pythium* spp. chronically infect creeping bentgrass roots at all temperatures, and become pathogenic when temperature and other environmental conditions favor *Pythium* pathogenesis (23).

In the summers of 2010 and 2011 in North Carolina, high temperatures exceeded averages from June to August. This persistent extreme heat led to a Pythium root rot epidemic throughout the southeastern United States. In those two years combined, a total of 120 samples sent into NCSU's Plant Disease and Insect Clinic were diagnosed as Pythium root rot, whereas only 45 samples were diagnosed with root rot in the following three years. Lower temperatures and more insistence on preventative fungicide treatments may have been a factor in the reduction of sample diagnoses observed after 2011. With a changing climate where extreme summer heat becomes normal, it is imperative that turf managers in the southeast United States base their creeping bentgrass management decisions on the results of scientific research. Otherwise without proper management higher temperatures may push managers to expensive renovations.

The distribution and diversity of *Pythium* spp. associated with Pythium root rot in North Carolina has been described (1), but the pathogenicity of many species on mature plants is still unknown. Furthermore, limited quantitative data exist demonstrating preventative chemical control of Pythium root rot in the field. With the lack of scientific research on management, golf course superintendents spend significant resources on chemical fungicides to blindly manage a very destructive group of pathogens. The work in this thesis will focus on the etiology and management of Pythium root rot in North Carolina, with the ultimate goal of improving Pythium root rot management strategies for golf course superintendents.

Background Information and Literature Review

Pythium taxonomy and phylogeny

Pythium and sister taxa *Phytophthora* are a part of class Oomycetes. They are classified in the Kingdom Chromista (= Stramenopila) with diatoms and brown algae. Once grouped with "true fungi" through traditional morphology-based taxonomy, molecular sequencing and other evidence supported removal of Oomycetes from the Kingdom Fungi. Cell wall composition, mitochondrial structure, flagella type and nuclear state of hyphae differ between Oomycetes and true fungi. True fungi produce mycelium with cell walls composed of chitin, mitochondrial cristae are flattened, have one whiplash flagellum, and vegetative hyphae are haploid or dikaryotic. *Pythium* and other Oomycetes, however, have cell walls composed of cellulose and beta glucans, tubular mitochondrial cristae, flagella that are heterkont, of two types, located posteriorly and anteriorly on asexual zoospores, and have mycelium with a diploid nuclear state. Oomycetes go through a sexual phase of reproduction with female (oogonium) and male (antheridium) structures each containing a single haploid nucleus. Fusion of the two structures gives rise to a single diploid oospore, and with germination, diploid mycelium (2).

Pringsheim established the genus *Pythium* in the family *Saprolegniaceae* in 1858 with the type species *P. monospermum*. It was moved into *Peronosporaceae* in 1881. In 1897, Schröter included the genus in the new family, *Pythiaceae*, in the order Peronosporales (57). Originally of interest to mycologists for taxonomic reasons, plant pathologists would soon value the work of Matthews (40); who constructed the first

workable key for identification of *Pythium* spp. Because of increased need to identify *Pythium* species Middleton enhanced the *Pythium* key in 1945 (20). Middleton's key highlighted morphological characteristics vital for identification, compiled host records, and illustrated the important pathogenic species. Later, compilations by van der Plaats-Niterink (57), and Dick (13) refined descriptions of morphological characteristics. Taxonomic separation is largely based on sporangial shape and size, oogonia size and ornamentation, the distance between the oogonia and oospore wall, antheridia numbers and attachment, and sporangia morphology. *Pythium* species are difficult to identify because that have few variable morphological characters. *Pythium irregulare*, for example, has variable oogonial morphology ranging from smooth to blunt conical or finger-like projections (17). The species has also been described as having variability in its morphology due to differences in temperature, light and nutrient agar (20). The discovery of heterothallism in the genus is another example of the limitations of morphological based identification. Most of the genus was believed to be homothallic, until heterothallism was found in *P. catenulatum* and *P. splendens*. It previously went undetected because of the medium used for isolation. Once sterols were introduced to media, mating reactions were stimulated for multiple new species (20).

With the advancement of molecular technologies, DNA sequence-based phylogenetic studies of *Pythium* were used as a less subjective and daunting endeavor than morphological classification. DNA sequence analysis could accurately distinguish between species, making it the primary tool used for identification. Various regions of DNA have been used in phylogenetic assays. Noncoding internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal DNA are the most popular genetic markers

(58), because this region is highly variable. ITS regions vary from 750-1050 bp, and contain the 5.8S, 18S and 28S coding regions. The nuclear rDNA region has also been used extensively. It is comprised of small and large subunits. Large nuclear ribosomal subunits are made up of D1 and D2 domains (34). The use of rDNA has limitations because the evolution of one gene does not necessarily mean the evolution of the entire genome. It requires a comparison of additional independent genes to determine if the gene phylogenies support or contradict each other (58). Mitochondrial encoded cytochrome oxidase II (*cox* II) and beta- tubulin genes increasingly have been used to study phylogeny in *Pythium*. *Cox* II genes code for an enzyme essential to the metabolic process of the electron transport. Beta- tubulin genes code for one of the tubulin proteins that make up microtubules of the cytoskeleton, mitotic spindles and flagella.

Many studies have been conducted using rDNA, demonstrating that *Pythium* is phylogenetically distant from other genera of *Oomycetes* (34). A study focused primarily on sequencing *cox* II demonstrated that *Phythiales* is monophyletic (34). Martin determined heterothallic *Pythium* spp. (*P. sylvaticum*, *P. heterothallicum*, *P. splendens*, and *P. catenulatum)* are polyphyletic because their separate placements within clades I, II, and III indicate that they arose independently in separate lineages (38). The most comprehensive study of the genera was conducted by Lévesque and de Cock (34), where 116 described species were sequenced using the ITS region. It revealed eleven clades within *Pythium.* Although most clades shared similar morphological characteristics, there were exceptions and phylogenetic relatedness was surprising (34). Through the combined datasets for the ITS, beta-tubulin, and *cox* II gene Villa et al. (58) revealed *Phytophthora undulata* is unaffiliated with any major clade but basal to Clade I which contains

filamentous *Pythium* spp. Instead of the clustering with other *Phytophthora* species*, P. undulata* was more closely related to *Pythium* spp. (9, 58), suggesting that *P. undulata* is an intermediate species in the evolution of *Pythium* to *Phytophthora.* Further evidence is needed to substantiate this claim.

Plant pathogenic species of *Pythium* typically cluster together. Lévesque placed most in two main clades (A and B). Most notably, *P. aphanidermatum* is placed within clade A, but exhibits the same morphological features as other pathogenic species. Many *Pythium* spp. that are pathogens of creeping bentgrass cluster in Clade B. All, including *P. aphanidermatum*, have inflated filamentous sporangia, smooth oogonia and no hyphal swelling. *Pythium irregulare*, another important plant pathogen, occupies Clade F. Its morphological characteristics consist of ornamented oogonia, hyphal swelling, and non-proliferating globose sporangia. Clade I contains *P. ultimum* var. *ultimum*, which is characterized by smooth oogonia and hyphal swellings (34).

There are approximately 120 species in the *Pythium* genus. Members vary from facultative plant pathogens to soil saprophytes, to even a mammalian pathogen, *P. insidiosum*. Other *Pythium* spp. have been reported as mycoparasites and have been utilized as biological controls, using their antagonism against plant pathogenic *Pythium*. *Pythium aphanidermatum* and *P. ultimum* have a very large host range and cause significant damage to many economically important food and ornamental crops (31).

Environmental factors that influence infection by *Pythium* spp.

Disease onset is based on the duration of time a susceptible host, virulent pathogen, and favorable environmental factors are present in a system. In turfgrass

systems the host is perennial and has limited resistance to *Pythium spp*. Moreover, many *Pythium* species are distributed throughout turfgrass systems (1), and have a wide range of optimal growth temperatures. Environmental factors that influence the behavior of *Pythium* spp. include moisture, temperature, pH, and the presence of soil microorganisms.

Soil moisture is the leading factor for infection. Research has shown a positive correlation between higher soil moisture and incidence of disease (5, 7, 15, 21, 41, 47, 48, 52). Differences in the biology of species can contribute to disease severity. For instance, root damage in peach infected by *P. vexans* was most severe under conditions of excess water, which was likely related to the production and dispersal of zoospores. On the other hand, disease severity did not vary according to soil moisture treatments, probably because the predominant species was *P. irregulare*, which does not readily produce zoospores (5). Soil moisture influences zoospore motility and the formation of oospores (39). Soil moisture can directly and indirectly select for different populations of soil-dwelling microorganisms. *Pythium* is insensitive to anaerobic environments, while other competing microorganisms are sensitive. When these conditions occur, studies have shown inoculum densities can peak (16).

Infection is also dependent on soil temperature. As is the case with moisture content, interspecies specialization is evident in the varied temperatures at which different *Pythium* spp. infect their hosts. Pathogenicity of *Pythium* spp. associated with creeping bentgrass varies greatly based on temperature. Abad et al. (1) characterized *Pythium* spp. based on their pathogenicity to creeping bentgrass, and showed highly aggressive species (*P. arrhenomanes*, *P. aphanidermatum*, *P. myriotylum, P. volutum,*

and *P. graminicola)* caused less disease at 16°C than at 28°C and 32°C. Disease caused by *P. vanterpoolii*, a species closely associated with creeping bentgrass, was most severe at 28°C, yet Craft and Nelson (43) demonstrated aggressiveness at 13°C. Disease induced by *P. torulosum*, another species closely associated with turfgrasses was most severe at 32°C and aggressiveness rapidly declined as temperature decreased (43). *Pythium ultimum* var. *ultimum* was most aggressive at 16°C indicating that this pathogen may be more prevalent in cooler climates (1).

Although environmental factors directly affect the growth and development of *Pythium* spp., these factors also have consequences on *Pythium* species. Just as pathogens have ideal conditions for growth, plants require environmental conditions to be within an optimal range. Extreme temperature and precipitation can hinder plant growth and predispose it to disease. Temperature and moisture can also negatively impact the microbial ecosystem of soils and the plant. At temperatures of ≥27°C the bacterial flora that generally outcompetes and impedes infection by *P. aphanidermatum* is instead, outcompete (54). Furthermore, immediately after irrigation *P. aphanidermatum* has a competitive edge over the same antagonistic bacteria. The addition of organic amendments in artificial environments has produced reductions in the severity of diseases associated with *Pythium* spp. Presumably this is due to increased competition between the microbes in the amendments and the soil matrix. The application of organic amendments reduced damping off in cucumber (37) and root rot in sand-based putting greens (10). Nelson and Craft demonstrated that microbial properties influenced the suppression of *Pythium* diseases in creeping bentgrass without impacting the turfgrass aesthetics (10). While these methods have proven effective in potting mixtures or

controlled environmental conditions, research under field conditions has not demonstrated the same effects. Although organic amendments stimulate microbes and serve as a source of additional microorganisms, even recently established turfgrass systems have extremely large microbial populations that overtime are challenged by changing environments along with nitrogen inputs into the system (49). More research is needed to characterize the specific microbial community structures of healthy golf greens compared to those that are prone to Pythium root rot.

Pathogenesis of diseases caused by *Pythium* spp.

Pythium is one of the most important genera of plant pathogens because of its broad host range and global distribution. Its large host range suggests that it can overcome many plant defenses in order to parasitize the plant. *Pythium* is a necrotrophic plant pathogen that consumes host material once it is dead. *Pythium* pathogenesis is still not understood. *Pythium* lacks the secretion of RXLR-like proteins found in *Phytophthora* and other true fungi, that interact with plant defenses often eliminating or dulling the plant defense response. Instead *Pythium* spp. secrete YxSL[KR], a highly conserved protein present across Oomycete species. YxSL[KR] has characteristics that suggest interaction with host cells. It is highly expressed during infection and thus is hypothesized to act as an effector (33). The absence of RXLR may be associated with the broad host range and lack of any gene-for-gene resistance found in *Pythium*-host interactions. It may also explain why *Pythium* generally is restricted to necrotrophic infection of plants with diminished defenses.

Although it is undetermined whether *Pythium* produces effectors it does produce cell wall degrading enzymes (CWDEs) that are used to cleave cell structure and are essential for *Pythium* to metabolize the host. CWDE's include: pectinase, cellulose, xylanase and protease (18). In a study of *P. myriotylum*, Geethu et al. (18) showed that fungal growth is dependent on CWDEs through cellular invasion. Proteases play a vital role in nutrition during necrotrophy, providing nitrogenous compounds and thereby assisting in tissue colonization. Other species such as *P. ultimum*, do not produce xylanase or chitinase, but are believed to have strong pectinase activity to breakdown carbohydrate (33).

Zoospores, sporangia, oospores and mycelium fragments can all initiate infection. Infection begins with encystment, then production of a germ tube, leading to the formation of penetration hyphae. It can also penetrate the cuticle, and grow through open wounds, or natural openings. Penetration by an appresorium is species specific (19). Formation has been observed at the tips of mycelial fragments (30), and used as another means of penetration. The long-term survival structures of the pathogen are called oospores. Oospore germination can be arrested for several years in adverse conditions or several weeks in favorable conditions (36). Sporangial production of zoospores serves as the inoculum for subsequent infections.

Pythium cause disease to a wide range of hosts. Seedlings, succulent plant tissue, fruits and roots can all be infected. Pre- and post-emergence damping-off caused by *Pythium* spp. are common diseases among agricultural crops, ornamentals, nurseries, and turfgrasses (20, 45, 50). *Pythium* spp. also infect mature plant roots causing root rot, stunting, and yield loss. Symptoms can be vastly different depending on host cultivar and

the pathogen. Some plants may exhibit minor stunting because the infection is limited to root tips (44), stopping in the epidermis due to plant inhibitory factors. More severe symptoms include a dramatic reduction in plant growth and destruction of fine roots and root tips. *Pythium myriotylum* causes premature leaf senescence in commercially grown caladium cultivars (11). In sugarcane production, *P. arrhenomanes* significantly reduces root and shoot weight, and lateral root growth (24). Postharvest damage of potato by *P. ultimum* and *P. aphanidermatum* can also occur by the pathogens entering through wounds and creating necrotic tissue inside the tuber (46).

Pythium diseases of turfgrass

Pythium spp. cause many diseases on turfgrasses. All turfgrasses are susceptible to infection by *Pythium* (6, 20), with stressed turf being the most susceptible. Common diseases are known as Pythium blight, grease spot, cottony blight, spot blight, crown and root rot, root dysfunction, and snow blight. Disease occurs on seed, seedlings, and mature plants in a variety of environmental conditions. Cold, wet conditions may be favorable for some diseases, but *Pythium* diseases are most obvious in hot, humid weather, when large patches of turfgrass can be damaged in less than one day. Most damage is seen on, but is not limited to, golf courses and athletic fields.

Pythium blight is a devastating disease of all turfgrass species. Its quick development and rapid spread can kill large swards of turf in days. Symptoms develop rapidly as small circular tan spots (1-6 inches diameter) that can coalesce during hot, humid weather. Excess nitrogen, leaf moisture, and hot humid weather favor disease development. *Pythium aphanidermatum* is the primary casual agent of Pythium blight

(25), but foliar blight can also be caused by *P. ultimum* (3), *P. graminicola*, *P. vanterpoolii* and *P. torulosum* (42).

Pythium spp. are also associated with turfgrass roots and crowns. Pythium root rot usually occurs in soils with higher water holding potential at any time during the year. Golf greens that are old and soil-based can have compacted root zones that may increase the plant's susceptibility to disease. Crowns and roots of infected plants become necrotic, and roots show reduced length and volume. Symptoms are typically apparent during periods of warm, humid weather. Affected turf typically has a thinning, water-soaked appearance that does not have any specific patterning. Many *Pythium* spp. have been associated with root rot (1, 43).

Pythium root dysfunction symptoms also appear during hot weather; however, field conditions are dry and soil has excellent drainage. The disease is most common in creeping bentgrass grown on newly established sand-based golf greens. Symptoms and plant death are more pronounced on creeping bentgrass under significant heat and drought stress (27). Roots affected by root dysfunction are tan, with bulbous tips that lack root hairs.

<u>*Pythium* species associated with turfgrass root diseases</u>

Pythium root dysfunction (PRD) was first described on sand-based putting greens in 1985. Recently renovated golf greens, consisting of high sand content growing media, were killed during seasonal temperature and humidity spikes. *Pythium aristosporum* and *P. arrhenomanes* were pathogenic on the roots of creeping bentgrass in the absence of necrosis causing reductions in shoot and root growth. Hodges and Coleman speculated

that the sand used in renovated greens was the source of the pathogen and that the ability of the *Pythium* species to function was related to lack of competition from other microbes (22).

Feng and Dernoeden continued research into Pythium-induced root dysfunction (14). Pathogenicity assays showed *P. aphanidermatum*, *P. aristosporum*, *P. ultimum* var. *ultimum*, *P. vanterpoolii*, *P. graminicola* and *P. volutum* were pathogenic on 'Crenshaw' creeping bentgrass. In the southeast United States *P. volutum* was identified as the causal agent of PRD (29), and the investigators hypothesized that disease development occurs during the spring and fall. Similarly, Feng and Dernoeden (14) showed *P. volutum* was highly aggressive at 18°C. It is the infection during the fall, winter, and spring that induces symptom expression during stressful conditions (27).

Since 1985, golf greens have transitioned to sand-based construction to allow water to move through the soil profile quicker, limiting fungal diseases. Over time, however, organic matter builds up through the decomposition of plant material. Organic matter changes the soil profile, and in doing so, favors hydrophilic microorganisms. These conditions favor the development of Pythium root rot. This disease develops during periods of prolonged soil wetness and is characterized by root and crown decay, chlorosis, and death (43). Over 27 species of *Pythium* can cause Pythium root rot. Highly aggressive species include: *P. aphanidermatum*, *P. myriotylum*, *P. arrhenomanes*, *P. ultiumum* var. *ultimum*, *P. vanterpoolii*, and *P. volutum*. Species predominantly associated with creeping bentgrass roots are *P. torulosum*, *P. arrhenomanes*, *P. graminicola* and *P. vanterpoolii* (1). Previous research (1, 43) suggests *P. arrhenomanes* and *P. graminicola* are the species most commonly involved in Pythium root rot;

nonetheless, *P. torulosum*, *P. aphanidermatum* and *P. aristosporum* are also pathogens of creeping bentgrass roots. There is potential for *P. torulosum* to co-infect a host with another *Pythium* spp. or pathogen; its association with, and virulence to, creeping bentgrass roots could be a culprit when growing conditions do not favor plant growth.

