ABSTRACT

KERNS, JAMES PATRICK. Biology and Management of Pythium root dysfunction in North Carolina. (Under the direction of Lane P. Tredway, and H. David Shew).

Pythium root dysfunction (PRD) has become an important disease of creeping bentgrass putting greens in the Southeastern U.S., yet very little is known about the etiology, epidemiology, and management of this disease. Seventy-five *Pythium* isolates were obtained from creeping bentgrass putting greens in NC, SC, GA, and VA. Using morphological and molecular identification techniques, 59 isolates were identified as *Pythium volutum* and 16 were identified as *P. torulosum*. A subsample of *P. volutum* and *P. torulosum* isolates were tested for pathogenicity in growth chamber experiments. All isolates of *P. volutum* examined were highly virulent toward creeping bentgrass roots, whereas isolates of *P. torulosum* were non-pathogenic. Isolates of *P. volutum* induced drastic reductions in creeping bentgrass root depth and root mass when infected plants were exposed to a four week high temperature regime.

Growth chamber experiments were conducted to determine the impact of temperature on infection of creeping bentgrass roots by *P. volutum*. This was conducted by varying the temperature during a four week infection period, after which the plants were exposed to a four week heat treatment. Symptoms characteristic of PRD developed in the 12^{o}C, 16^{o}C, 20^{o}C, and 24^{o}C infection temperature treatments, but not in the 28^{o}C and 32^{o}C treatments. Root depth and root mass was reduced prior to heat exposure in only the 12^{o}C, 16 ^{o}C, and 20 ^{o}C treatments. After a four week exposure to 32 ^{o}C/26 ^{o}C (day/night), considerable reductions in root depth and root mass were observed in all infection temperature treatments except for the 28 ^{o}C and 32 ^{o}C treatments.

Field experiments were conducted to evaluate fungicides for preventative control of PRD. Applications of pyraclostrobin provided the best and longest lasting preventative suppression of PRD symptoms. Azoxystrobin and cyazofamid provided moderate levels of preventative suppression and the standard *Pythium* fungicides were not effective against PRD. In vitro assays were conducted to determine the sensitivity of *P. volutum*'s to fungicides. *Pythium volutum* isolates were highly sensitive to pyraclostrobin and cyazofamid, moderately sensitive to azoxystrobin, and the least sensitive to mefenoxam.

Growth chamber experiments were performed to evaluate the effects of creeping bentgrass cultivar, organic matter content, and irrigation frequency on development of PRD. 'Crenshaw', 'Syn-96', and 'G-6' were the least susceptible cultivars when compared to 'Penncross'. The popular cultivars 'A-1' and 'A-4' were moderately susceptible and 'LS-44', 'G-2' and 'Penncross' were the most susceptible cultivars. Organic matter added at the time of establishment did not have an effect on PRD development. Symptoms of PRD were most severe when creeping bentgrass was irrigated 6 times a week when environmental conditions were conducive for infection by *P. volutum*. When creeping bentgrass was irrigated 3 or 4 times a week, PRD symptoms were less severe and turf quality did not decline.

Another series of growth chamber experiments were established to determine the effects of *P. volutum* infections on creeping bentgrass nitrate uptake, evapotranspiration, and photosynthesis. Nitrate uptake was elevated in creeping bentgrass plants that were infected with *P. volutum* when compared to the non-inoculated controls. Evapotranspiration was similar among inoculated and non-inoculated plants.

Biology and Management of Pythium Root Dysfunction in North Carolina

by
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DEDICATION

This work is dedicated to my mother (1949-2003) who left this world all too early. She is the person who convinced me to pursue a PhD. Thank you for all the sacrifices you made to help me achieve my goals. I know you are watching, but I wish you could be here. I love you very much!

BIOGRAPHY

The author of this text, James P. Kerns, was born January 30, 1980, in Wheaton IL to David and Evelyn Kerns. He graduated from Lee Senior High School in Sanford, NC in May of 1998. While in high school, he worked for Billy and Jimmy Parrish at Quail Ridge Golf Club. During his time at Quail Ridge, he learned that universities offered degrees that focused on turfgrass management. As a result, he decided to enroll at North Carolina State University to pursue a Bachelor of Science in Agronomy with a concentration in Turfgrass Management.