Chemical control of *Pythium* root rot on turfgrass

Oomycetes are genetically distant from true fungi, and require unique fungicides to control the diseases they cause. Fungicides effective against Oomycetes generally have a narrow spectrum of activity, and typically are only effective against oomycete pathogens (32). The first fungicides to control Oomycetes were introduced in the 1970's. The introduction of fosetyl-Al, metalaxyl, and propamocarb to commercial markets brought excellent control against *Pythium* and *Phytophthora*. Metalaxyl and propamocarb provided excellent control of Pythium blight. Fosetyl aluminum was the first truly systemic (ambimobile) fungicide for control of Pythium blight and Pythium root dysfunction. Currently, many active ingredients can prevent Pythium blight, Pythium root dysfunction and Pythium root rot, including mefenoxam, fluopicolide, cyazofamid, etridiazole, propamocarb, potassium phosphate, fosetyl-aluminum, azoxystrobin, fluoxastrobin, and pyraclostrobin (8, 9, 28). For prevention of root diseases, fungicides must be watered in after application.

Mode of action classification of these active ingredients reveals how these fungicides interfere with the metabolic processes of *Pythium* spp. The mode of action is directly related with the risk of *Pythium* developing resistance to an active ingredient.

Mefenoxam interferes with the function of RNA polymerase and limits production of rRNA, depriving the cell of ribosomes and ultimately, disrupting the production of proteins essential for cell structure and metabolism. While it disrupts mycelium growth, it does not affect germinating spores, which contain a bounty of ribosomes. Mefenoxam and metalaxyl have high resistance risk and resistance has been observed in many crop production systems (35, 53). Preventative applications of mefenoxam are done at a 14 to 21 day interval to control *Pythium* diseases. Mefenoxam is an acropetal penetrant and is distributed toward leaf tips through the xylem (32).

QoI fungicides (azoxystrobin, fluoxastrobin, pyraclostrobin) are site-specific inhibitors that disrupt Complex III of the respiratory electron transport chain. QoI's target the outside location of cytochrome bc_1 in Complex III, inactivating the ubiquinol oxidase enzyme. Ubiquinol oxidase enzyme inactivation reduces metabolism and inhibits spore germination, mycelial growth and reduce spore production. Risk of resistance is high for QoI's because of the potential for alteration of the target site, which avoids interruption of the electron transport chain. Resistance has been observed for common turfgrass pathogens including *P. aphanidermatum* and cross-resistance is possible. Drench application of azoxystrobin and pyraclostrobin at 28-day intervals is effective for prevention of Pythium root dysfunction (28). QoI's also have non-disease related physiological effects that increase yield and vigor in some crops (4).

QiI fungicide (cyazofamid) also disrupts Complex III of the electron transport chain, but it targets the inside location of cytochrome bc_1. Spore germination, mycelial growth and spore production are all inhibited by this active ingredient. Cyazofamid is only affective against oomycetes (32). Cyazofamid is a relatively new fungicide;

therefore, risk of resistance development is unknown but is assumed to be medium to high based upon QoI's high risk of resistance. Resistance to cyazofamid has been documented in *Phytophthora capsici* (26). Preventative applications for *Pythium* diseases are commonly done on 21 to 28 day intervals. Cyazofamid is a local penetrant, requiring product to be watered in for fungicide to reach plant roots (32).

Active ingredients have also been mixed into a single product. Propamocarb and fluopicolide were combined to form the product Stellar. Both fungicides have limited effectiveness to *Pythium* species. Propamocarb induces membrane leakage by altering functional groups in membrane phospholipids disrupting the membrane function. Fluopicolide interferes with the assemblage of spectrin-like proteins that are needed for the elongation of hyphal tips. Both fungicides have a moderate risk of resistance development. Preventative applications are made every 14 to 21 days. Propamocarb moves through the xylem to leaf tips. Fluopicolide penetrates the plant locally, and does not translocate (32).

Other fungicides used as preventative treatments for *Pythium* diseases have unknown mode of actions. Etridazole is an aromatic hydrocarbon. The active ingredient is believed to cause breakdown of lipids found in the cell membrane. Other studies suggest an interruption of mitochondrial respiration or the enzymes used in cell wall biosynthesis. This fungicide remains on the surface of the plant. Etridazole is believed to be at low risk of resistance development. Labels suggest application every 14 days. Phosphonate fungicides also have an unknown mode of action. Phosphonates (fosetyl-aluminum and potassium phosphonate) are true systemic fungicides (8). Once the active ingredient enters the plant it is circulated throughout the plant in the phloem and xylem.

As a result these products do not need to be drenched into the soil. Resistance risk is low; phosphonates have also been shown to stimulate the plant's defense mechanisms. Applications are given every 14 to 21 days (32).

Although Pythium root rot on turfgrasses was first described in the 1940's, and reinvestigated in the 1990's there is little knowledge on the management and biology of this disease. Previous work has shown *P. torulosum*, a suspected causal agent, to be a weak pathogen against most hosts, but an aggressive pathogen of creeping bentgrass. Pathogenicity assays typically are performed on creeping bentgrass seedlings, but fail to reveal effects on mature plants. Prevention of Pythium root rot at a field site has never been demonstrated and has severely limited our understanding of what fungicides are efficacious against Pythium root rot. Therefore, increased understanding of the biology of this disease may improve our ability to manage it. In order to obtain this information this thesis focused on the following objectives:

1. Identify the pathogens that cause Pythium root rot in North Carolina using morphological and molecular techniques, and determine their pathogenicity on seedlings and established, mature creeping bentgrass plants.
2. Determine fungicide sensitivity in culture of common species and conduct field trials to develop effective control practices for Pythium root rot.

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CHAPTER 2 - CHARACTERIZATION AND PATHOGENICITY OF *PYTHIUM* SPP. ASSOCIATED WITH PYTHIUM ROOT ROT OF CREEPING BENTGRASS

ABSTRACT

Four *Pythium* spp. were obtained from roots or crowns of creeping bentgrass and other turfgrass species with symptoms of Pythium root rot. Species isolated include *P. torulosum*, *P. vanterpoolii, P. aphanidermatum* and *P. arrhenomanes*. *Pythium torulosum* was the most prevalent species isolated comprising 85% of isolations, followed by *P. vanterpoolii*, comprising 12% of isolates. Pathogenicity of nine *P. torulosum* isolates, seven *P. vanterpoolii* isolates, two *P. arrhenomanes* isolates and one *P. aphanidermatum* isolate was analyzed on post-emergent and mature creeping bentgrass. To test post-emergent pathogenicity 7-day old 'A-1' creeping bentgrass seedlings grown in cups (5.7cm x 11.6cm) were inoculated and placed in 100% humidity. After seven days, isolates of *P. arrhenomanes*, *P. vanterpoolii* and *P. aphanidermatum* had caused wilt or necrosis to 100% of inoculated plants. In pathogenicity tests of mature plants, the root zone of 5-week old 'A-1' creeping bentgrass plants was inoculated and plants were incubated at 34°C/28°C. *Pythium aphanidermatum* caused 100% plant death by one week after inoculation, but *P. torulosum*, *P. vanterpoolii* and *P. arrhenomanes* had not caused disease after 5 weeks. These results demonstrated that pathogenicity on post-emergent seedlings may not correlate with pathogenicity on mature plants, and also determined that *P. aphanidermatum* is pathogenic on mature creeping bentgrass.

INTRODUCTION

Creeping bentgrass (*Agrostis stolonifera* L.) is a cool-season grass species that is planted primarily on golf course putting greens in the Southeastern United States. This grass species is ideal for golf course putting greens because of its tolerance of low mowing heights, its aggressive stolon growth and its uniform playing surface (20). Most of the Southeastern United States is located within the transition zone, a region where cool and warm season grasses can grow. However, hot and humid weather in summers causes cool season grasses, like *A. stolonifera*, to lose root mass, resulting in loss of plant vigor and also predisposing plants to abiotic and biotic problems (18). Furthermore, *A. stolonifera* grows quickly and in the process produces organic matter that has high water holding potential. Not only does this water make the environment more suitable for fungal pathogens, it also holds heat that can be detrimental to root health. The accumulation of organic matter in the topsoil of putting greens often is associated with poor health of creeping bentgrass growing on golf greens (7). Golf course managers must actively manage their creeping bentgrass putting greens to maintain plant health. Managers aerify greens in the spring and late summer by mechanically removing plugs of soil and turf to allow air to penetrate the topsoil, alleviating soil compaction and improving soil drainage and drying. Managers also are encouraged to topdress greens with a thin layer of sand after aerification to fill holes. They are also encouraged to perform regular applications of sand during the active growing season to dilute the buildup of organic matter in the topsoil (2). Neglecting these recommended cultural practices can cause creeping bentgrass to succumb to disease.

Beginning in the early 1990's, increasing numbers of reports describing Pythium root and crown rot of grasses used on golf courses were published. Susceptible grasses included species of *Agrostis*, *Festuca*, *Lolium*, and *Poa* (15). Pythium root rot symptoms begin as a slight necrotic and thinning appearance in small areas of turf. As symptoms progress, small symptomatic areas can coalesce into larger areas appearing more pronounced and leading to the loss of the established turfgrass stand. *Pythium* species associated with root rot have been isolated from turfgrasses and characterized, but defining any single species as the principal cause of disease is difficult due to the large number of *Pythium* species present on rotted roots (1, 8). Abad et al. (1) isolated 33 *Pythium* species from turfgrasses, and characterized eight species (*P. myriotylum*, *P. arrhenomanes, P. aphanidermatum, P. aristosporum, P. volutum, P. graminicola, P. vanterpoolii, P. ultimum* var. *ultimum*) as highly aggressive on post-emergent creeping bentgrass. Based on frequency of isolation and aggressiveness, *P. arrhenomanes* was identified as an important root pathogen of creeping bentgrass in North Carolina. *Pythium torulosum* and *P. vanterpoolii* were also commonly associated with both healthy and diseased turfgrass samples, indicating that a complex of *Pythium* species may be involved in root rot development. Pathogenicity assays consisted of pre-emergent pathogenicity on germinating creeping bentgrass seeds, and post-emergent pathogenicity on week-old creeping bentgrass seedlings. Pathogenicity of *Pythium* spp. on established creeping bentgrass plants has not been determined.

Pythium graminicola was characterized as the principal pathogen of creeping bentgrass in the Northeast United States, based upon the large percentage of isolates identified as *P. graminicola* and pathogenicity on creeping bentgrass. Pathogenicity

assays in the lab, growth chamber, and field demonstrated that *P. graminicola* is pathogenic in all environments (16). Pathogenicity assays also demonstrated that *P. aphanidermatum*, *P. aristosporum*, *P. torulosum*, and *P. vanterpoolii* caused root rot at both 13°C and 28°C. However, pathogenicity of *P. vanterpoolii* and *P. torulosum* was tested on pre-emergent seeds and not on established bentgrass turf. Hsiang et al. (9) demonstrated similar findings by leaf culture pathogenicity assays; *P. graminicola, P. aphanidermatum*, *P. torulosum,* and *P. ultimum* caused substantial necrosis of leaf disks. They also demonstrated in a greenhouse assay that *P. torulosum* caused moderate symptoms of Pythium root rot on 2 to 3 week old creeping bentgrass (9). The study was conducted at ideal growing temperatures (20°C to 25°C) for creeping bentgrass. It is possible that plants under high temperature stress could develop more severe symptoms of Pythium root rot.

Many *Pythium* species are associated with creeping bentgrass roots. Interactions among species of *Pythium* and other organisms could enhance infection or symptoms of disease and need to be characterized (12). Hodges (7) hypothesized that black layer accumulation and its association with reduced growth of creeping bentgrass may predispose the host to infection by *Pythium torulosum*. Sulfate-reducing bacteria, *Desulfovibrio desulfuricans*, require an anaerobic environment and an organic nutrient source to metabolize, and are the primarily cause of black layer in sand-based golf greens. Cyanobacteria provide such an environment for *D. desulfuricans* to thrive. The greatest effect on growth was caused by the combination of *D. desulfuricans* and *P. torulosum*. In this combination, black layer was not produced due to the absence of cyanobacteria in the soil. This provided evidence that *D. desulfuricans* is active within

the soil, even without black layer. But more importantly, showed the presence of *D. desulfuricans* in the soil enhanced aggressiveness of *P. torulosum*. These findings show a complex of species affecting creeping bentgrass. These types of interactions may be occurring between two or more *Pythium* species as well. Abad et al. (1) noted the recovery of multiple species from the same infected roots. *Pythium* species (*P. torulosum* or *P. catenulatum*) were often found in combination with other species. Pathogenicity assays were performed at 22°C, which is optimal conditions for creeping bentgrass to grow, and suggests that higher temperatures could cause symptoms much more severe than those observed in this study.

Previous research provides evidence that single species of *Pythium,* as well as the interactions between *Pythium* species and other organisms have potential to cause Pythium root rot. Diseases can also be enhanced by abiotic stresses, such as organic matter accumulation (2), excessively wet soil (13), drought (11), or excessive heat (1). These complex interactions in the root zone of golf course putting greens have not been well characterized and epidemics are still occurring. In North Carolina the summers of 2010 and 2011 produced persistent high temperatures ≤ 34°C. These summers combined brought 120 samples into North Carolina State University's Plant Disease and Insect Clinic that were diagnosed as Pythium root rot. With a changing climate where extreme temperatures become normal, investigating the etiology of this disease is imperative.

To further improve our ability to diagnose and manage root diseases of creeping bentgrass on sand-based putting greens, additional research is needed to determine the distribution of *Pythium* species causing Pythium root rot. The objectives of this study were to (i) determine the distribution of *Pythium* species causing Pythium root rot in

North Carolina; (ii) determine the pathogenicity and virulence of *Pythium* species on post-emergent creeping bentgrass seedlings and mature creeping bentgrass plants.

MATERIALS AND METHODS

Collection of *Pythium* species. *Pythium* isolates were obtained from golf course putting green samples sent into the Plant Disease and Insect Clinic (PDIC) at North Carolina State University between 2014 and 2015. Samples were also collected from Lake Wheeler Turfgrass Research Station in Raleigh, NC at the beginning of each month from March 2014 until October 2015. Samples diagnosed with Pythium root rot had visible oospores embedded in roots, and necrotic lesions on primary roots. Samples, which consisted of an 11 cm core of turf and associated soil, were cleaved and individual plants were excised from the sample. Individual plants were washed thoroughly under running tap water for four hours, blotted dry, and cut into pieces. Twenty roots, measuring 1 cm long, were placed on sterilized P_{10}ARP agar (17 g/liter corn meal agar amended with pimaricin, ampicillin, rifampicin, and PCNB (Terraclor 75% WP, Southern Ag Insecticides, Inc.), clarified V8 juice cholesterol agar (SV8). Similar samples were placed within the root zone of 'A-1' creeping bentgrass seedlings grown in calcined clay (Turface Allsport; Profile Products LLC, Buffalo Grove, IL) and incubated at room temperature in saturated conditions until plants wilted (11). Baited roots were washed and plated on P_{10}ARP agar. Plates were incubated in the dark at 23°C and colonies and resembling *Pythium* were transferred to potato dextrose agar (PDA). If bacterial contamination occurred, a mycelial plug was transferred to a fresh PDA plate and the top of a 1.5 ml microcentrifuge tube was placed over the mycelial plug. Bacteria-free

Pythium mycelium grew through the PDA and a mycelial plug was transferred to a new PDA plate. For extended storage of isolates, mycelial plugs were placed on a water agar medium and stored at 18°C. Stored cultures were routinely transferred every two months. *Pythium* isolates were also provided from the collection of B.B. Shew and E.C. Lookabaugh at North Carolina State University, Department of Plant Pathology. These included *Pythium vexans* and *P. myriotylum* isolated from chrysanthemum; *P. aphanidermatum* isolated from poinsettia; *P. irregulare* and *P. ultimum* var. *ultimum* from unknown hosts.

Morphological identification of *Pythium* spp. All isolates believed to be *Pythium* species were transferred to grass-leaf cultures to induce production of sporangia, antheridia, oogonia, and oospores. Grass-leaf cultures consisted of a petri dish filled with 10 ml sterile deionized water and 10 to 20 pieces (1 cm long) of autoclaved (30 minutes) creeping bentgrass leaves (1, 11, 13). Two (0.5 cm^2) mycelial cuttings placed over the leaves and cultures were incubated at room temperature under continuous fluorescent light for 3 to 5 days. All isolates produced oogonia, oospores, antheridia in culture; most produced sporangia. *Pythium* species were identified using the keys and descriptions of van der Plaats-Neterink (19), Dick (3) and Abad et al. (unpublished data). Identification was based on morphology and dimensions of 20 reproductive structures and colony morphology on PDA.

Phylogenetic analysis using ITS sequencing. Internal spacer region (ITS1-5.8S-ITS2) of the rDNA gene of the isolates was sequenced for DNA-based identification. Four mycelial plugs from individual isolates were placed in 50 ml of autoclaved SV8 broth (100 ml V8 Juice, 400 ml deionized water, and 1 g $CaCO_3$) and grown at room

temperature in shake culture (100 rpm) for 7 days. Mycelium was poured onto filter paper in a Buchner funnel and rinsed with sterile tap water during aspiration. The mycelium was harvested from filter paper and placed in individual 1.5 ml microcentrifuge tubes. Genomic DNA was extracted using the Easy-DNA Kit (Invitrogen Corp., Carlsbad, CA), quantified by spectrophotometry at 260 nm, and standardized to 100 ng/µl.