As an undergraduate, Jim worked for Dr. Charles Peacock and Dr. Tom Rufty as an undergraduate research assistant. Working in a research setting convinced him to continue his studies and ultimately decided he wanted to be a faculty member at a Land Grant University. After graduation in 2002, he accepted an assistantship at Texas A&M University in College Station, TX. Under the direction of Dr. Don Vietor and Dr. Richard White, Jim focused on the effects of composted dairy manure applications on soil physical and biological properties.

During the summer of 2004, Jim decided to pursue a PhD in Plant Pathology with Dr. Lane Tredway. Without question this was the best academic decision Jim ever made! His degree requirements were completed in March of 2008 with the approval and acceptance of this dissertation. He has accepted an Assistant Professor position in the Department of Plant Pathology at the University of Wisconsin-Madison.

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I would like to thank my father for his constant support and guidance during my entire college career. Without the support of my mother and father none of this would have been possible. I thank my brother and sisters for their support and guidance as well. I'm blessed to have such a wonderful and supportive family. Finally, I would like to express my deepest thanks and appreciation to my wife, Anna, for her unwavering support, patience, and understanding during my graduate career.

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CHAPTER 1- INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Creeping bentgrass is used throughout the state of North Carolina on golf course putting greens. It is an ideal cool-season turfgrass for putting greens because it tolerates extremely close mowing (28). However, North Carolina is in the transition zone for cool and warm season turfgrasses, and the hot, humid summers often result in plants that have reduced vigor and increased susceptibility to diseases. In addition, the United States Golf Association recommends a high sand-based rootzone for putting green construction, which has low water and nutrient holding capacity (28). This environment may place additional stress on bentgrass during the summer months, which may predispose creeping bentgrass to diseases that are encouraged by physiological stress.

In 2002 an undescribed fungal disease began to be an important problem on creeping bentgrass roots in the Southeast. Symptoms of the disease initially appear as areas of wilt, drought stress, or chlorosis ranging in size from approximately 6 to 40 cm in diameter. If these areas are left uncontrolled, turf density declines, the color of the affected turf will shift to yellow or orange, and eventually much of the grass may die. Examination of affected plants revealed necrotic crown and root tissue that was similar to symptomology of take-all patch, a disease caused by *Gaeumannomyces graminis* var. *avenae*. However, symptoms appeared in June, July, and August, which is later than would be expected for take-all patch in North Carolina. Isolations from symptomatic tissues attempted during the onset of disease symptoms yielded a diverse collection of microorganisms and saprophytes, especially *Curvularia* spp. Although *Gaeumannomyces graminis* could not be isolated from diseased

tissue and the onset of symptoms was not consistent with take-all patch in North Carolina, many turfgrass pathologists diagnosed the problem as take-all patch.

During a period of unseasonably warm and dry weather in the fall of 2003 and spring of 2004, symptoms of the disease reappeared in multiple areas. Examination of root tissue revealed bulbous root tips, loose cortical structure, and an absence of root hairs. A significant amount of *Pythium* hyphae and oospores were present in the cortical tissue of the roots as well. Isolations from multiple samples from 12 golf courses in NC, SC, GA, and VA yielded a collection of 75 *Pythium* isolates. Since take-all patch is historically not a problem outside of the NC Mountains, and isolations primarily indicated the presence of *Pythium* in the roots, we concluded that take-all patch was not the disease in question. Tentative identification indicate that *Pythium volutum* was the species isolated from diseased tissue in 2003 and 2004.

Similar symptomology to that observed in NC was described by Hodges and Coleman in 1985 (15). Those symptoms also were associated with newly constructed (<8 years old), high-sand-content putting greens. In addition, symptoms seemed to be induced by heat, drought stress, and close mowing. To distinguish this condition from Pythium root rot, Hodges and Coleman named the disease Pythium root dysfunction (PRD). Over 40 golf courses in NC and many golf courses in other states have sent disease samples to the Plant Disease and Insect Clinic at NC State with symptoms similar to the ones observed by Hodges and Coleman (15). Based on the similarity between symptoms reported for PRD in Iowa and the symptoms observed in NC, Pythium root dysfunction is most likely the disease that was first present in 2002 and subsequent years.

Very little is known about the distribution of *Pythium* species associated with PRD in the Southeastern U.S. Furthermore, little information exists on the factors that influence disease development and management of PRD. As a result, golf course superintendents have reported spending $10,000 to $20,000 a month in attempts to control this disease. The work presented in this dissertation will focus on the etiology, epidemiology, and management of PRD in NC, with an ultimate goal of improving PRD management strategies for golf course superintendents.

BACKGROUND INFORMATION AND LITERATURE REVIEW

History of *Pythium* Taxonomy:

The genus *Pythium* was established in 1858 by Pringsheim, who placed the genus in the Family *Saprolegniaceae* (14). *Pythium monospermum* is the type species for the genus. By 1897, the genus was moved into a new family called the *Pythiaceae* by Schroter and this classification remained until the mid-1980s (14). At first, species of *Pythium* and *Phytophthora* were solely the interest of taxonomic mycologists, but later it was discovered that these organisms caused root diseases in many plants. Matthews devised the first workable key of the known *Pythium spp*. in 1931 (14). However new species were being discovered quite frequently, which quickly made Matthews work obsolete. As a result, Middleton complied an extensive monograph of the genus that contained host information and illustrations of the most important species in 1945 (14). The most up to date morphological treatment of the genus *Pythium* were conducted by van der Plaats Niterink in 1981 and by Dick in 1990 (20). The lack of consensus on the important characteristics for identification, the high variability among structures and considerable overlap among species, and the absence of diagnostic features for many species have contributed to errors in

identification of many isolates. Therefore molecular tools should be used in conjunction with morphology to obtain an accurate identification to species within the genus *Pythium*.

Few regions have been used in DNA-sequence-based phylogenetic studies of the Oomycetes. Nuclear rDNA is the main region that has been used for phylogenetic studies of genera and species of *Pythium* (20). Based on phylogenetic analysis of the D1 and D2 regions of the large nuclear ribosomal subunit, it was demonstrated that *Pythium* was phylogenetically isolated and distant from other genera of the *Oomycetes* (20). Phylogenetic analysis based on partial sequences of the mitochondrial cytochrome oxydase II gene of 15 different genera of *Oomycetes* revealed that *Pythiales* were also monophyletic (20). The internal transcribed spacer (ITS) is another region that has been widely used for systematic studies in the *Oomycetes*. This is primarily because of the universal primers developed by White et al. (31) that amplify the ITS region throughout all taxa. The ITS region of *Oomycetes* vary from 750-1050 bp, which is much longer than the usual 300-700 bp of the Eumycota (19). Variability of the ITS region in the genus *Pythium* was deemed appropriate for studies examining species level differences (20).

Middleton considered all the species of *Pythium* as homothallic organisms, partly because all the species he worked with were in fact homothallic. Consequently the taxonomic separation of species in his monograph is based on sporangial and oogonial characteristics. Yet in 1967 Hendrix and Campbell (15) described a new species *P. sylvaticum* from southern US forests, which was heterothallic. Further studies lead to the discovery of more species that were heterothallic such as *P. catenulatum* and *P. splendens* (14). The inability of previous researchers to detect heterothallism was related to the media used for culture of *Pythium* spp. The mating reaction rarely occurs on cornmeal, PDA, or

other similar agars. Agars containing sterols are needed to stimulate the mating reaction such as hemp seed extract agar (14).

The genus *Pythium* and the closely related genus *Phytophthora* are no longer recognized as true fungi. Based on molecular and morphological characteristics *Pythium* and *Phytophthora* were placed in a separate kingdom called Stramenopila (or Chromista) along with diatoms and brown algae (25). Until recently, the oomycetes were considered "true fungi" because they absorb nutrients through cell walls and they produce filamentous strands of hyphae or mycelium. However, results from a number of phylogenetic studies provide overwhelming evidence that the members of the oomycetes share a common ancestor with the heterokont algae or Chromista. Furthermore, oomycetes are distinct from true fungi with respect to cell wall composition, nuclear state of vegetative hyphae, type of flagella produced, and mitochondrial structure. True fungi have chitin in their cell walls, have haploid or dikaryotic vegetative hyphae, have a whiplash flagella, and have flattened cristae in the mitochondria. Whereas the oomycetes have cellulose and beta glucans in their cell walls, have diploid vegetative hyphae, have two types of flagella, and their mitochondria have tubular cristae (25).