PCR amplification of the ITS1, 5.8S, and ITS2 regions of the ribosomal DNA was performed using ITS4 (5' TCCTCCGCTTATTGATATGC) and ITS5 (5' GGAAGTAAAAGTCGTAACAAGG) primers (21). Reactions were 15 µl in volume and composed of 1.5 mM 10X PCR Buffer (Bioline USA, Taunton, MA), 0.75 mM dNTP mix (Bioline), 0.75 µM of each primer, 0.09 µl *Taq* DNA polymerase (Bioline), 10.16 µl nuclease-free water, and 1 µl of 1 ng/µl extracted DNA. Thermal cycling conditions consisted of an initial denaturation step at 94°C for 2 minutes; followed by 30 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 45 seconds; and a final extension step at 72°C for 5 minutes. Positive PCR amplification was confirmed by gel electrophoresis before purifying samples using ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA); 2 µl of ExoSAP-IT was added to 6 µl PCR product and subjected to thermal cycling conditions of 37°C for 30 minutes followed by 80°C for 20 minutes. Purified samples were sequenced using BigDye v3.1 protocols obtained from the Duke University Center for Genomic and Computational Biology. Sample reactions 10 µl in volume were prepared and sequencing reactions were performed using thermal cycling conditions of an initial denaturation step at 96°C for 2 minutes; followed by 40 cycles of 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes. The resulting product volume was

submitted to Duke University Genome Sequencing Laboratory for sequencing. Results for each sample submission were aligned and edited with CLC Genomics Workbench v7.5 (CLC bio: A QIAGEN Company, Boston, MA). Identification was obtained using the Basic Local Alignment Search Tool (BLASTn) utility in the National Center for Biotechnology Information (NCBI) Database. Reference sequences of identified *Pythium* spp. were obtained from GenBank for comparison. A phylogenetic tree was constructed in CLC Genomic Workbench using the UPGMA algorithm from genetic distances calculated using Kimura-80 model. Bootstrap values were calculated in CLC Genomic Workbench based on 10,000 random samples of the data set, and held to a 50% threshold.

Pathogenicity assays on post-emergent creeping bentgrass. 'A-1' creeping bentgrass was used to test the pathogenicity of 19 isolates of *Pythium* species collected from turfgrass. Twelve isolates from creeping bentgrass cultivars and seven from bermudagrass cultivars were tested. Included were eight isolates of *P. torulosum* (LW1, LW5, LW6, LW8, LW9, LW10, LW11, LW12, and LW14), seven *P. vanterpoolii* (WRGC1, RBR, Pinehurst, DMC22, DMC15, Lambert, and P1), two *P. arrhenomanes* (Sedgefield and WRGC5), and one *P. aphanidermatum* (CBG; Table 2.1). In post-emergence tests, 3 oz. cups (Dixie cups, Georgia Pacific, Atlanta, GA), with holes for drainage, were filled with calcined clay (Turface Allsport) or growing mix consisting of approximately 35% sphagnum peat moss, aged-pine bark and dolomite lime (Metro-Mix 360, Sun Gro Horticulture, Agawam, MA). Cups were seeded with 'A-1' creeping bentgrass (10 g m^{-2}), incubated at 25°C/22°C (12-hour day/night cycles) and misted twice daily for rapid germination.

Seven days after seeding, plants in each cup were trimmed to a height of 1 cm and

inoculated with one of the 19 isolates. Inoculum was prepared by placing two 6-mm mycelial plugs into sterile water (10 ml) containing five to ten sterilized 1-cm long creeping bentgrass leaves. Inoculum was then incubated under continuous fluorescent light at room temperature (~25°C). Inoculations were performed by placing five to ten *Pythium*-colonized grass blades approximately 5-mm deep into the soil of each cup followed by saturating the cup with 10 ml of water used to culture the grass blade inoculum. For non-inoculated controls, sterile grass blades were placed in the soil at approximately 5-mm depth. Grass in cups was transferred into humidity boxes where they were arranged in a complete randomized block design with 3 replications with peat moss for soil, and 3 replications with calcined clay substrate. Forty cups were arranged randomly within three blocks. Humidity boxes were placed atop lab workbenches at room temperature (23 to 25°C). After seven days, disease was visually assessed on a scale of 1-5 (1 = no disease, 3 = 50% disease, and 5 = 100% of seedlings wilted or necrotic). Symptoms included seedling chlorosis, necrosis and wilting. Individual plants were removed for root symptom observation and isolation of the pathogen on P_{10}ARP medium. Post-emergence experiments were conducted twice.

Statistical analysis was performed using SAS (v9.4; SAS Inc., Cary, NC). Analysis of variance was conducted using PROC GLIMMIX to estimate the effects of growth substrate, experiment, and isolate on disease severity. Tukey-Kramer was used to separate means for comparison of the *Pythium* isolates.

Pathogenicity assays on mature creeping bentgrass. 'A-1' creeping bentgrass was used to test the pathogenicity of the 19 isolates of *Pythium* previously described (Table 2.2). In preparation for the pathogenicity assay, cone-tainers (3.8 cm x 20 cm)

containing a 50:50 (% wt : wt) mix of sand and 60% sphagnum peat moss (Sunshine AG-Lite, Sun Gro Horticulture, Agawam, MA) were filled within approximately 1.5 cm of the top. The last 1.5 cm were filled with the 60% sphagnum peat moss substrate and seeded with 'A-1' creeping bentgrass (10 g m^{-2}). The sphagnum peat moss layer was used imitate the accumulated organic matter often found in putting greens prone to Pythium root rot problems. Cone-tainers were placed in a greenhouse at 26°C/22°C (12-hour day/night cycles) and misted twice daily. Following germination, creeping bentgrass was maintained in the greenhouse by irrigating twice daily with a one-weekly complete nutrient solution containing 106.23 mol m^{-3} of nitrogen, 10.41 mol m^{-3} of phosphorous, and 111.03 mol m^{-3} potassium. Turf was manually trimmed weekly to a height of 5 mm.

Five weeks after seeding, each cone-tainer was infested with one of the 19 *Pythium* isolates. Inoculum was prepared by placing two 6-mm mycelial plugs into sterile water (10 ml) containing fifteen to twenty sterilized 1-cm long creeping bentgrass leaves. Inoculum was then incubated under continuous fluorescent light at room temperature (~25°C). Inoculations were performed by gently removing contents of the cone-tainer, cutting the root system at a 1-cm depth, and removing the turf plug. The contents of *Pythium* grass-blade inoculum (15 to 20 grass blades with 2 mycelial plugs and 10 ml deionized water) were placed on top of the cut soil surface and the turf plug was replaced on top of the inoculum in the cone-tainer. Non-inoculated controls were included in each experiment by cutting the roots at 1-cm depth and placing sterilized grass blades on top of soil, and then replacing the removed turf plug on the grass blades.

Inoculated cone-tainers were placed in water-filled trays, covered with humidity domes and kept in the greenhouse for 7 days to allow *Pythium* to infect the roots, and also

for the cut roots to recover. After 7 days cone-tainers were transferred to a growth chamber and arranged in a randomized complete block design with 3 replications per isolate. Growth chamber conditions consisted of a 12 hour day/night cycle at 34°C/28°C to mimic summer field conditions with misting twice daily and complete nutrient solution once weekly. Plants were maintained at 5 mm height with weekly trimmings. Plants were visually assessed for foliar symptoms of chlorosis, necrosis and wilting weekly on a 1-5 scale (1 = no disease, 3 = 50% disease, and 5 = 100% of seedlings wilted or necrotic). After 5 weeks, cone-tainers were destructively sampled to visually assess root symptoms, and to isolate the pathogens on P_{10}ARP. Growth chamber experiments were conducted twice.

Statistical analysis was performed using SAS v9.4. Analysis of variance was conducted using PROC GLIMMIX to estimate effects of experiment and isolate on disease severity. Tukey-Kramer was used to separate means for comparison among the *Pythium* isolates.

RESULTS

Morphological identification of *Pythium* species. *Pythium* isolates were collected during the summer of 2014 and 2015. Isolates collected from Lake Wheeler Turfgrass Research Station were all *P. torulosum*. Of the 105 *Pythium* isolates obtained from turfgrasses, 90 were identified as *P. torulosum*, 13 as *P. vanterpoolii*, two as *P. arrhenomanes*, and one as *P. aphanidermatum* (Table 2.3). The majority of *P. torulosum* isolates were collected from location 1, the Lake Wheeler Turfgrass Research Field Lab when P_{10}ARP was used as the isolation medium (Table 2.3). *Pythium vanterpoolii* was

isolated from turf grown at 7 different golf courses, and from creeping bentgrass and bermudagrass. *Pythium arrhenomanes* isolates were associated with bermudagrass cultivars from two different golf courses, but was not isolated from bentgrass. One isolate of *P. aphanidermatum* was collected in Wake Co., NC from the seed of 'A-1' creeping bentgrass, where it had caused damping-off of seedlings. *Pythium torulosum* isolates were characterized by single monoclinous antheridia arising close to the oogonium, lobate sporangia, small plerotic oospores 15 to 18 μm in diameter, and rosette colony morphology on (PDA) (Table 2.5, Figure 2.1). *Pythium vanterpoolii* isolates were characterized by 1-2 monoclinous antheridia, lobate and catenulate sporangia, small plerotic oospores 13 to 22 μm in diameter, and radiate, semi-fluffy colony morphology on PDA (Table 2.5, Figure 2.1). *Pythium arrhenomanes* isolates were characterized by seven to eight diclinous antheridia, lobate sporangia, aplerotic oospores between 27 and 35 μm in diameter, and a radiate, semi-fluffy growth pattern on PDA (Table 2.5, Fig. 2.1). *Pythium aphanidermatum* isolates were characterized by one sac-shaped antheridia, lobate sporangia, aplerotic oospores 20 to 27 μm in diameter, and fluffy fast growth in PDA culture (Table 2.5, Fig. 2.1). Isolates of *P. irregulare, P. myriotylum, P. vexans* and *P. ultimum* var. *ultimum* were previously identified by the collector.

Phylogenetic analysis using ITS sequence. According to ITS sequence data, *Pythium* species collected during 2014-2015 clumped closely with reference isolates from GenBank, with the exception of one *P. aphanidermatum* isolate (Paph), and *Pythium vexans* isolate (Ed-mum-27). However, morphological characteristics classified (Paph) as *P. aphanidermatum* and Ed-mum-27 as *P. vexans*. *Pythium* species fit into clades similar to those determined by Lévesque et al. *Pythium vanterpoolii, P. myriotylum, P.*

arrhenomanes, P. volutum, P, torulosum and P. aphanidermatum were all closely clumped (Fig 2.2). Three more clades were formed by GenBank *P. vexans* reference sequence, *Pythium ultimum* var. *ultimum* and *P. irregulare*; consistent with Lévesque et al. All ten isolates of *P. torulosum* were placed in the same monophyletic group as *P. torulosum* ITS reference sequence obtained from GenBank (Fig. 2.2). *Pythium vanterpoolii* isolates clumped together with GenBank ITS reference sequence. *Pythium arrhenomanes* isolates (WRGC5 and Sedgefield) were similar to the GenBank reference sequence.

Pathogenicity of isolates on creeping bentgrass: Post-emergence There were no significant differences in isolates between experiments and data from both experiments was averaged. There was no significant difference ($P < 0.05$) between the disease severity ratings on seedlings grown in sphagnum peat moss compared to calcined clay growing substrate. Disease ratings ranged from 1.0 to 5.0 by 7 days after inoculation. Seedlings expressed symptoms of wilt, root necrosis, and foliar necrosis and chlorosis in both calcined clay and sphagnum peat moss growing substrates (Fig 2.1, Fig. 2.7). Mycelial growth was visible on the foliage of creeping bentgrass in all isolates of *P. vanterpoolii*, *P. arrhenomanes*, and *P. aphanidermatum*. Mycelial growth was also visible in creeping bentgrass foliage inoculated with *P. torulosum* isolate LW6. Four isolates of *P. vanterpoolii* (DMC15, DMC22, P1, Lambert), both isolates of *P. arrhenomanes* (WRGC5 and Sedgefield), and *P. aphanidermatum* isolate CBG all caused 100% disease severity of creeping bentgrass seedlings (Table 2.1). Three isolates of *P. vanterpoolii* (RBR, WRGC1, and Pinehurst) caused slightly less than 100% disease severity (means = 4.7 to 4.8), but differences among isolates of *P. vanterpoolii* were not

significant (Table 2.1). *Pythium torulosum* isolates were pathogenic to creeping bentgrass seedlings, but disease severity was low (0 to 20%) and inconsistent. One *P. torulosum* isolate (LW6) caused more disease (mean = 1.8 $\pm$ 1.1) than the uninoculated check (mean = 1.1 $\pm$ 0.2). All isolates of *P. torulosum*, *P. vanterpoolii*, *P. arrhenomanes*, and *P. aphanidermatum* were isolated from foliage and roots 7 days after inoculation.

Pathogenicity on mature creeping bentgrass. There were no significant differences in isolates between experiments and data from both experiments was averaged. In the first experiment, foliar symptoms developed and mycelium was visible on foliage of creeping bentgrass plants that had been inoculated with *P. aphanidermatum* isolate CBG within three days after they were moved from the greenhouse to growth chambers. After six days in the growth chamber, *P. aphanidermatum* infected plants were severely blighted and were removed from growth chamber. *Pythium aphanidermatum* was isolated from the roots and foliage of each sample. In the second experiment, creeping bentgrass growing in cone-tainers infested with *P. aphanidermatum* expressed foliar symptoms four days after inoculation before being moved to the growth chamber. Foliar mycelium was again visible. After four days in the growth chamber, severe blighting occurred and cone-tainers were removed from the growth chamber. *Pythium aphanidermatum* was isolated from the removed samples. Cross-contamination was observed on two cone-tainers that had not been infested with *P. aphanidermatum*. Contaminated plants were removed and isolation confirmed *P. aphanidermatum* was the pathogen. Plants inoculated with *P. aphanidermatum* isolate CBG consistently exhibited a 5 on the disease rating scale (Table 2.2). Foliar symptoms did not develop on plants inoculated with isolates of *P. vanterpoolii*. No isolates significantly differed from one

another or the non-inoculated control (Table 2.2). Similarly, foliar symptoms did not develop on creeping bentgrass plants inoculated with *P. torulosum* or *P. arrhenomanes*. Plants inoculated with isolates of *P. arrhenomanes* or *P. torulosum* were not significantly different from the uninoculated controls (Table 2.2). However, upon dissection of the creeping bentgrass plants, oospores were observed congregated in the root cortex of plants inoculated with *P. torulosum* (Fig. 2.5) and *P. vanterpoolii* (Fig. 2.6). *Pythium torulosum*, and *P. vanterpoolii* were also isolated from roots on P_{10}ARP media.

DISCUSSION

Numerous studies have characterized *Pythium* spp. associated with root rot and many are potential pathogens of creeping bentgrass seedlings and mature, established turfgrass (1, 7, 9, 11, 15). Predominant *Pythium* spp. associated with creeping bentgrass roots in North Carolina include *P. torulosum*, *P. catenulatum*, *P. vanterpoolii* and *P. arrhenomanes* (1). In our study, 105 isolates of *Pythium* were collected from 10 locations. *Pythium torulosum* was the predominant species isolated, comprising 85% of isolates collected. However, 97% of *P. torulosum* isolates came from a single location. The remaining isolates came from a single golf course with bermudagrass putting greens. Its small geographic distribution is contrary to previous findings (1, 4, 5, 15, 17), where it was the predominant.

Pythium vanterpoolii was isolated 13 times from six locations and was associated with both creeping bentgrass roots and bermudagrass foliage. Its presence in numerous locations is similar to previous surveys. The exclusive use of selective media (P_{10}ARP) for isolations may have resulted in under-recovery of *P. vanterpoolii* in previous studies

(1, 15). We successfully isolated *P. vanterpoolii* from roots by baiting with creeping bentgrass seedlings, whereas isolation on P_{10}ARP sometimes failed.

We were unable to isolate *P. arrhenomanes* from creeping bentgrass roots, but it was isolated from foliage of bermudagrass growing in two locations. SV8 agar was not useful when root rot symptoms were severe and was prone to contamination from *Fusarium* and *Mortierella* especially during the summer months.

After Pythium root rot epidemics in the summers of 2010 and 2011, there was a greater emphasis and concerted effort to apply preventative root rot fungicides. This management practice may have made it more difficult to isolate *Pythium* from roots in our study. It also is possible that the fungicides commonly used to control root rot caused a shift in *Pythium* populations, favoring the species we recovered in this survey. Difficulty selecting for *Pythium* on P_{10}ARP medium also hindered recovery. *Fusarium* and *Mortierella* were isolated frequently from infected roots, suggesting that their effects on creeping bentgrass roots should be investigated. Nevertheless, isolations in our study support conclusions from previous research on *Pythium* species associated with creeping bentgrass roots.

In previous experiments, *P. arrhenomanes* and *P. aphanidermatum* were both highly aggressive on creeping bentgrass seedlings at temperatures between 28°C and 32°C (1). Similarly, in our experiments both species caused 100% disease severity and foliar necrosis or wilt at 25°C. However, contrary to previous studies, which indicated that *P. vanterpoolii* was moderately aggressive at 28°C (1, 4), isolates of *P. vanterpoolii* were highly aggressive at 25°C in our study, causing 100% disease severity. Optimal growth in culture was reported at 25°C (Table 2.2), which may explain the high

aggressiveness observed in our study. Most *P. torulosum* isolates caused little or no disease on creeping bentgrass, but one isolate was pathogenic with low aggressiveness, as was shown in similar studies (1, 4). It is possible *P. torulosum* would be more aggressive near its optimal growth temperature of 30°C (Table 2.2). Pythium root rot often is associated with persistent hot and humid weather that can cause suppression in host defense signaling (5). Pathogenicity of *P. vanterpoolii, and P. arrhenomanes* on creeping bentgrass seedlings may demonstrate the ability of *Pythium* to overcome weak plant immunity under favorable conditions. It was expected that *P. aphanidermatum* would quickly consume the seedlings. However, *P. vanterpoolii* and *P. arrhenomanes* are not known to for their aggressiveness, like *P. aphanidermatum*, and therefore plant age may play a role in whether a *Pythium* species is determined pathogenic.