Presently there are over 120 recognized species of *Pythium* and they have a world wide distribution (21). Many of the species are pathogenic on economically important crop plants, turfgrasses, and ornamentals and induce major losses due to pre- and post-emergence damping-off by infecting germinating seeds or young succulent tissue. However, mature plants can also be attacked through infection of root tips, feeder roots, fruits, and in some cases foliage. Infection of mature plants via roots typically results in reduced plant vigor and yield, yet plant death rarely occurs (21). In some cases, pathogenic species of *Pythium* may

be isolated from roots that appear healthy, yet their colonization causes a reduction in plant growth without the typical necrotic appearance normally associated with *Pythium* infection.

Environmental factors that influence infection by *Pythium* spp:

Common factors that influence infection by *Pythium* species include inoculum density, soil moisture, soil temperature, pH, cation composition, light intensity, and presence of other microorganisms. In general, soil temperature and soil moisture are the most important factors that influence infection (14). The factor that is most important depends on the *Pythium* species involved and the host. High soil moisture is generally considered to be required for disease development, consequently *Pythium* infections are often considered as the cause of plant decline in areas that are low-lying or water-logged. However, experiments that examined the response of *P. vexans*, and *P. irregulare*, it was discovered that neither species induced disease at 90% saturation capacity (SC) (3, 4). *Pythium vexans* induced disease at 70% SC and *P. irregulare* induced disease at 50% SC. The results from these studies clearly show that saturated soils do not favor disease development.

In general, *Pythium* species are one of the more tolerant microorganisms to anaerobic conditions. Species of *Pythium* thrive in an environment with reduced microbial competition at high soil moisture. For example, the inoculum densities of *P. vexans* and *P. irregulare* were higher in field soils as CO_2 concentration increased from 0.03 % to 15% (21). At high soil moisture, *Pythium* species are primarily saprophytes, but as soil moisture decreases other fungi predominate (19). In particular, *P. ultimum* propagules were shown to convert from thick-walled oospores to thinner walled germinable oospores when O_2 levels in soils dropped (18). In order to germinate and infect, *Pythium* species need some level of free-water in the

rootzone. However, the soil moisture content required for propagule germination and infection varies among species of *Pythium*.

Temperature can have as much of an affect on infection and disease development as soil moisture. Occasionally the optimal temperature for in vitro growth of *Pythium* species correlates well with disease development, yet with certain *Pythium* species this relationship does not occur. For example, an unnamed *Pythium* spp. with an optimal temperature of 30^{o}C *in vitro* induced more disease in rice at 20^{o}C than at 30^{o}C (22). Species such as *P. irregulare* and *P. ultimum* have lower optimal temperatures for growth and typically infect and cause diseases when soil temperatures are cooler. *Pythium aphanidermatum* and *P. myriotylum* on the other hand, have higher optimal temperatures for growth and induce symptoms when soil temperatures are higher (22). However, generalizations about the influence of temperature on disease severity should be made with caution because variation among isolates of the same species has been reported. For instance, Nelson and Craft (23) reported varying levels of aggressiveness on turfgrasses for specific isolates of *P. graminicola* and *P. aphanidermatum*. Hodges and Campbell (17) also reported isolate specific variation in temperature moderated pathogenicity assays for *P. aristosporum*, *P. torulosum*, *P. vanterpoolii*, and *P. graminicola*.

Pythium species are considered primary colonists, therefore, organic matter which serves as a nutrient source, can moderate *Pythium* activity. Organic matter can also serve as a nutrient source for microorganisms that are antagonistic to *Pythium* spp, thus organic matter additions may enhance or suppress disease activity. For example, Pythium root rot of wheat was less severe when manure or pea vine residue was incorporated into soils (26). Yet, incorporation of lettuce debris initially enhanced the inoculum density of *P. ultimum* and

subsequently increased incidence of root rot. Suppression of Pythium root rot of creeping bentgrass was observed when certain composts were added to the potting mixture at the time of establishment (8). Furthermore, studies have documented reduced incidence of post-emergence damping-off of greenhouse crops when slightly decomposed peat material was added to the potting mixtures (5). The effect of organic matter additions on PRD severity has not been determined and warrants investigation.