Our research also showed that *P. aphanidermatum* was highly aggressive on roots of mature creeping bentgrass. This was expected given that our studies were conducted at 34°C and the optimal temperature range for growth of *P. aphanidermatum* is 31°C to 37°C (Table 2.2; 14, 16). *Pythium vanterpoolii*, *P. torulosum* and *P. arrhenomanes* did not cause symptoms on mature bentgrass plants under our experimental conditions. However, oospores were observed in roots, indicating that inoculum was present (Fig. 2.6, Fig 2.7). It is possible that large numbers of oospores could injure or disrupt functions in roots and cause noticeable symptoms under certain conditions (4).

Conditions in our study may not have stressed plants enough to predispose them to disease. The use of sphagnum peat moss in the top 1.5 cm of soil may have provided better growing conditions for creeping bentgrass than in sand-based soils typical of putting greens. Peat moss has more available nutrients than sand, which may have

reduced the plants response to heat stress and maintained plant immunity. Heat stress, as simulated in the growth chamber, is only one of many stresses on creeping bentgrass putting greens. Traffic from golfers and mowers, frequent mowing at extremely low heights, intense UV radiation, and extreme fluctuations in temperature and moisture are only a few of the additional stresses present on a putting green. Hodges (7) showed that anaerobic conditions can lead to an interaction between a sulfate-reducing bacterium, *Desulfovibrio desulfuricans* and *P. torulosum* that can reduce root growth significantly (7). It also is possible interactions among the tested species, or other *Pythium* species not tested, may produce more severe disease than infections by a single species. Further research should be conducted to determine the effects of multiple *Pythium* spp. on established creeping bentgrass, and should examine both disease severity and root growth. Plant age may have was also been a factor in resisting *P. torulosum*, *P. vanterpoolii*, and *P. arrhenomanes*. More established, mature plants have a more robust physiology that makes it harder for *Pythium*

Our results demonstrate that pathogenicity of *Pythium* spp. on creeping bentgrass seedlings may not correlate with pathogenicity on established plants. While, post-emergent pathogenicity assays still hold valuable information of what species are pathogenic, plant age also appeared to be a leading variable in whether disease occurred. Our results support previous research characterizing *Pythium aphanidermatum* as pathogenic and highly aggressive on mature creeping bentgrass (17). Further surveys of diseased creeping bentgrass roots are needed to determine if interactions between *Pythium* and other organisms, including bacteria and true fungi, affect disease

development. More research is also needed to determine pathogenicity of other *Pythium* species on mature creeping bentgrass plants.

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Table 2.1. Pathogenicity of individual isolates of various *Pythium* species to post-emergent creeping bentgrass.

***Pythium* species**	**Isolate number**	**Disease rating[y]**
P. vanterpoolii	DMC15	5a[z]
	DMC22	5a
	P1	5a
	Lambert	5a
	RBR	4.8a
	WRGC1	4.7a
	Pinehurst	4.7a
P. arrhenomanes	Sedgefield	5a
	WRGC5	5a
P. aphanidermatum	CBG	5a
P. torulosum	LW6	1.8b
	LW9	1.6bc
	LW12	1.5c
	LW11	1.5c
	LW14	1.4c
	LW8	1.3c
	LW10	1.2c
	LW5	1.2c
	LW1	1.2c
Non-inoculated check	-	1.1c

[y] Disease rating was determined 7 days after inoculation on a 1 to 5 scale (1 = no disease, 3 = 50% disease, and 5 = 100% of seedlings wilted or necrotic).

[z] Means in column followed by the same letter are not significantly different according to Tukey-Kramer.

Table 2.2. Pathogenicity of individual isolates of various *Pythium* species to mature creeping bentgrass.

***Pythium* species**	**Isolate number**	**Disease rating[y]**
P. vanterpoolii	DMC15	1.2b[z]
	DMC22	1.2b
	P1	1b
	Lambert	1b
	RBR	1.2b
	WRGC1	1.2b
	Pinehurst	1.3b
P. arrhenomanes	Sedgefield	1.2b
	WRGC5	1.2b
P. aphanidermatum	CBG	5a
P. torulosum	LW6	1b
	LW14	1b
	LW12	1b
	LW11	1.2b
	LW9	1.2b
	LW8	1.2b
	LW10	1b
	LW5	1.2b
	LW1	1.2b
Non-inoculated check	-	1.2b

[y] Disease rating was determined 35-days after inoculation on a 1 to 5 scale (1 = no disease, 3 = 50% disease, and 5 = 100% of seedlings wilted or necrotic).

[z] Means in column followed by the same letter are not significantly different according to Tukey-Kramer.

Table 2.3. Origin of *Pythium* isolates, grass variety planted on putting green, isolation method and medium, number of isolates collected at each location, and the prevalence of *Pythium* species.

Location	Golf Course Location	Variety	Isolation Method	Isolation Media	No. Isolates	*P. torulosum*[y]	*P. arrhenomanes*[y]	*P. vanterpoolii*[y]
1	Wake Co, NC	Dominant Plus	Direct plating	PARP	88	88	-	-
2	Rutherford Co, NC	Dominant Plus	Baiting	PARP	4	-	-	4
3	Moore Co, NC	Champion[z]	Direct plating	PARP	1	-	-	1
4	Guilford Co, NC	Champion[z]	Direct plating	PARP	1	-	1	-
5	Fulton Co, GA	A-1	Direct plating	PARP	1	-	-	1
		A-1	Baiting	PARP	1	-	-	1
6	Cleveland Co, NC	Mini verde[z]	Direct plating	PARP	2	2	-	-
7	Wake Co, NC	A-1	Direct plating	PARP	1	-	-	-
8	Maricopa Co, AZ	Bermuda[z]	Direct plating	PARP	3	-	1	2
9	Maricopa Co, AZ	Bermuda[z]	Direct plating	PARP	2	-	-	2
10	Wake Co, NC	A-1	Baiting	PARP	2	-	-	2
					Totals:	90	2	13

[y] Number of isolates collected of *Pythium torulosum*, *Pythium arrhenomanes*, and *Pythium vanterpoolii*.

[z] Depicts *Pythium* isolates isolated from a Bermuda grass putting green.

Table 2.4. Designation of host from which *Pythium* species were isolated, number of isolates obtained and optimal growth conditions in culture.

Host	No. of isolates	*Pythium* species	Optimal growth temperature[x]
Creeping bentgrass	88	*P. torulosum*	30°C
	8	*P vanterpoolii*	25°C
	1	*P. aphanidermatum*	31-37°C
	1	*P. volutum*[z]	22-25°C
Bermudagrass	5	*P. vanterpoolii*	-
	2	*P. torulosum*	-
	2	*P. arrhenomanes*	25-31°C
Chrysanthemum	1	*P. myriotylum*[y]	28-31°C
	1	*P. vexans*[y]	28°C
Poinsettia	2	*P. aphanidermatum*[y]	-
Unknown	1	*P. ultimum* var *ultimum*[y]	25-30°C
	1	*P. irregulare*[y]	25-30°C

[x] Optimum growth temperature of *Pythium* species *in vitro* (14, 16).

[y] Species supplied from *Pythium* collection of B.B. Shew and E.C. Lookabaugh.

[z] *P. volutum* obtained from research conducted in 2008 on Pythium root dysfunction of creeping bentgrass by J.P. Kerns and L.P. Tredway.

Table 2.5. Designation of *Pythium* species, species isolate, number of isolates per haplotype, and morphological characteristics of *Pythium* species.

***Pythium* Species**	**Species Isolate[y]**	**Arrangement of Antheridia**	**No. of Antheridia**	**Average oogonium diameter (µm)[z]**
P. aphanidermatum	Met-pom	Monoclinous	1	23.1 ± 2.0
	33	Monoclinous	1	25.1 ± 1.5
	CBG	Monoclinous	1	24.9 ± 1.9
P. vanterpoolii	P1	Monoclinous	1	18.1 ± 3.2
	Pinehurst	Monoclinous	1	17.1 ± 2.3
	RBR	Monoclinous	1	19.0 ± 2.7
	DMC15	Monoclinous	1	18.9 ± 2.2
	DMC22	Monoclinous	1	20.4 ± 2.5
	Lambert	Monoclinous	1	16.4 ± 3.2
	WRGC1	Monoclinous	1	19.8 ± 3.1
	Peachtree	Monoclinous	1	21.6 ± 2.0
P. torulosum	LW1	Monoclinous	1	15.9 ± 1.9
	LW5	Monoclinous	1	16.4 ± 1.5
	LW6	Monoclinous	1	16.3 ± 1.7
	LW8	Monoclinous	1	16.5 ± 1.7
	LW9	Monoclinous	1	16.0 ± 1.7
	LW10	Monoclinous	1	16.5 ± 1.5
	LW11	Monoclinous	1	15.6 ± 1.6
	LW12	Monoclinous	1	16.4 ± 1.5
	LW14	Monoclinous	1	16.1 ± 1.9
	Riverbend	Monoclinous	1	16.3 ± 1.6
P. arrhenomanes	Sedgefield	Diclinous	14	31.3 ± 3.3
	WRGC5	Diclinous	8	33.0 ± 2.2
P. irregulare	Rw-pet-10	Monoclinous	1	17.1 ± 1.7
P. volutum	OC6	Diclinous	6	28.5 ± 2.6
P. vexans	Ed-mum-27	Monoclinous	1	21.9 ± 1.8
P. myriotylum	Ed-mum-22	Diclinous	7	25.4 ± 2.5

[y] Isolate depicted in phylogram.

[z] Diameter in µm based on measurement of 20 oospores per isolate, average and standard deviation calculated from all isolates in each species.

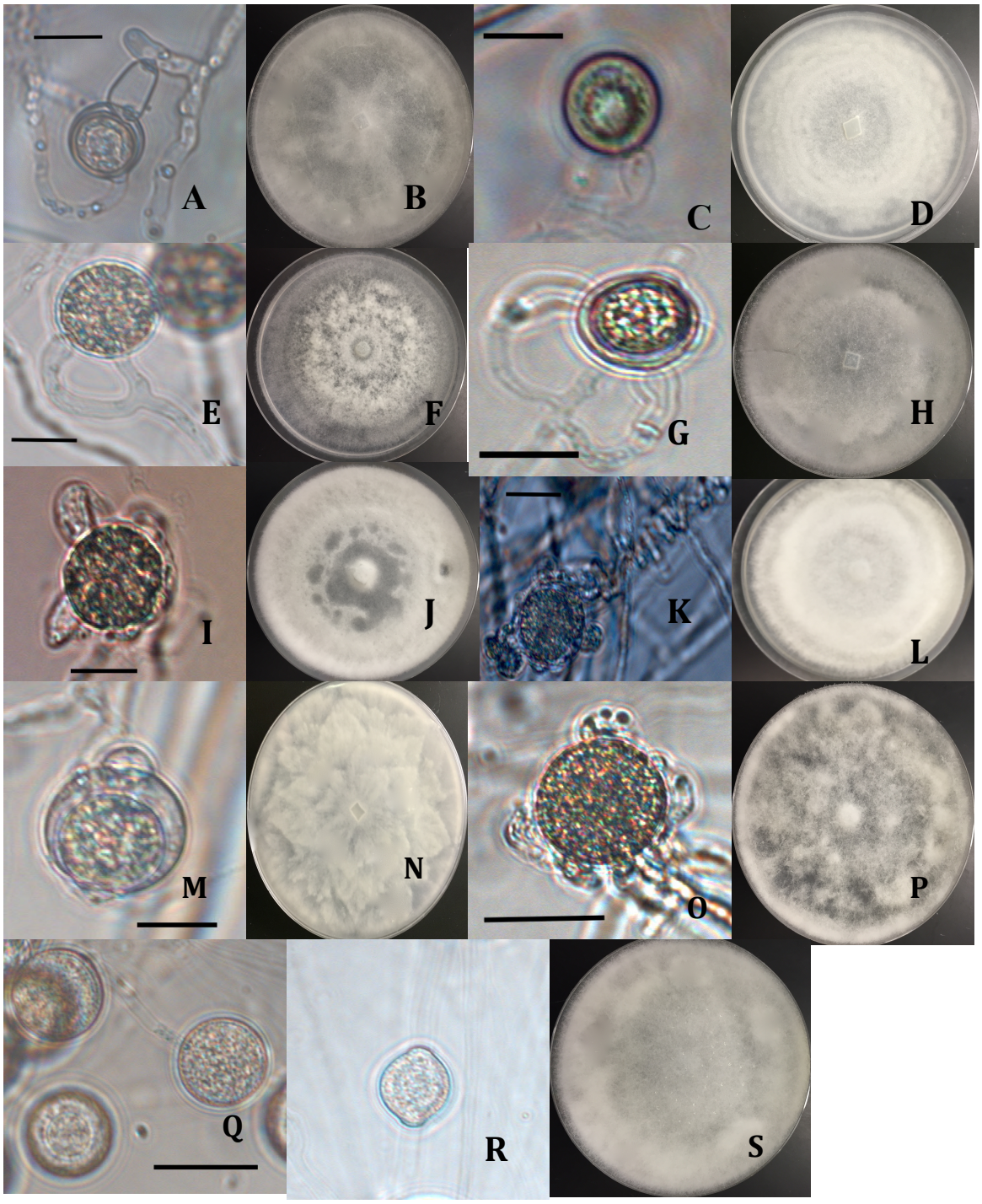

Fig. 2.1. Antheridial and oospore characteristics and colony morphology of *Pythium* spp. on PDA. **A.** *P. aphanidermatum* oogonium with sac-shaped antheridium, Bar =20 µm; **B.** *P. aphanidermatum* colony morphology; **C.** *P. torulosum* oogonium with monoclinous

antheridium, Bar =10 μm; **D.** *P. torulosum* colony morphology; **E.** *P. vanterpoolii* oogonium with monoclinous antheridium, Bar =10 μm; **F.** *P. vanterpoolii* colony morphology; **G.** *P.irregulare* oogonium with multiple monoclinous antheridia Bar =10 μm; **H.** *P. irregulare* colony morphology; **I.** *P. arrhenomanes* oogonium with multiple diclinous antheridia, Bar =20 μm; **J.** *P. arrhenomanes* colony morphology; **K.** *P. volutum* oogonium with multiple diclinous antheridia wrapping around oogonial stalk, Bar =20 μm; **L.** *P. volutum* colony morphology; **M.** *P. vexans* oogonium with monoclinous antheridium, Bar =10 μm; **N.** *P. vexans* colony morphology; **O.** *P. myriotylum* oogonium with multiple diclinous antheridia, Bar =20 μm; **P.** *P. myriotylum* colony morphology; **Q.** *P. ultimum* var. *ultimum* terminal spherical sporangia, Bar =20 μm; **R.** *P. ultimum* var. *ultimum* intercalary sporangia; **S.** *P. ultimum* var. *ultimum* colony morphology.

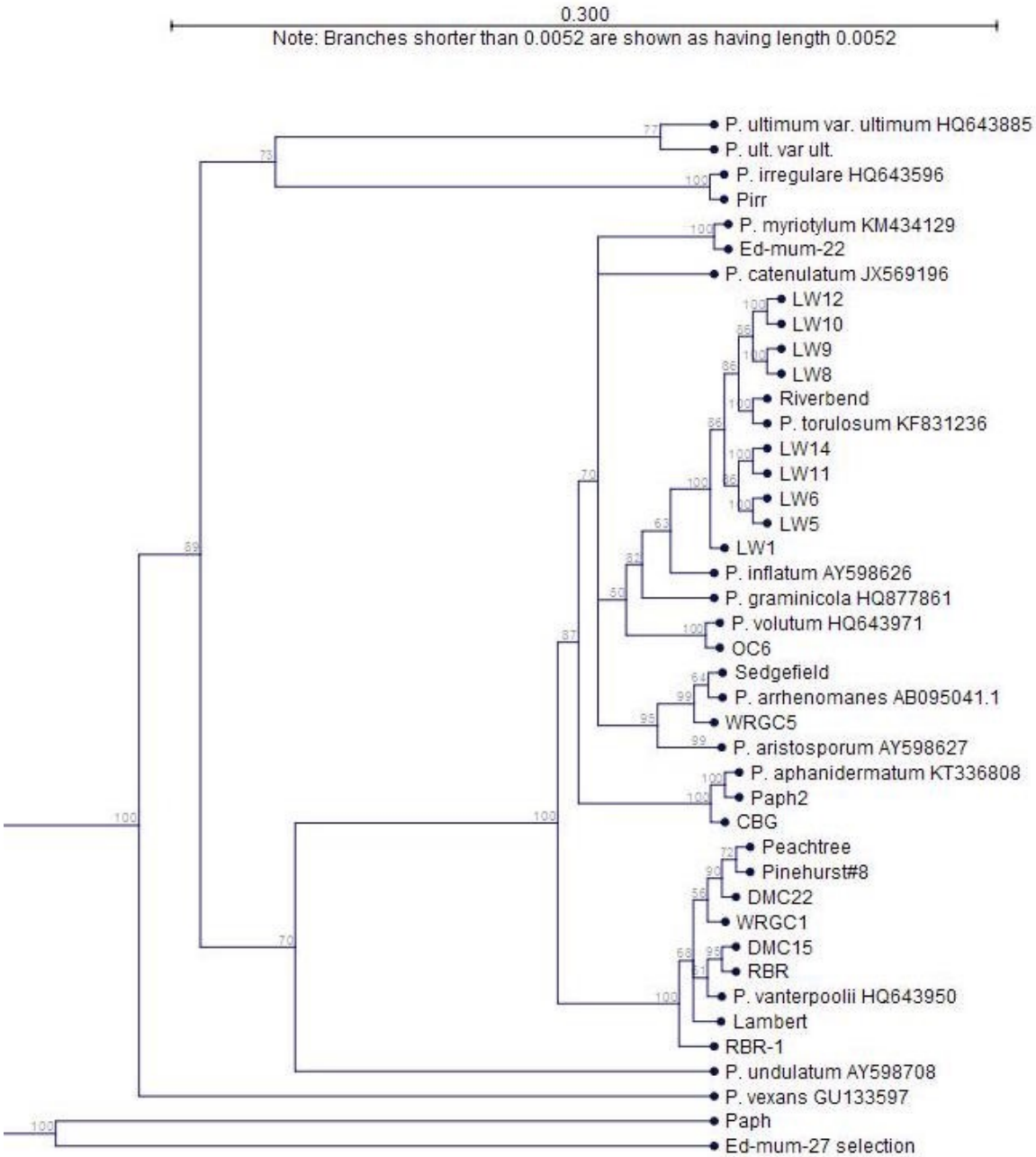

Fig. 2.2. UPGMA phylogram of *Pythium* species produced from sequences of rDNA regions ITS1, 5.8S, and ITS2. Scale bar indicates horizontal distance corresponding to genetic distance as calculated by the Kiumra-80 model. Bootstrap values are indicated adjacent to the nodes and are based on resampling the data set 10,000 times. Bootstrap threshold is set to 50%. Two letters with six-digit code follow GenBank reference species.

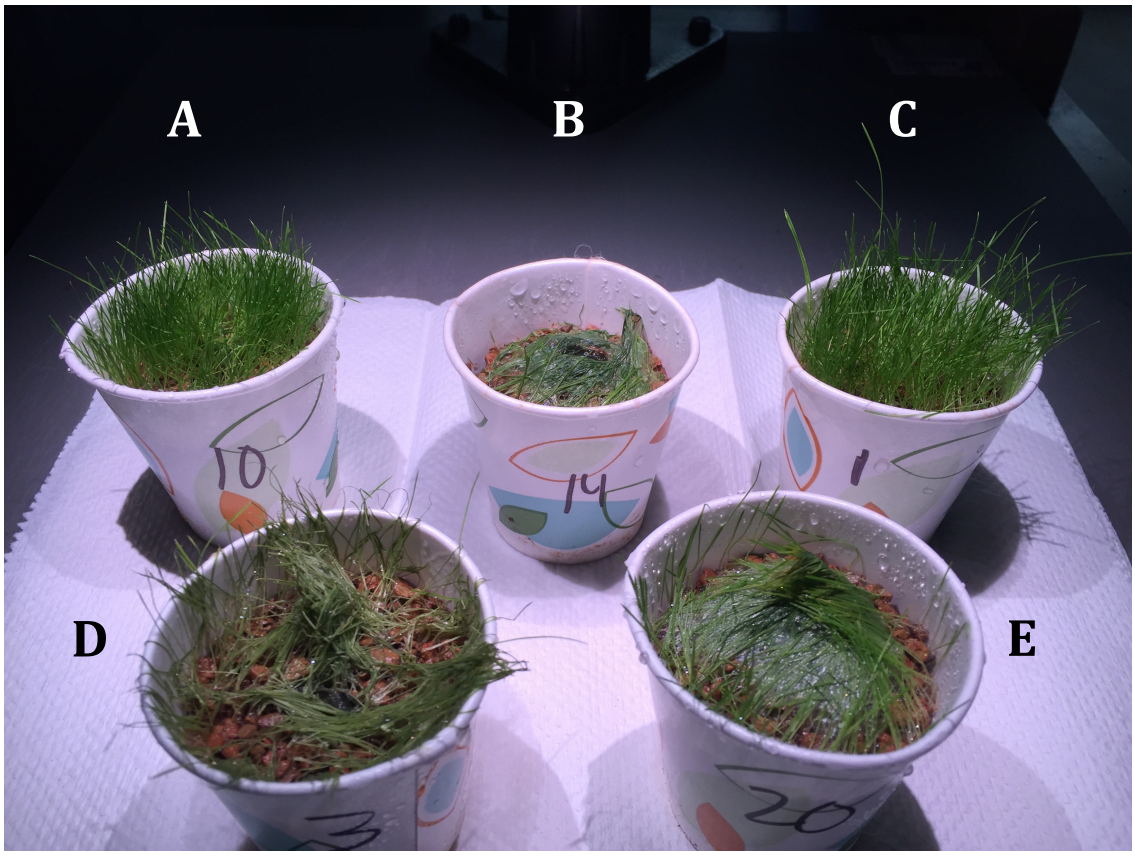

Fig. 2.3. Pathogenicity of four *Pythium* spp. at 25°C to creeping bentgrass grown in calcite clay 7-days after inoculation. **A**, *P. torulosum*, isolate LW14; **B**, *P. vanterpoolii*, isolate RBR; **C**, Non-inoculated control; **D**, *P. aphanidermatum*, isolate CBG; **E**, *P. arrhenomanes*, isolate WRGC5. 3-day old inoculum was placed 5mm into soil. Inoculum consisted of 20 sterile grass leaf blades in 10 ml of sterilized DI H_2O, with two 6mm. in diameter hyphal plugs.

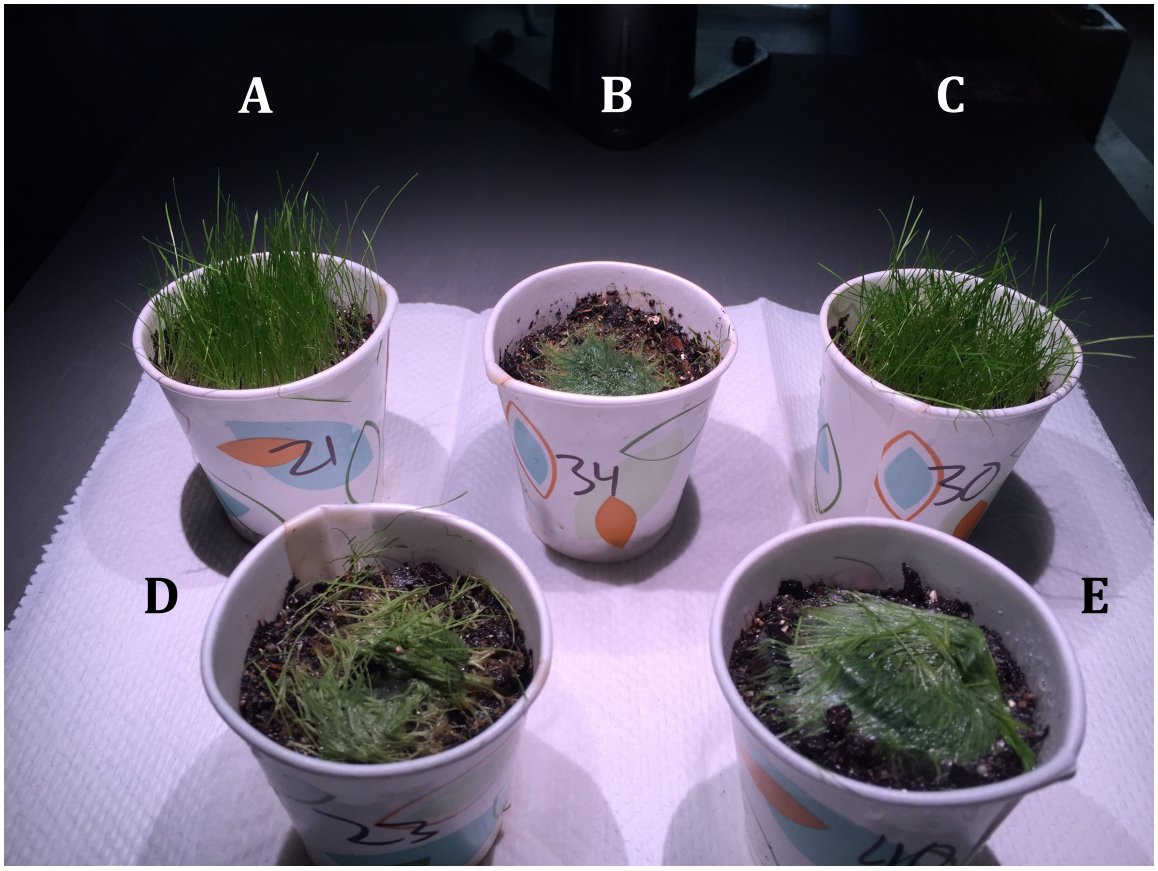

Fig. 2.4. Pathogenicity of four *Pythium* spp. at 25°C to creeping bentgrass grown in potting mix 7-days after inoculation. **A**, Non-inoculated control; **B**, *P. vanterpoolii*, isolate RBR; **C**, *P. torulosum*, isolate LW14; **D**, *P. aphanidermatum*, isolate CBG; **E**, *P. arrhenomanes*, isolate WRGC5. 3-day old inoculum consisted of 20 sterile grass leaf blades in 10 ml of sterilized DI H_2O, with two 6-mm. in diameter hyphal plugs.

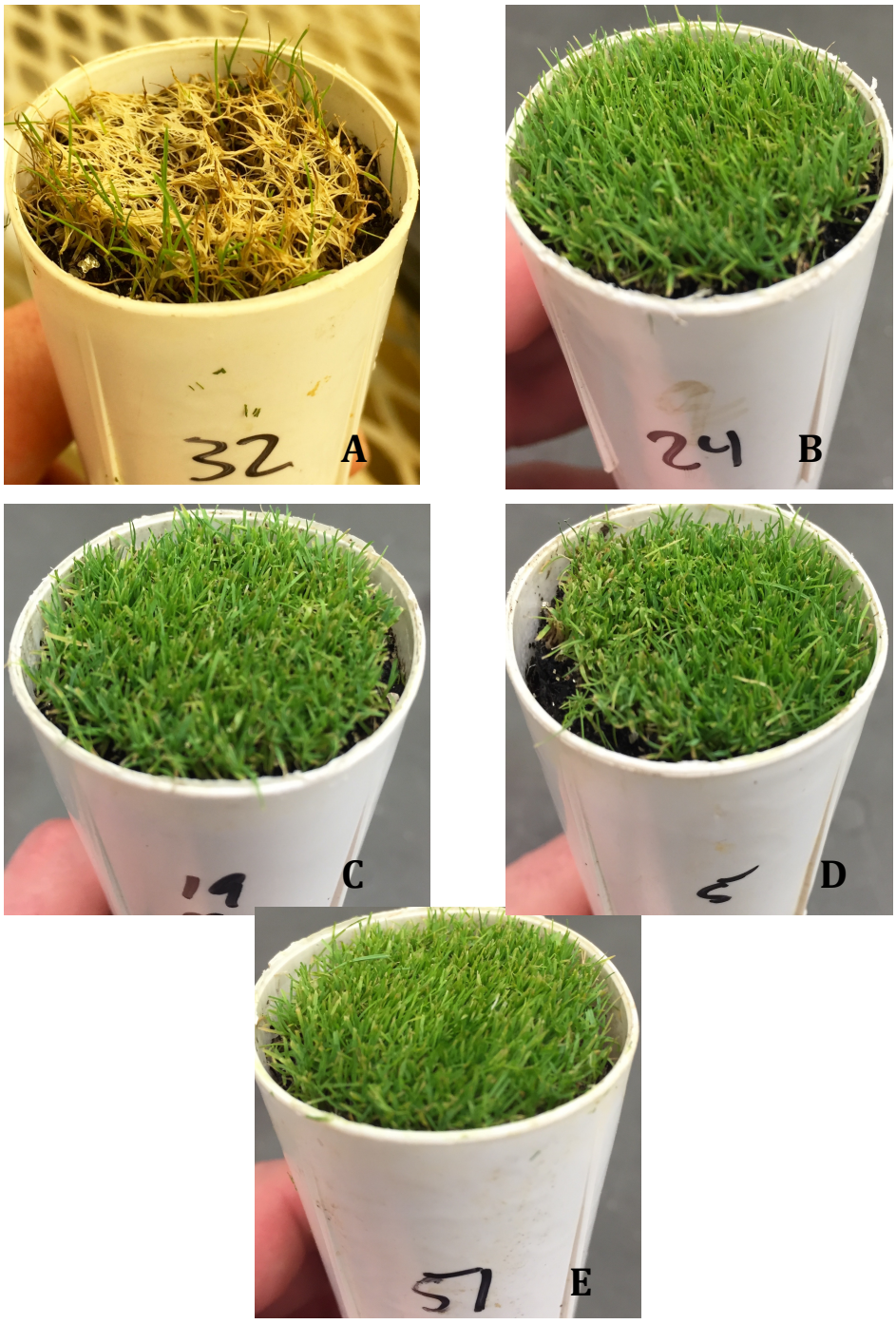

Fig. 2.5. Pathogenicity of four *Pythium* spp. to mature creeping bentgrass plants. **A.** *Pythium aphanidermatum* isolate CBG; **B.** *Pythium arrhenomanes* isolate WRGC5; **C.** *Pythium torulosum* isolate LW12; **D.** *Pythium vanterpoolii* isolate P1; **E.** Non-inoculated control.

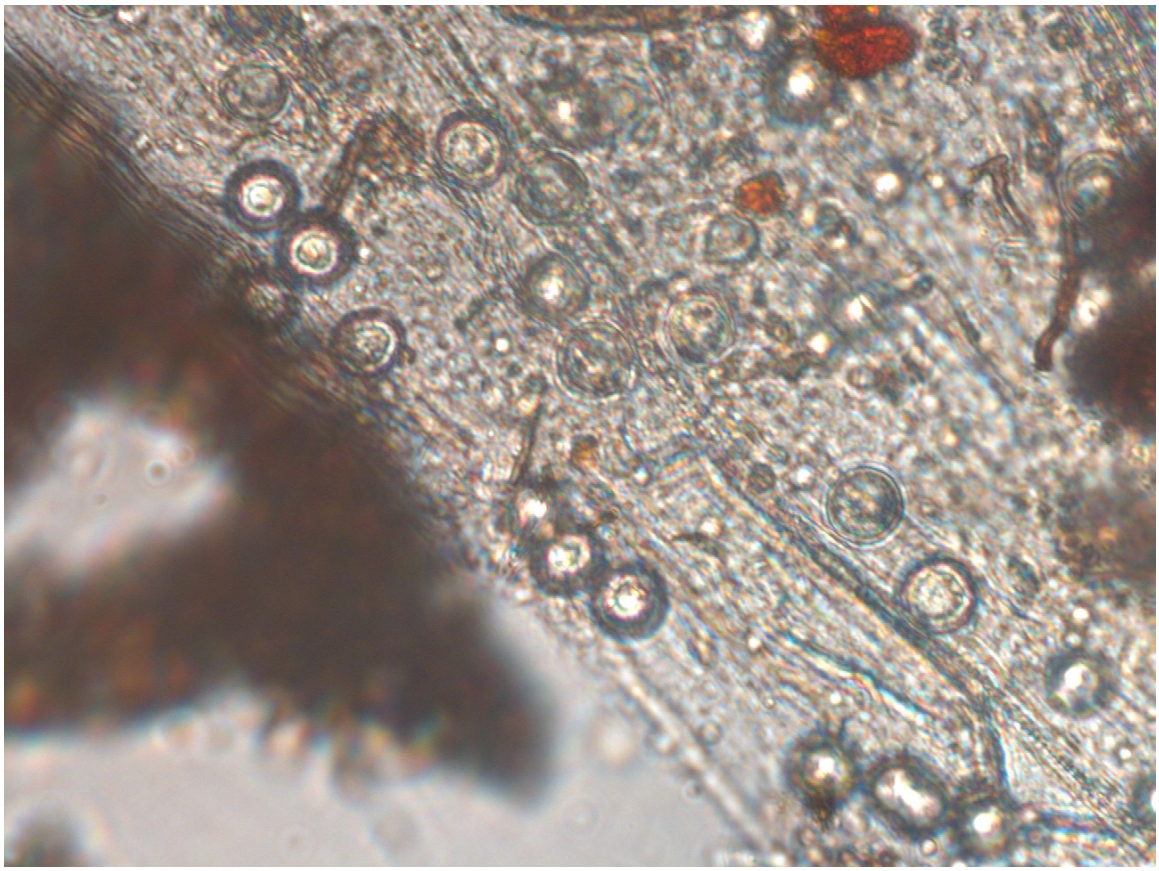

Fig. 2.6. Root cortex of 70-day old creeping bentgrass root containing oospores of *Pythium torulosum* 35-days after inoculation with infested grass leaf blade inoculum of *P. torulosum*.

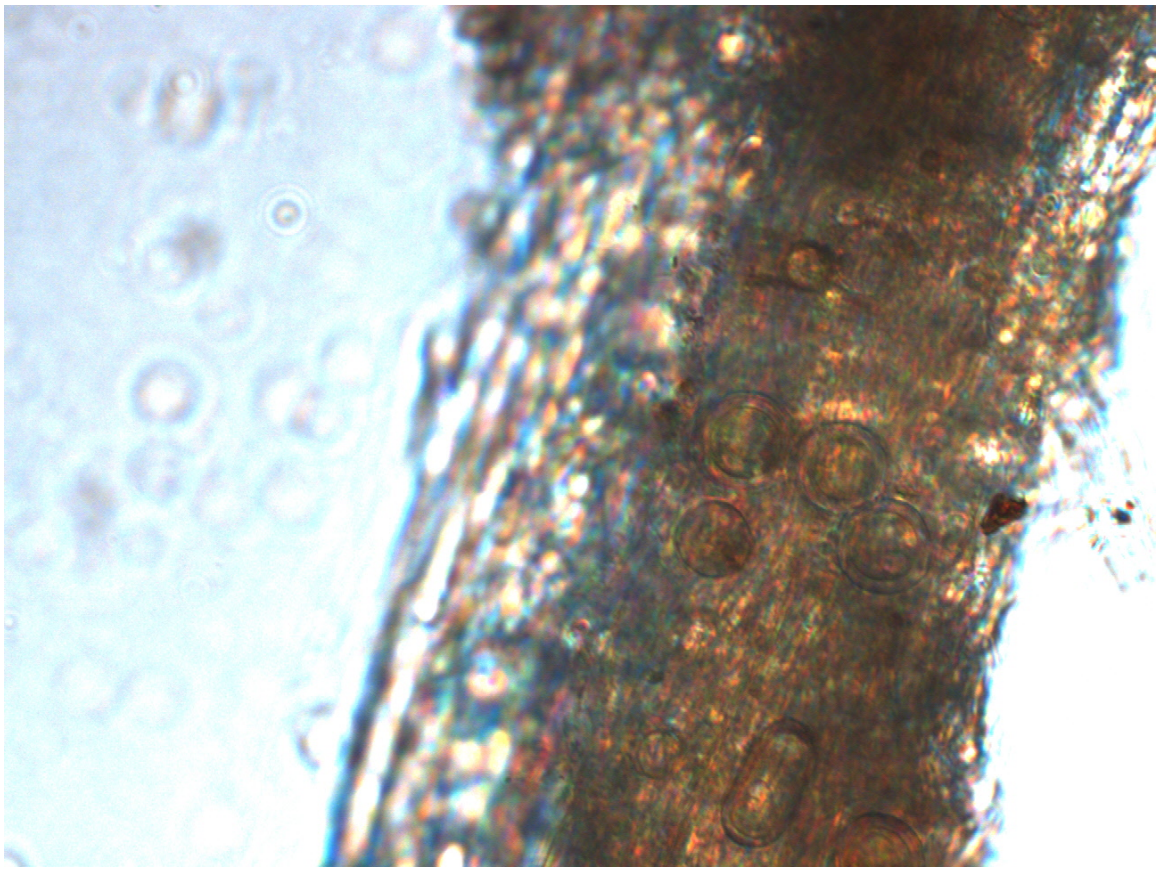

Fig. 2.7. Root cortex of a 70-day old creeping bentgrass root containing oospores of *Pythium vanterpoolii* 35-days after inoculation with infested grass leaf blades of *P. vanterpoolii*.