Diseases incited by *Pythium* spp.

Most species of *Pythium* infect juvenile or very succulent tissues, which restrict their parasitism to seedlings, feeder roots, or root tips of older plants. They also can infect watery fruits or stems. *Pythium* spp commonly infect seed and the emerging radicals causing seed rot or pre-emergence damping-off. They also can infect seedlings at ground level, causing them to collapse or fall over, which is a common symptom of post-emergence damping-off (14). Pre- and post-emergence damping-off are very important in a wide range of agricultural crops, ornamentals, nurseries, and turfgrasses (14). As plants mature and main roots and stems develop secondary wall thickenings, infection is typically restricted to lateral roots, root hairs, and root tips. Infections of these tissues result in stunted and chlorotic plants that usually do not recover even if conditions for disease development become unfavorable.

A great deal of research has focused on the decline of perennial crops induced by *Pythium* spp. The gradual to sudden deterioration of mature plants and poor survival of replants is a common symptom in peaches, turf, citrus, apples, pines, and other perennial crops. The classic example of this symptom is observed in shortleaf and loblolly pines that are affected with littleleaf disease. Although the primary causal agent is *Phytophthora*

cinnamomi, various *Pythium* species are also associated with the destruction of fine roots and root tips (15). This leads to reduced nitrogen uptake from the soil and is known as feeder root necrosis. Peach decline has been shown to be a feeder root problem that is induced by a variety of factors, which include infection by *Pythium* spp (3). Similarly, citrus decline is associated with soils with high population densities of oomycetes (14).

Even though *Pythium* spp. are normally considered root pathogens, they can infect the stems and foliage of some plants. Greenhouse and nursery crops that are overcrowded, well-watered, and well-fertilized are subject for foliar infections by *Pythium* spp (14). Similar conditions are required for *Pythium* spp. to induce foliar blight of turfgrasses (27). Succulent fruits such as squash, watermelons, peppers, oranges, and cucumbers are also commonly attacked by *Pythium* spp. Various *Pythium* species can cause post-harvest rot of multiple fruits and vegetables, as well as bulbs and tubers of horticultural plants.

<u>*Pythium* induced diseases of Turfgrasses:</u>

Species of *Pythium* incite numerous diseases of turfgrasses, which include Pythium blight, crown and root rot, root dysfunction, pre-and post-emergence damping-off, and snow blight (27). All species of turfgrasses are susceptible to *Pythium* spp., but cool-season grasses are the most commonly damaged. Diseases caused by *Pythium* spp. in turfgrasses can occur across a wide range of environmental conditions, yet the most severe problems are observed during hot, humid weather. Under ideal conditions for disease development entire stands of turfgrasses can be wiped out in a single day. Most *Pythium* induced diseases are usually associated with golf course turf and athletic fields. However, Pythium blight and damping-off can be problematic in home lawns established with cool-season grasses.

When environmental conditions favor the growth of *Pythium* spp. more than the growth of seeds or seedlings, *Pythium* may cause seed death that results in pre-emergence damping-off (27). Seedlings are also susceptible to attack by *Pythium* species and are easily killed if they emerge in a weakened state. The pathogens induce a water-soaked lesion that girdles the plant; as a result the seedling collapses, turns yellow, and dies. The incidence of these two diseases can be sporadic across the seedbed.

Symptoms of Pythium blight develop as small circular spots (2-5 cm dia.) that occur rapidly in hot, humid weather. On putting greens, tees, and fairways, symptoms initially appear as small tan or brown spots that can expand at a startling pace. In higher cut perennial ryegrass and tall fescue stands, spots may be copper colored or may have a gray water-soaked appearance (27). This disease has probably received the most attention of researchers because it can destroy large areas of turfgrass in a few days. Conditions that favor disease development include warm, humid nights, prolonged leaf wetness, and elevated nitrogen levels in turfgrass leaves (27). *Pythium aphanidermatum* is considered as the main causal agent of Pythium blight, but *P. torulosum*, *P. graminicola*, *P. myriotylum*, and *P. ultimum* can also incite foliar blight of turfgrasses.