CHAPTER 3 - PREVENTATIVE AND CURATIVE CONTROL OF PYTHIUM ROOT ROT IN CREEPING BENTGRASS PUTTING GREENS AND SENSITIVITY OF *PYTHIUM* SPP. TO FUNGICIDES

ABSTRACT

Pythium root rot, caused by various *Pythium* spp., is a major disease of creeping bentgrass in the transition zone. Twenty isolates from nine species were evaluated for sensitivity *in vitro* to ten commercially available fungicides. Performance of fungicides for preventative management of the disease was evaluated for two years in Raleigh, NC on a 'Dominant Plus' creeping bentgrass putting green. Fungicides initially were applied when soil temperatures consistently reached 18 to 21^{o}C and were re-applied every 21 days thereafter. Curative activity of fungicides was evaluated in a single year. *Pythium* species were highly variable in their sensitivity to the fungicides tested. Most species were highly sensitive to cyazofamid. Applications of cyazofamid and tank-mixtures of cyazofamid and strobilurins (azoxystrobin, pyraclostrobin, and fluoxastrobin) provided superior protection compared to other fungicides tested. Curative applications of cyazofamid significantly reduced disease severity compared to other fungicides. Due to its preventative and curative ability, golf course managers should base Pythium root rot management on cyazofamid.

INTRODUCTION

Creeping bentgrass (*Agrostis stolonifera* L.) is a cool-season grass that is planted on golf course putting greens in temperate and subtropical climates. It is the preferred

species for golf course greens because its aggressive growth, fine texture, and adaptation to low mowing heights produce a highly desirable putting surface (23). Although creeping bentgrass is planted in subtropical climates, it is not adapted to the persistent heat and humidity typical of summers in the Southeastern United States. As a result, roots decline in response to high soil temperatures in summer. Decline in roots results in a loss of vigor and an increased susceptibility to disease (20). In addition, while aggressive growth in bentgrass is desirable for maintaining a dense and uniform putting surface, it also leads to rapid accumulation of organic matter. Although desirable in many agricultural systems, organic matter accumulation in putting greens typically is associated with poor creeping bentgrass health that predisposes the turf to disease (9).

North Carolina's geographic location impacts growth of plants adapted to colder and warmer environments. This region of the country has winter temperature averages that drop below freezing and summer temperatures with extreme heat and humidity. This makes this region available for growing cool-season plants, but summer temperatures can impact plant health. For these reason it is an optimal region for numerous root diseases of creeping bentgrass. Take-all patch caused by *Gaeumannomyces graminis* var. *avenae* is common on creeping bentgrass grown in northern temperate regions of the United States (20). Symptoms generally appear in late spring and early summer after a cool and wet spring. Summer patch, caused by *Magnaporthe poae*, has been observed throughout the temperate regions of the United States when soil temperatures reach 24°C in the summer (6, 22). Root diseases caused by *Pythium* spp. in creeping bentgrass include Pythium root dysfunction and Pythium root rot. In 1985, Hodges and Coleman (10) described Pythium root dysfunction and demonstrated the pathogenicity of *P. arrhenomanes* and *P.*

aristosporum on creeping bentgrass roots in high sand content golf greens. Feng and Dernoeden (7) similarly concluded that *P. aristosporum* was an important cause of root dysfunction in the Mid-Atlantic region of the US. In 2008, Kerns (14) demonstrated the pathogenicity of *P. volutum* on mature creeping bentgrass roots.

Pythium root rot of turfgrasses has been observed for many decades. Initial symptoms are slight necrosis and thinning of small areas of turf. These areas can expand and coalesce into large areas, which may die as the disease progresses. Symptomatic roots often are shallow in soil and have tan or necrotic lesions, which may also be present on crowns or stolons. The oospores are diagnostic but may be difficult to see near necrotic lesions. Conversely, oospores often can be found on asymptomatic roots. Numerous studies have characterized species associated with root rot, yet defining a single species as the principal pathogen is difficult due to the large number of *Pythium* species present on rotted roots (11). Nelson and Craft (19) and Hsiang et al. (12) characterized *P. graminicola* as the principal pathogen of Pythium root rot diseases in the upper North East United States. Based on the frequency of isolation and pathogenicity of *P. graminicola* on established creeping bentgrass they considered it the principal pathogen causing Pythium root rot. Additionally, pathogenicity assays identified five other species: *P. aphanidermatum*, *P. aristosporum*, *P. vanterpoolii*, *P. ultimum*, and *P. torulosum*, as root pathogens of bentgrass (9, 12, 19). Abad et al. (1) isolated 33 *Pythium* spp. from turfgrasses, and characterized eight pathogenic species as highly aggressive and nine as moderately aggressive on post-emergent creeping bentgrass. Based on isolation and pathogenicity *P. arrhenomanes* was identified as an important pathogen in North Carolina. *Pythium torulosum* is pathogenic on mature creeping bentgrass roots (9).

By 10 weeks after inoculation, *P. torulosum* had reduced root dry weight by 35% relative to non-inoculated control plants, although no foliar symptoms were evident. Large numbers of oospores also were observed on older roots. This demonstrated that root infections by *P. torulosum* reduce growth in the absence of other symptoms. It is possible that disease symptoms may have developed under different or more stressful conditions, such as higher temperatures. In addition, an interaction between *P. torulosum* and sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, exhibits an additive effect resulting in decreased root mass. It is postulated that microbial populations within the accumulation of organic matter in the topsoil might play a bigger role in bentgrass mortality than previously thought.

Summer decline of bentgrass greens is believed to be a complex of factors that influences plant death. Nonetheless, *Pythium* spp. are believed to be a cornerstone to this decline complex (1). Without proper green maintenance as well as management of soil inhabiting *Pythium* spp. large areas of creeping bentgrass can be killed very quickly.

As the desire for perfect putting greens continues, the need for fungicide recommendations and appropriate timing for preventative control will increase, especially in areas of the country where creeping bentgrass is partially adapted. Previous studies on Pythium root dysfunction of creeping bentgrass showed that pyraclostrobin, azoxystrobin and cyazofamid were effective at preventing root dysfunction when applied at favorable temperatures for infection (15). However, several fungicides commonly used to control *Pythium* spp., for example, mefenoxam, propamocarb, fosetyl-Al and etridiazole, did not suppress disease. Research into effective timing and fungicide efficacy is needed to determine what is effective for management of Pythium root rot.

Cyazofamid belongs to group 21 of the FRAC mode of action codes. It is a QiI (quinone inside inhibitor) fungicide that interrupts the respiratory electron transport chain in the mitochondria at Complex III by targeting cytochrome bc_1, effectively starving the pathogen of ATP. Its fungicidal activity is limited to Oomycetes. Its unique mode of action avoids cross-resistance to other commercially available oomycete fungicides and inhibits the growth of fungal isolates that are resistant to mefenoxam and strobilurins (13). Cyazofamid has been demonstrated to inhibit growth of *Phytophthora infestans* (13)*, Psuedoperonospora cubensis* (18) and *Pythium volutum* (15).

The site-specific nature of cyazofamid, however, leaves it susceptible to being overcome by pathogens. Insensitivity to cyazofamid has been reported in populations of *Phytophthora capsici* in the southeastern United States (13, 16). Although no reports of resistance of *Pythium* spp. to cyazofamid have been observed. *Pythium* reproduce similarly to *P. capsici*, sexual recombination occurs allowing for a greater chance of the active ingredient to be overcome.

Strobilurins (azoxystrobin, fluoxastrobin, and pyraclostrobin) belong to group 11 of the FRAC mode of action codes. Strobilurins are similar to cyazofamid in the way that QoI (quinone outside inhibitor) fungicides interrupt the respiratory electron transport chain in the mitochondria at Complex III by targeting cytochrome bc_1. QoI compounds are effective against most fungal pathogens on turfgrass, inhibiting spore germination and mycelial growth. Strobilurins have demonstrated good efficacy against *P. aphanidermatum* (4).

Similarly to cyazofamid, strobilurin fungicides have site-specific activity, which can be overcome by *Pythium* spp. Although resistance has not been observed in *Pythium*

spp. it has been documented in *Plasmopara viticola* (3). Cross-resistance between strobilurin fungicides is expected based on similar mode of actions. Determining the sensitivity of *Pythium* spp. to commercially available fungicides may help diversify available active ingredients for management practices. Data of *Pythium* spp. sensitivity to active ingredients will also provide a foundation for future monitoring if *Pythium* spp. develop resistance to cyazofamid.

Research is needed to evaluate fungicides for preventative and curative effects to improve management of Pythium root rot. The objectives of this study are to (i) determine the *in vitro* sensitivity of *Pythium* spp. to commercially available fungicides, and (ii) evaluate fungicides for preventative and curative control of Pythium root rot in field experiments.

MATERIALS AND METHODS

Sensitivity of *Pythium* spp. to fungicides. The sensitivity of 10 *Pythium* spp. (*P. vanterpoolii*, *P. torulosum*, *P. arrhenomanes, P. aphanidermatum*, *P. irregulare*, *P. ultimum* var. *ultimum*, *P. volutum*, *P. vexans*, *P. myriotylum* and *P. catenulatum*) to the fungicides cyazofamid, mefenoxam, etridiazole, azoxystrobin, fluoxastrobin, pyraclostrobin, fluazinam, propamocarb, fluopicolide, chlorothalonil, fosetyl-Al, and potassium phosphite was determined in mycelial growth assays. Isolates of *Pythium* were obtained from golf course putting green samples sent into the Plant Disease and Insect Clinic (PDIC) at North Carolina State University between 2014 and 2015. Samples were cleaved from turfgrass samples (11 cm cores) and individual plants were excised from the soil. Individual plants were washed thoroughly under running tap water for four hours, blotted dry, and cut into pieces. 20 roots, measuring 1 cm. long, were placed on sterilized

P_{10}ARP agar (17 g/liter corn meal agar amended with pimaricin, ampicillin, rifampicin, and PCNB [Terraclor 75% WP, Southern Ag Insecticides, Inc.], clarified V8 juice cholesterol agar (SV8), or placed within the root zone of 'A-1' creeping bentgrass seedlings grown in calcined clay (Turface Allsport; Profile Products LLC, Buffalo Grove, IL) and incubated at room temperature in saturated conditions until plants wilted (14). *Pythium* isolates were also provided from the collection of B.B. Shew and E.C. Lookabaugh at North Carolina State Univeristy, Department of Plant Pathology. These included *Pythium vexans*, *Pythium myriotylum, Pythium aphanidermatum*, *Pythium irregulare* and *Pythium ultimum* var. *ultimum*. Hyphal plugs (6mm) from the edge of actively growing colonies on potato dextrose agar (PDA) were placed in the center of Petri dishes containing PDA amended with five concentrations (0, 0.001, 0.01, 0.1, 1, and 10 μg of active ingredient (a.i.) kg^{-1}) of commercially formulated fungicides. Salicylhydroxamic acid (SHAM) was added to the media amended with strobilurin (azoxystrobin, pyraclostrobin, fluoxastrobin) fungicides at a concentration of 50 μg/ml (3). A PDA + SHAM control was included for comparison of strobilurin fungicides. Fungicide and SHAM solutions were added to autoclaved PDA after cooling to 55°C.

Pythium cultures were incubated in the dark at 23°C for 1 to 2 days. Species with rapid growth rates were incubated for 1 day. The diameter of each colony was measured in two perpendicular directions and the mean diameter was adjusted after subtracting the diameter of the hyphal plug. Relative growth (RG= [the mean adjusted colony diameter on fungicide-amended medium/the mean adjusted colony diameter on non-amended medium]) was determined for each isolate, fungicide and fungicide concentration. Each

fungicide-isolate combination was replicated three times and the experiment was repeated twice.

The 50% effective concentration (EC_{50}) values were estimated by linear regression using PROC REG (SAS Institute Inc., Cary, NC) based on the relative inhibition value (RI = 1 – RG) on log_{10}-transformed fungicide concentration. The EC_{50} values for all isolates were subjected to an ANOVA (PROC GLM) and mean separations using the Waller-Duncan k-ratio t-test (k=100).

Preventative control of Pythium Root Rot. Three field studies were conducted on creeping bentgrass maintained as golf course putting greens. Two-year field assays were conducted at the Lake Wheeler Road Field Lab in Raleigh, NC on 'Dominant Plus' creeping bentgrass. A two-year study was conducted at Highlands Country Club in Highlands, NC on 'A-1/A-4' creeping bentgrass. An additional one-year study was also conducted at Cowan's Ford Golf Club in Stanley, NC on 'A-4' creeping bentgrass. Greens were constructed to USDA standards (22) with a root zone mix of 85% sand to 15% sphagnum peat moss. Plots in Raleigh, NC were mowed daily with clippings collected and maintained at 4 mm. In Highlands, NC and Stanley, NC plots were mowed daily with clippings collected and maintained at 3 mm.

Inoculations were performed at the Raleigh site on May 15, 2014 and May 22, 2015 when soil temperatures reached 22°C to 24°C consistently. Grass-leaf cultures containing 70 to 80 grass leaf blades and one plug each of *Pythium ultimum* var. *ultimum*, *P. arrhenomanes*, and *P. aphanidermatum* in a petri dish were incubated under continuous fluorescent light at room temperature for three days. A cup cutter was used to

place two equidistant 7.62 –cm deep holes in each plot. Inoculum was placed in holes and covered with the removed soil plug. The study was irrigated 3 times a day totaling 1.9 cm of water a day for the duration of the study.

Fungicide applications were conducted on 05-22-2014, 06-12-2014, 07-03-2014, 07-24-2014, and 08-14-2014 in 2014, following inoculation, and continued on 05-29-2015, 06-19-2015, 07-10-2015, 07-31-2015, and 08-21-2015 in 2015. All fungicides were applied at high-labeled rates (Table 3.1). Treatments were applied in water equivalent to 150 ml m^{-2} with a CO_2-powered boom sprayer equipped with air induction nozzle (TeeJet AI9508E, R&D Sprayers, Opelousas, LA) at 3.5 Kg cm^{-2}. Following fungicide applications, the study was irrigated with 6 mm of water within an hour after application. Individual plots were 1.67 m^2 (0.91m x 1.83m) and arranged in a randomized complete block design with 4 replications.

Disease severity and turf quality was evaluated visually during the 2014 and 2015 summer in Raleigh on the dates of: 07-15-2014, 07-24-2014, 08-01-2014, 08-08-2014, 06-19-2015, 06-26-2015, 07-02-2015, 07-10-2015, 07-17-2015, 07-25-2015, 07-31-2015, 08-06-2015, 08-17-2015, 08-21-2015, and 08-28-2015; in Highlands on 7-31-2014, 8-25-2015; and in Charlotte on 8-25-2015. Disease severity was visually assessed as the percentage of the plot area exhibiting symptoms consistent with Pythium root rot (Figure 3.1). Turf quality was assessed by estimating the overall uniformity, density, and color of the plot at each rating. Turfgrass quality was rated on a 1 to 9 scale (9 = best, 5 = minimal acceptance, and 1 = bare ground). Symptomatic turf samples were sent to the Plant disease and insect clinic (PDIC) at North Carolina State University for confirmation of Pythium root rot diagnosis. Isolation of *Pythium* spp. present at Raleigh, NC field site

was performed by washing and plating roots on PARP selective media; infected roots were also placed 5 mm below the soil of 'A-1' creeping bentgrass seedlings to bait *Pythium* from infected roots, followed by plating newly infected seedlings on PARP. A sample from non-treated control plots was taken in August 2014 and once every month from the beginning of February 2015 to September 2015 to determine *Pythium* spp. active during time of symptom expression. Turf quality and AUDPC values were subjected to ANOVA (PROC GLM) and mean separations using the Waller-Duncan *k*-ratio *t*-test (k=100) in SAS v9.4.

Curative control of existing Pythium root rot. A one-year field experiment was conducted in 2015 alongside the preventative Pythium root rot study in Raleigh, NC on 'Dominant Plus' creeping bentgrass. The golf green was constructed and maintained as previously described.

Fungicide treatments were applied as previously described.. Following application the study was irrigated with 6 mm. of water within an hour after application. Individual plots were 1.67m^2 (0.91 m x 1.83 m) and arranged in a randomized complete block design with 4 replications. Fungicide treatments and application dates were initiated when symptoms of Pythium root rot were visible (Table 3.1) and rates were as previously described, except that cyazofamid was applied at 0.191 ml m^{-2}. Plots were 1.67m^2 and arranged in a randomized complete block design with four replications.

Disease severity was visually evaluated on 06-26-2015, 07-02-2015, 07-10-2015, 07-17-2015, 07-25-2015, 07-31-2015, 08-06-2015, 08-17-2015, 08-21-2015, and 08-28-2015 the percent area of the plot exhibiting symptoms consistent with Pythium root rot. Turf quality was visually accessed on the dates disease severity was rated. Turf quality

and AUDPC were subjected to ANOVA (PROC ANOVA) and mean separations using the Waller-Duncan *k*-ratio *t*-test (*k*=100) in SAS v9.4.

RESULTS

Sensitivity of *Pythium* spp. to fungicides. There was no significant difference between experiment repetitions, which allowed experiments to be combined for analysis. Fungicides, isolates, and fungicide x isolate interactions affected growth of *Pythium* species ($P < 0.001$). The 20 isolates tested varied in sensitivity to fungicides across and within species of *Pythium* (Table 3.2). *Pythium torulosum*, and one isolate (Pinehurst) from *P. vanterpoolii* were most sensitive to cyazofamid, followed by fluazinam, etridiazole and chlorothalonil, respectively. Of the six isolates of *P. vanterpoolii* tested all were highly sensitive to cyazofamid, etridiazole and fluazinam. *Pythium arrhenomanes* and *P. aphanidermatum* isolates were insensitive to strobilurin fungicides. *Pythium volutum* was highly sensitive to most fungicides except propamocarb and fluopicolide, which generally were ineffective at inhibiting mycelial growth of all *Pythium* spp. (Table 3.2). Fosetyl-Aluminum and potassium phosphite fungicides did not reduce mycelial growth of the isolates tested and were not included in presented data.

Preventative control of Pythium root rot. In 2014 and 2015, symptoms resembling Pythium root rot developed in June and continued to develop throughout the summer at the Lake Wheeler Turfgrass Research Station (Fig 3.1). Samples were collected and Pythium root rot was confirmed by microscopic examination. Applications of cyazofamid alone or mixtures of cyazofamid with azoxystrobin, fluoxastrobin, or pyraclostrobin reduced Pythium root rot severity when compared to the non-treated

control in 2014 and 2015 (Figs 3.2, 3.3). Applying a QoI along with cyazofamid did not statistically improve control. No other treatments were effective in 2014. In 2015, application of mefenaoxam, pyraclostrobin, fluoxastrobin, and etridiazole also reduced disease relative to the control but none provided control similar to cyazofamid alone or in mixture (Fig. 3.3). Cyazofamid-based treatments also maintained acceptable turf quality throughout the summers of 2014 and 2015 (Fig 3.4).

Studies located at golf courses in North Carolina did not develop disease symptoms during the summer. Acceptable turf quality was maintained at Highlands, NC with no significant separation from the non-treated control (Fig. 3.5) The Stanley, NC study did not maintain turf quality due to drought conditions and fungicide applications did not improve turf quality compared to the non-treated control (Fig. 3.6).

Creeping bentgrass samples sent to the PDIC at North Carolina State University were diagnosed with Pythium root rot, based upon presence of oospores on roots. *Pythium torulosum* was consistently isolated from symptomatic tissue taken from non-treated control plots in August 2014 and once every 4 weeks from the beginning of February 2015 to September 2015.

Curative control of Pythium root rot. Disease symptoms began to develop in June 2015 and disease continued to increase throughout the summer. Biweekly applications of cyazofamid resulted in significant reduction in disease relative to the non-treated control (Fig. 3.7). Summer disease progression was unaffected by the other fungicides. Disease was uniform throughout research plots on when fungicides were first applied on June 26. A week after the first application, cyazofamid and etridiazole had less

disease than the non-treated control (Fig. 3.8). Etridiazole sometimes suppressed disease for 7 days, but it did not prevent disease over a 14-day application interval. Disease progression reached an apex on July 31, 2015. After this peak cyazofamid was less effective at controlling disease; disease ratings in plots treated with cyazofamid were highest on August 17, 21, and 28.

DISCUSSION

Pythium root rot has been reported on turfgrasses since the 1940's (17), but the species causing root rot were not identified until a resurgence of the disease in the 1980's (1, 9, 11, 12, 19). Based on this work, *P. graminicola*, *P. aphanidermatum*, *P. arrhenomanes*, *P. torulosum*, *P. vanterpoolii*, *P. aristosporum*, and *P. ultimum* have been identified as pathogens of creeping bentgrass roots (1, 7, 8, 9, 10, 11, 12, 14, 19). However, in-depth research on effective management strategies for the control of this disease is lacking. This study focuses on evaluating the efficacy of commercially available fungicides in the field and *in vitro* to further develop a management strategy for growers.

In the Southeastern United States, disease often is severe when high temperatures (≥ 32°C) persist throughout the summer. It is thought that with persistent heat, creeping bentgrass becomes stressed and root-dwelling *Pythium* spp. then exploits the plant's weakened health (1). Numerous studies have found that *P. torulosum* is present on healthy plants but also can be a weak pathogen of creeping bentgrass roots. In our studies, 100% of isolations from diseased areas were identified as *P. torulosum*. It is possible that environmental conditions enhanced the ability of *P. torulosum* to cause disease on a weak host. Preventative applications of cyazofamid suppressed root rot

symptoms, and also alleviated aboveground disease symptoms. Furthermore, *P. torulosum* isolates were highly sensitive (EC_{50}, LW1 = 0.098; LW5 = 0.042; LW10 = 0.056; LW12 = 0.453) to cyazofamid. When preventative applications were initiated, soil temperatures fluctuated between 24°C to 25°C. Both air and soil temperatures fluctuate day-to-day, but soil temperatures are more stable. For better preventative control of Pythium root rot, turfgrass managers may want to use soil temperature as a guide for preventing this disease. Based on this study preventative applications of cyazofamid are strongly recommended to begin as soil temperatures reach 24°C to 25°C.

Cyazofamid has a site-specific mode of action that targets cytochrome bc_1 in mitochondria, interrupting the electron transport chain and the production of ATP. Based on its performance in the field compared to other active ingredients, development of resistance to cyazofamid is a major concern. Isolates of *Phytophthora capsici* have been reported as resistant to cyazofamid in all stages of its life cycle except zoospore germination (13, 16). It is important for growers to rotate or tank-mix fungicides with different mode of actions to maintain the efficacy of cyazofamid. Furthermore, growers may apply only a total of 0.86 ml m^{-2} of cyazofamid per year, further emphasizing the need for alternative fungicides for management of Pythium root rot. A management program centered on applying cyazofamid as a protectant or curative was effective in our trials (Fig. 3.7). This research demonstrated low rates (0.191 ml m^{-2}) were effective at decreasing disease severity and increasing turf quality. Applying an initial preventative application at a high rate (0.287 ml $m.^{-2}$) and continuing with a low rate application coupled with a QoI or mefenoxam at the onset of disease symptoms would allow growers

to distribute applications throughout the summer to maintain adequate putting green aesthetics, while also applying a lawful amount of product a year.

Cyazofamid was comparatively more successful at preventing Pythium root rot symptoms than other active ingredients tested. No plant health or greening effects from chemical interaction with plant metabolites was observed based on field results from Highlands, NC and Stanley, NC where no disease was present (Fig. 3.5 and 3.6). Mefenoxam, etridiazole and the strobilurin fungicides reduced disease and improved turf quality in 2015. No plant health or greening effect was observed. Although QoI greening effect has been observed to increase yield in wheat (2), health effects have not been observed in turf (15). Isolates of *P. torulosum* were insensitive to mefenoxam and the strobilurins, while being slightly sensitive to etridiazole. It would be reasonable to see a reduction in disease based on the performance of etridiazole in reducing the mycelial growth of *P. torulosum*, but performance of mefenoxam and strobilurins in the field suggests that other sensitive species of *Pythium* may have been present. Future studies should continue to monitor the diversity of *Pythium* spp. found at field sites throughout the year.

A large number of *Pythium* spp. have been associated (1, 7, 8, 9, 10, 11, 12, 14, 19) and are pathogenic to the roots of creeping bentgrass. The diversity of *Pythium* species that cause root rot can complicate management practices. Active ingredients in fungicides do not affect all species in the genera. Selection pressures in the field may result in a different species predominating from location to location. Species assembled for fungicide sensitivity are a snapshot in time and space. Fungicide sensitivity and insensitivity may have been directly influenced by a grower's primary fungicide of

choice, essentially selecting for species insensitive to the active ingredient. Broad sensitivity of most pathogenic species to an active ingredient increases the value as a management tool. All fungicides that were tested demonstrated some variable levels of activity against *Pythium* spp. (Table 3.2). However, a number of species tested had one isolate representing species sensitivity, it is expected that fungicide sensitivity on a species level may change with an increased number of isolates per species tested. All fungicides labeled for control of Pythium root rot should be considered for managing the disease on golf course putting greens. Field and *in vitro* data show that the best products for managing this disease are cyazofamid, etridiazole, azoxystrobin, fluoxastrobin, pyraclostrobin, and mefenoxam. Fluazinam, which performed well against a number of *Pythium* spp. and may produce results in the field.

Our results demonstrate that preventative control of Pythium root rot can be achieved when cyazofamid is applied days when soil temperatures reach 24°C. Cyazofamid also performs well as a curative application at low volumes. For best results preventative fungicide programs should be based on cyazofamid for its broad effectiveness against most *Pythium* species associated with turfgrass roots. With an initial preventative application, and additional applications in small volumes when conditions worsen turfgrass managers can maintain adequate turf quality throughout periods of the summer that are particularly damaging to creeping bentgrass. Predominant species can vary from location to location and therefore etridiazole, fluazinam and mefenoxam should also be considered as effective fungicides at limiting the largest number of *Pythium* spp.

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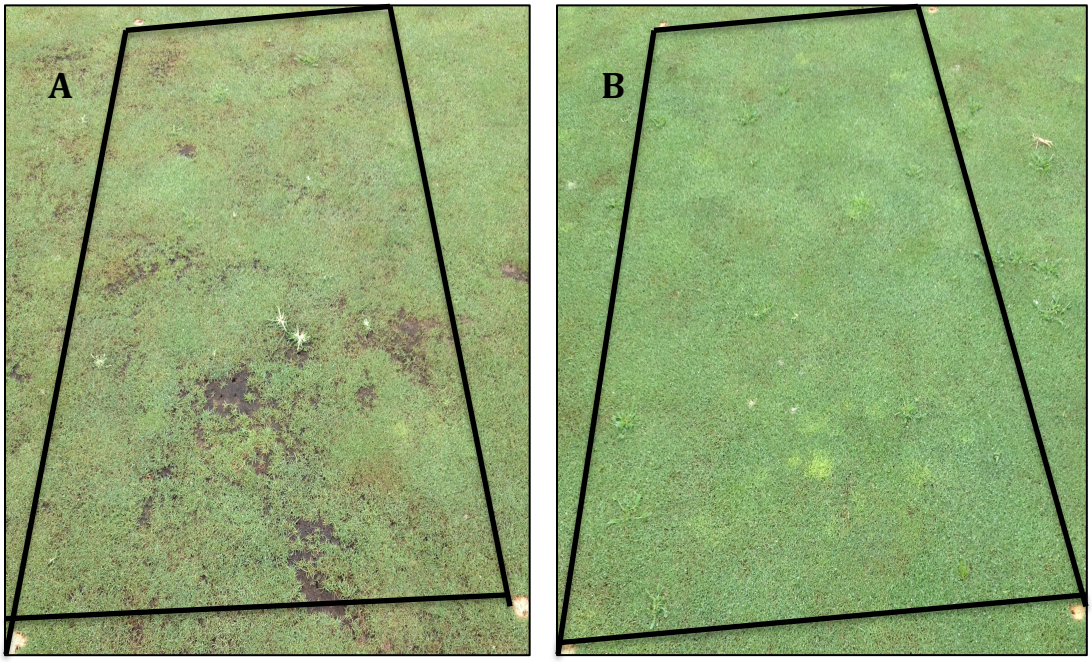

Fig. 3.1. Preventative fungicide field assay at Lake Wheeler Turfgrass Research Station in Raleigh, NC. Pictures taken September 9, 2014. **A,** Pythium root rot symptoms on area of "Dominant Plus" creeping bentgrass treated with propamocarb. **B,** Area of "Dominant Plus" creeping bentgrass expressing no Pythium root rot symptoms treated with cyazofamid.

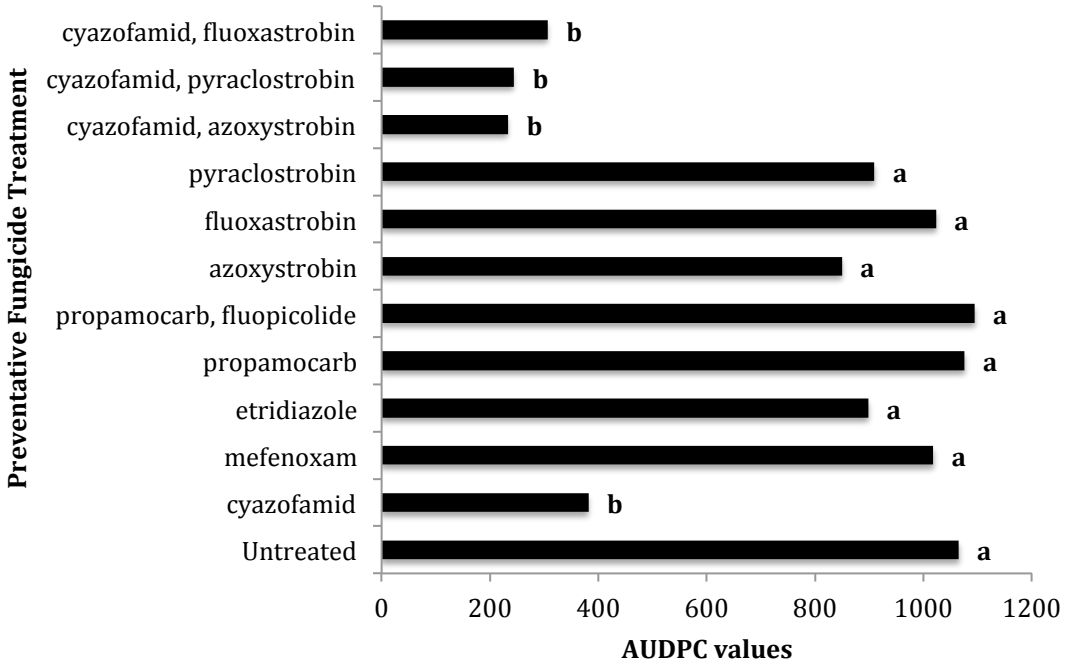

Fig. 3.2. Area under disease progress curve (AUDPC) values in response to preventative fungicide applications in 2014. AUDPC values were calculated from disease data collected on 07-15-2014, 07-24-2014, 08-01-2014, 08-08-2014 at the Lake Wheeler Turfgrass Research Station in Raleigh, NC. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio t-test (k=100).

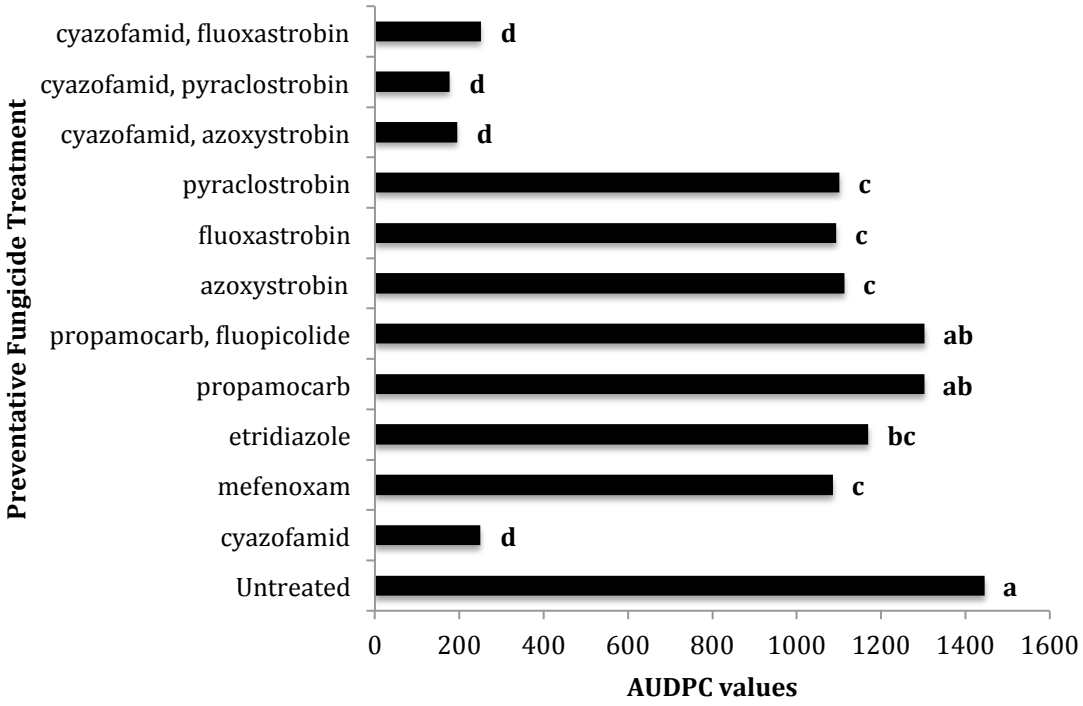

Fig. 3.3. Area under disease progress curve (AUDPC) values in response to preventative fungicide applications in 2015. AUDPC values were calculated from disease severity data collected weekly for 11 rating dates at the Lake Wheeler Turfgrass Research Station in Raleigh, NC. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio, t-test (k=100).