Pythium snow blight of cool-season grasses commonly occurs in the colder regions of Japan and North America. This type of foliar blight is favored by high soil fertility and saturated soils. Symptoms may appear as small orange or tan spots that progress into large areas of uniform blight. Infected leaves are light tan or brown and are laden with oospores (27). Roots are typically unaffected, but crowns can be completely rotted, which results in rapid death of the turfgrass. *Pythium* species associated with snow blight include *P. aristosporum*, *P. graminicola*, *P. horinouchiense*, *P. iwayami*, and *P. paddicum*.

Various species of *Pythium* are associated with turfgrass roots and crowns as well. The symptoms of these diseases are typically general decline of turf in no particular pattern. Crown and root rot usually occur in poorly drained soils and can develop during any season. The crowns may develop severe necrosis and have a water-soaked appearance, and their roots are necrotic and display reduced length and volume (1, 23). Typically in NC, symptoms of Pythium root and crown rot appear during warm, humid conditions on sites that have poor drainage.

The symptoms of Pythium root dysfunction typically appear during hot, dry conditions on sites with excellent drainage. Symptoms develop during the summer when creeping bentgrass is subjected to heat and drought stress. Symptoms initially appear as areas of wilt or chlorosis in a patch that ranges in size from 6 to 40 cm in diameter. Symptoms may also appear as irregular areas of wilt or drought stress (27). With Pythium root dysfunction, the roots appear healthy and may not show noticible reductions in root depth and root mass (11, 15). Affected roots have a light tan color, have bulbous root tips, and lack root hairs.

Pythium species associated with Root Diseases in Turfgrass:

Pythium root rot typically occurs during hot, moist conditions and induces severe necrosis of creeping bentgrass roots and crowns. Nelson and Craft (23) obtained 121 isolates of *Pythium* species from rotting crown and root tissue. Of these isolates, only 46 were pathogenic towards creeping bentgrass roots. Species that were highly aggressive included *P. gramincola*, *P. aristosporum*, and *P. aphanidermatum*. In contrast, *P. torulosum* was isolated most frequently, but was not pathogenic toward creeping bentgrass seedlings.

Nelson and Craft (23) concluded that *P. gramincola* was the principle root rotting species affecting creeping bentgrass species in New York State.

Abad et al. (1) found 33 species of *Pythium* associated with Pythium root rot of creeping bentgrass species in North Carolina. The predominant species recovered were *P. arrehenomanes*, *P. catenulatum*, *P. intermedium*, *P. oligandrum*, *P. periilum*, *P. torulosum*, and *P. vanterpoolii*. Of the 33 species only eight were identified as highly aggressive to seedlings of creeping bentgrass. *Pythium arrhenomanes*, *P. aristosporum*, *P. aphanidermatum*, *P. graminicola*, *P. myriotylum*, *P. tardicrescens*, *P. vanterpoolii*, and *P. volutum* were shown to induce 61 to 100% disease in pre-and post-emergence damping-off pathogenicity tests. Among the eight highly aggressive species was *P. volutum,* but it represented 2 isolates out of 222 obtained. This was the first report of *Pythium volutum* as a causal agent of root rot of turfgrass and was documented to be most virulent when temperatures were 28^{o}C or 32^{o}C. Abad et al. (1) suggested that *P. arrehenomanes*, the most virulent and frequently isolated highly virulent species, may be the most important root and crown rotting pathogen in NC.

Hodges and Coleman (16) initially described Pythium root dysfunction on sand-based putting greens in Iowa in 1985. The primary symptom observed was turf death characteristic of Pythium blight, but *Pythium spp.* could not be isolated from the foliage. In contrast, two *Pythium* species were isolated from creeping bentgrass root tissue. Hodges and Coleman (15) readily isolated *P. arrhenomanes* and *P. aristosporum*, which they found to be pathogenic to secondary roots of creeping bentgrass (16). Roots infected with these two species of *Pythium* were white with some buff-colored lesions. Roots also were devoid of root hairs and had bulbous root tips. They named the disease Pythium-induced root

dysfunction because the pathogens failed to cause substantial necrosis of roots, yet infection did result in reductions in creeping bentgrass shoot and root growth. The authors speculated that *P. arrhenomanes* and *P. aristosporum* infections impaired nutrient and water uptake of creeping bentgrass roots, which ultimately lead to reductions in growth.