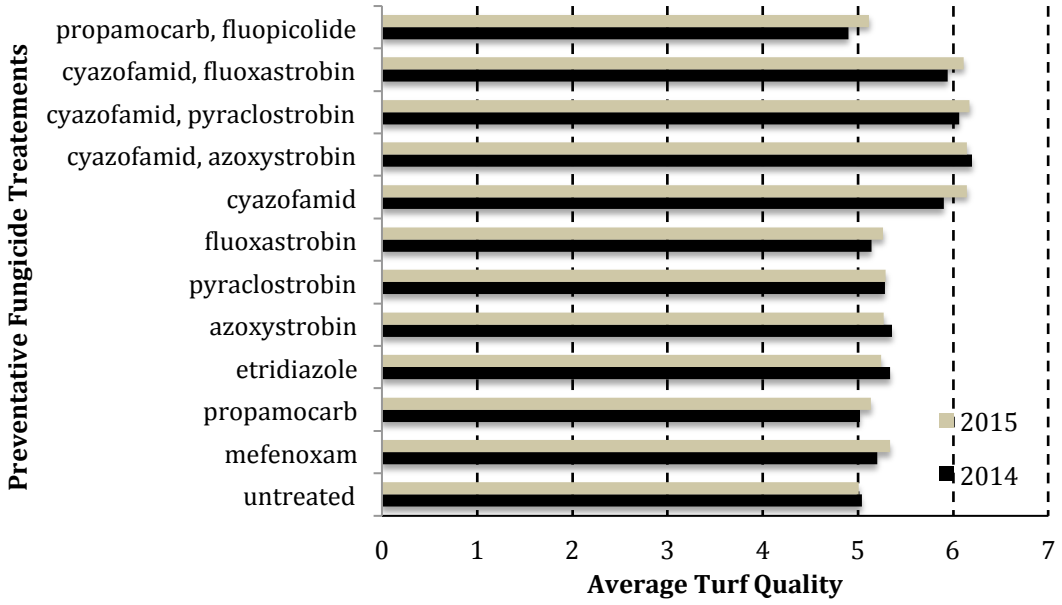

Fig. 3.4. Impact of preventative fungicide applications on turfgrass quality in 2014 and 2015. Turf quality was visually estimated on a scale of 1 to 9 (1=bare ground, 6=minimally acceptable, and 9=best). Bars represent the average of five turf quality rating dates in 2014 and 11 rating dates in 2015.

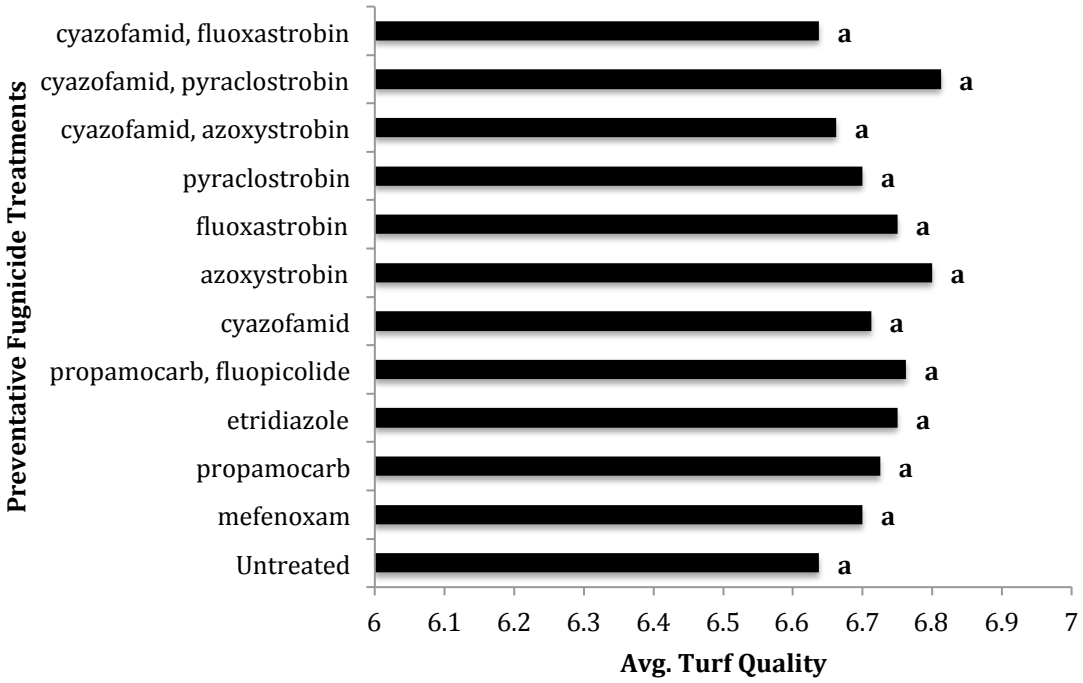

Fig. 3.5. Impact of preventative fungicide applications on turf quality in 2015 at Highlands Country Club in Highlands, NC. Turf quality was visually estimated on a scale 1 to 9 (1=bare ground 6=minimally acceptable and 9=best). Bars represent the average of 2-turf quality rating dates. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio, t-test (k=100).

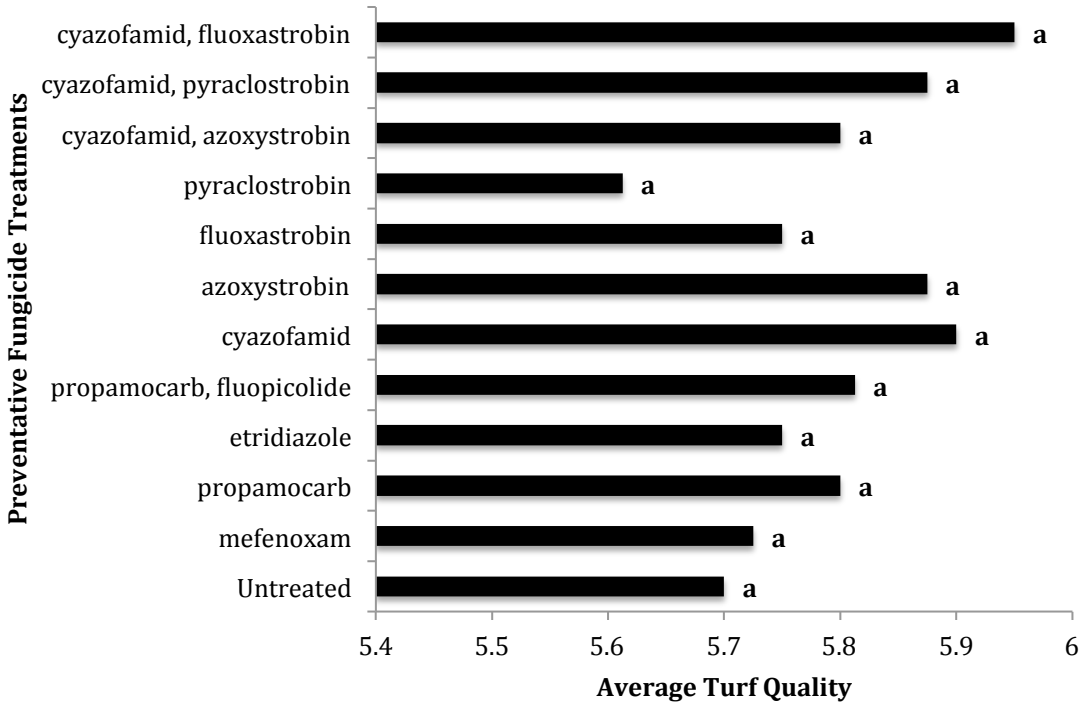

Fig. 3.6. Impact of preventative fungicide applications on turf quality in 2015 at Cowan's Ford Golf Club in Stanley, NC. Turf quality was visually estimated on a scale 1 to 9 (1=bare ground 6=minimally acceptable and 9=best). Bars represent the average of 2 turf quality rating dates. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio, t-test (k=100).

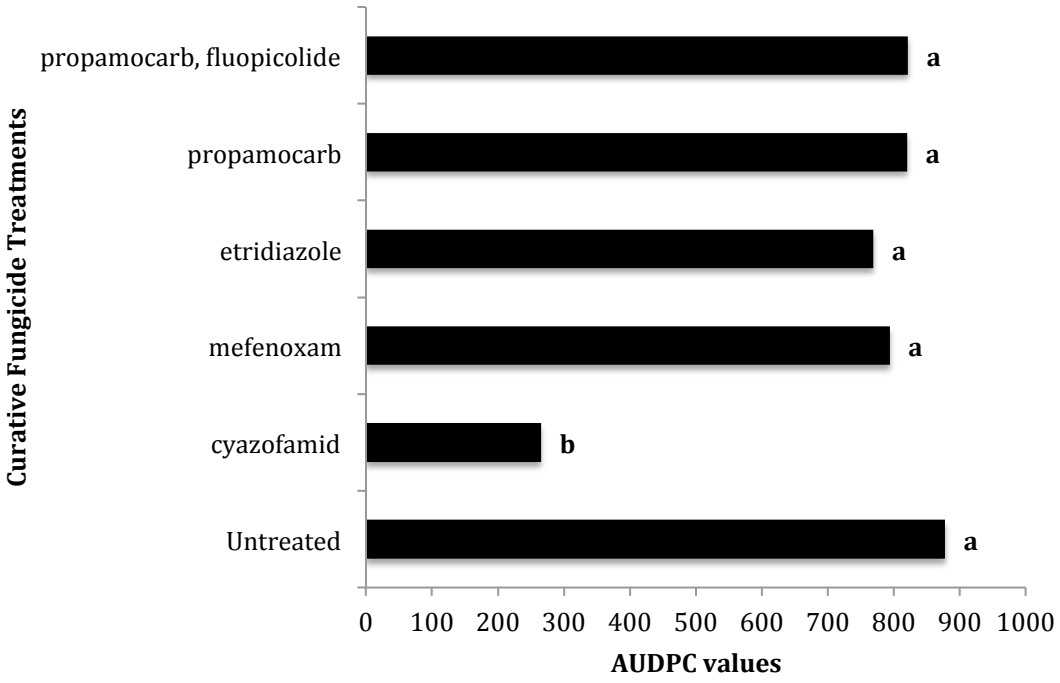

Fig. 3.7. Area under disease progress curve (AUDPC) values in response to curative fungicide applications in 2015. AUDPC values were calculated from the percent disease data collected weekly for 10 rating dates at the Lake Wheeler Turfgrass Research Station in Raleigh, NC. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio, t-test (k=100).

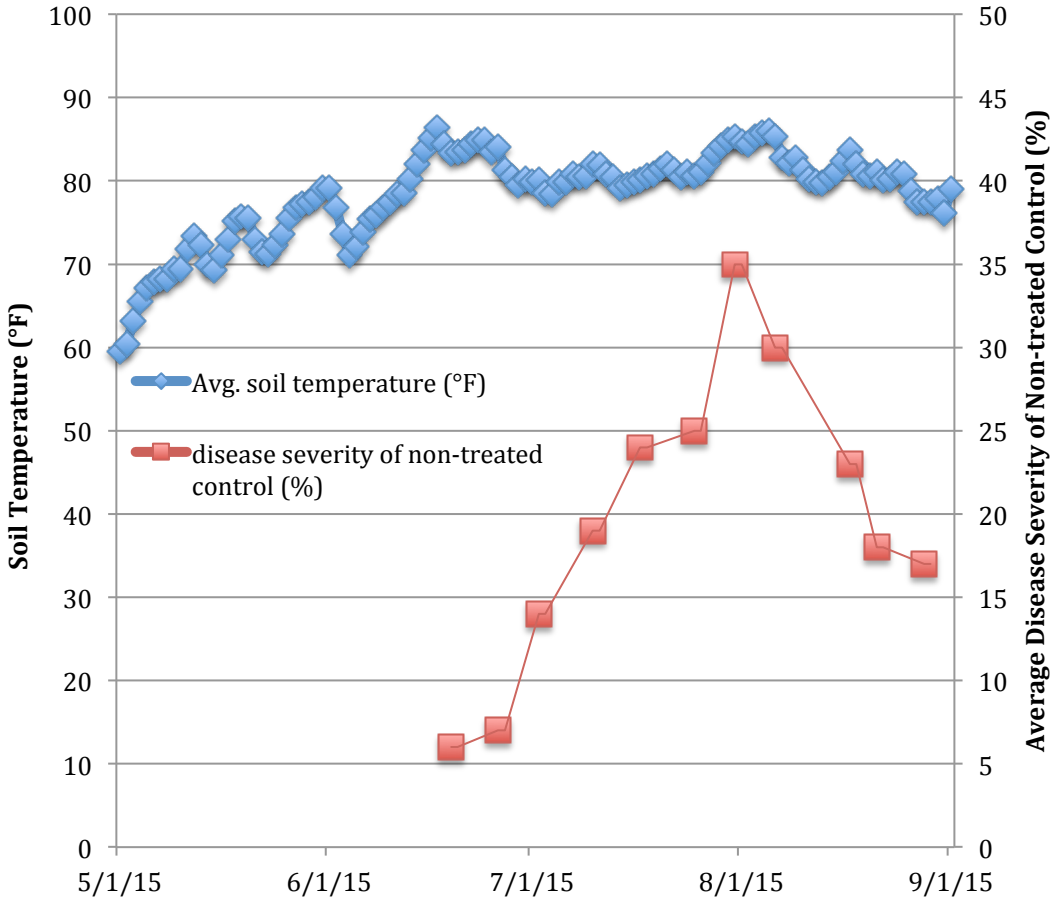

Fig. 3.8. Pythium root rot disease progression 2015. Average disease ratings of non-treated plots over time related to average soil temperature. Percent disease ratings collected 11 times at the Lake Wheeler Turfgrass Research Station in Raleigh, NC.

Table 3.1. Treatments, rates and application dates for preventative and curative control of Pythium root rot in 2014 and 2015.

Fungicide	Trade name	Rate[y] (ml or g m^{-2})
Control	**--------**	**--------**
cyazofamid	**Segway**	**0.287**
mefenoxam	**Subdue Maxx**	**0.318**
etridiazole	**Terrazole**	**0.955**
azoxystrobin	**Heritage**	**0.122[w]**
pyraclostrobin	**Insignia**	**0.223**
fluoxastrobin	**Disarm**	**0.127**
propamocarb	**Banol**	**0.637**
propamocarb, fluopicolide	**Stellar**	**0.382**

[w] Rate is in g. m^{-2}.

[x] Applications were applied on 05-22-2014, 06-12-2014, 07-03-2014, 07-24-2014, 08-14-2014, 05-29-2015, 06-19-2015, 07-10-2015, 07-31-2015, 08-21-2015.

[xy] Applications were applied in Highlands, NC and Stanley, NC on 05-25-2015, 06-22-2015, 07-20-2015.

[y] Treatments were applied in 610 mL H_2O m^{-2}, and watered in with irrigation immediately after application.

[z] Fungicides tested in curative control assay, in which cyazofamid was applied at 0.191 ml m^{-2}.

Table 3.2. *In vitro* sensitivity of mycelium of *Pythium* spp. (number of isolates) isolates to commercially available fungicides.

***Pythium* species**	**Fungicides[x]**									
	EC_{50} Concentrations µg ml^{-1}									
	cyazofamid	**fluazinam**	**etridiazole**	**azoxystrobin[y]**	**fluoxastrobin[y]**	**pyraclostrobin[y]**	**mefenoxam**	**chlorothalonil**	**propamocarb**	**fluopicolide**
P. aphanidermatum (2)										
P. aph	9.895 a[z]	0.380 de	0.439 def	>10 a	>10 a	>10 a	0.074 e	3.390 de	>10 a	6.640 b
P. aph2	0.035 d	0.559 de	2.310 a	>10 a	>10 a	>10 a	0.226 e	3.094 def	>10 a	>10 a
P. irregulare (1)										
P. irr	4.098 b	>10 a	0.755 d	0.9354 b	3.336 b	0.643 b	0.202 e	>10 a	>10 a	>10 a
P. arrhenomanes (2)										
WRGC5	0.039 d	0.198 de	1.368 c	>10 a	>10 a	>10 a	3.116 b	8.907 ab	1.141 c	0.956 d
Sedgefield	0.004 d	0.110 e	0.518 def	>10 a	>10 a	>10 a	0.204 e	1.212 efg	>10 a	>10 a
P. vanterpoolii (6)										
RBR	0.012 d	0.237 de	0.241 ef	0.0608 c	0.06 c	0.271 b	1.965 bcd	9.137 ab	>10 a	>10 a
P1	0.058 d	0.267 de	0.799 d	>10 a	>10 a	>10 a	>10 a	0.997 fg	>10 a	>10 a
Lambert	0.031 d	0.292 de	1.945 ab	0.1637 c	0.116 c	0.06 b	0.485 e	3.501 d	>10 a	>10 a
DMC15	0.044 d	0.241 de	0.775 d	.0733 c	0.116 c	0.047 b	2.547 bc	7.615 bc	>10 a	>10 a
DMC22	0.026 d	0.212 de	0.642 de	.0904 c	0.113 c	0.047 b	0.618 e	>10 a	6.468 b	3.341 c
Pinehurst	0.074 d	0.432 de	1.287 c	>10 a	>10 a	>10 a	>10 a	6.276 c	>10 a	>10 a
***P. ultimum* var. *ultimum* (1)**										
P. ult	0.367 d	3.01 c	0.383 def	0.1284 c	0.163 c	0.139 b	>10 a	7.491 bc	>10 a	>10 a
P. volutum (1)										
OC6	0.002 d	0.058 e	1.341 c	0.0431 c	0.095 c	0.041 b	1.833 cd	0.678 g	>10 a	>10 a
P. torulosum (4)										
LW1	0.098 d	0.819 d	1.532 bc	>10 a	>10 a	>10 a	>10 a	7.173 bc	>10 a	>10 a
LW5	0.042 d	0.195 de	0.223 ef	>10 a	>10 a	>10 a	>10 a	1.935 defg	>10 a	>10 a
LW10	0.056 d	0.210 de	0.532 def	>10 a	>10 a	>10 a	>10 a	1.729 defg	>10 a	>10 a
LW12	0.045 d	0.212 de	0.257 ef	>10 a	>10 a	>10 a	>10 a	2.08 defg	>10 a	>10 a
P. vexans (1)										
Ed-mum-27	>10 a	5.229 b	0.526 def	0.1649 c	0.263 c	0.92 b	0.3576 e	0.127 g	>10 a	3.966 c
P. myriotylum (1)										
Ed-mum-22	1.078 c	0.591 de	0.148 f	0.076 c	0.058 c	0.403 b	0.168 e	>10 a	6.407 b	>10 a

[x] Commercial formulations of fungicides.

[y] SHAM (50 µg ml^{-1}) was added with fungicides to reduce alternative oxidase pathway.

[z] Values followed by the same letter within a column are not significantly different according to Waller-Duncan k-ratio t-test (k=100).