Feng and Dernoeden (11) inspected 109 putting green samples for the presence of *Pythium* oospores in response to an outbreak of symptoms similar to Pythium-induced root dysfunction. The authors documented 28 *Pythium* species that were isolated from symptomatic tissue. Only three of the *Pythium* species (*P. aristosporum, P. aphanidermatum, and P .volutum*) were highly virulent to 'Crenshaw' creeping bentgrass seedlings. In addition, Feng and Dernoeden (11) frequently isolated *P. torulousm* from creeping bentgrass crowns and roots, yet this species was not pathogenic to creeping bentgrass seedlings. The authors only found one isolate of *P. volutum*, but observed that it was highly virulent to creeping bentgrass seedlings when temperatures were 18^{o} C.

Pythium volutum in Other Cropping Systems:

Pythium root dysfunction has not been documented in other cropping systems. However, many other diseases caused by *Pythium volutum* are prevalent in traditional cropping systems. For example, wheat grown in the Pacific Northwest is constantly plagued by Pythium root rot (9). Yet, the species responsible for the root rot was unknown, until Washington State researchers Chamswarng and Cook (9) collected 350 isolates from soil and wheat roots. Of the 350 isolates collected, only ten species could be identified, but all ten species were pathogenic to wheat seedlings. The species that were rated as the most virulent were *P. aristosporum* and *P. volutum*. These species caused severe root necrosis that resulted in significant reductions in wheat yield and biomass (9). The authors concluded that

these two *Pythium* species were the main causal agents of Pythium root rot in the Pacific Northwestern United States.

Pythium volutum is also a significant problem in tobacco float cultures. Tobacco seedlings are grown in polystyrene seed trays filled with a soil-less medium and floated in a nutrient solution (12). In this environment, damping off and root rot can be difficult to control. Though *P. aphanidermatum* and *P. myriotylum* were documented as being the major causal agents of root rot in tobacco float cultures, *P. volutum* was documented as being an aggressive pathogen of tobacco as well (12). Seedling disease caused by *P. volutum* in tobacco was more prevalent when the nutrient solution temperatures were between 15 and 20^0 C. In contrast, the other *Pythium* species caused more disease when the solution temperatures were greater than 20^0 C (12). The University of Kentucky Extension Agency also reported that Pythium root diseases were difficult to manage in tobacco float cultures. The authors found that *P. volutum* was the most prevalent species when temperatures were below 17 ^{0}C. In addition, they concluded that *P. volutum* was the most aggressive *Pythium* species they isolated (24).

Chemical control of Pythium root dysfunction:

Systemic control of *Oomycete* induced diseases became viable in the mid to late 1970's (7). The products that were introduced were propamocarb, metalaxyl, and fosetyl-Al. These chemistries have excellent activity against a broad range of *Oomycete* genera and have systemic properties (7). For Pythium diseases of turfgrasses, multiple fungicides are labeled for Pythium foliar blight or Pythium root rot, which include mefenoxam (more active enantiomer of metalaxyl), propamocarb, ethazole, fosetyl-Al, azoxystrobin, pyraclostrobin, and cyazofamid. For Pythium blight control, fungicides are applied on a 7 to 14 d interval

when conditions become favorable for disease development (30). Under intense Pythium blight pressure, applications of menfenoxam provided the best control of Pythium foliar blight of perennial ryegrass (30). For Pythium root rot control, fungicides are applied as soon as symptoms develop and are generally applied as a soil drench. Although data is limited on Pythium root rot control, ethazole or mefenoxam applied on 7 to 10 intervals are recommended.

Many fungicides are available for control of *Pythium* diseases in turfgrass, however, all of these may not be effective for controlling *Pythium* root dysfunction (29). Three studies were conducted at Myers Park Country Club in Charlotte, NC to evaluate curative fungicide efficacy for this disease. Treatments included standard Pythium fungicides (mefenoxam, fosetyl-Al, propamocarb, and ethazole), QoI's, or combinations of the two. The standard *Pythium* fungicides alone showed no activity against the disease and the only treatment that showed efficacy in early 2004 was a combination of azoxystrobin and thiophanate methyl. However, when pyraclostrobin was introduced into fungicide research trials later in 2004, significant reductions in disease severity were observed (29).

This indicates that pyraclostrobin may be controlling the pathogen, but *P. volutum* sensitivity to pyraclostrobin has not been examined. Furthermore, there is evidence of increased yields, higher growth rates, and greater plant vigor with QoI's. When QoI fungicides are applied to wheat and barley there are clear benefits in regard to yield and grain size (2). The chemicals for which this has been documented include azoxystrobin, kresoxim-methyl, trifloxystrobin, picoxystrobin, and pyraclostrobin (2). Yet, when compared to a triazole-based disease management program, similar disease control is attained. This effect has been termed the "greening effect" because the QoI's maintain green leaf area late in the

season (2). Non-disease related physiological effects of the QoI's have been attributed to "greening" effects seen in the field. Two major theories have been offered to explain this phenomenon. First, a variety of physiological processes in the host plant may be affected such as carbon dioxide compensation point, leaf senescence, nitrate reductase activity, and many others (2). Second, the greater yield and quality may be due to the prevention of sporulation of primary pathogens as well as secondary saprophytes and thereby preventing induction of any energy sapping host-defense responses (2).

Although PRD was first described in 1985 and subsequently re-examined in 1999, very little is known about the biology and management of this disease. Previous work has shown that *P. volutum*, the suspected causal agent of PRD in NC, is a virulent pathogen of creeping bentgrass. Yet pathogenicity assays were performed with creeping bentgrass seedlings, thus the impact of *P. volutum* infections on mature creeping bentgrass plants remains unknown. Curative suppression PRD symptoms has been demonstrated in NC, however, our observations indicate that *P. volutum* is active and infects creeping bentgrass in the fall and spring coinciding with the time of optimal growing conditions for creeping bentgrass. Therefore understanding the basic biology of PRD may provide information that will improve our ability to manage PRD. In order to obtain this information this dissertation focused on the following objectives:

1. Identify the pathogen that causes Pythium root dysfunction in North Carolina using morphological and molecular techniques and conduct pathogenicity assays under growth chamber conditions using mature creeping bentgrass plants.

2. Evaluate the effects of temperature during infection of creeping bentgrass roots by *P. volutum* and determine the optimal temperature for growth of *P. volutum*.

3. Determine fungicide sensitivity in culture, greenhouse, and field trials to develop effective control practices for Pythium root dysfunction.

4. Determine the relative susceptibility of eight commonly used creeping bentgrass cultivars to PRD and assess the impact of organic matter content and irrigation frequency on PRD development.

5. Evaluate the impact of *P. volutum* infections on creeping bentgrass photosynthesis, evapotranspiration, and nitrate uptake.

REFERENCES:

1. Abad, Z.G., Shew, H.D., and Lucas, L.T. 1994. Characterization and pathogenicity of *Pythium* species isolated from turfgrass with symptoms of root and crown rot in North Carolina. Phytopathology 84: 913-921.

2. Bartlett, D.W., Clough, J.M., Godwin, J.R., Hall, A.A., Hamer, M., and Parr-Dobrzanski, B. 2002. Review The strobilurin fungicides. Pest Manag. Sci. 58:649-662.

3. Biesbrock, J.A. and Hendrix, F.F. 1970. Influence of soil water and temperature on root necrosis of peach caused by *Pythium* spp. Phytopathology 60:880-882.

4. Biesbrock, J.A. and Hendrix, F.F. 1970. Influence of continuous and periodic soil water conditions on root necrosis of holly caused by *Pythium* spp. Can. J. Bot. 48: 1641-1645.

5. Boehm, M.J. and Hoitink, H.A.J. 1992. Sustenance of microbial activity in potting mixes and its impact on severity of Pythium root rot of poinsettia. Phytopathology 82: 259-264.

6. Campbell, W.A. and Hendrix, F.F. 1967. *Pythium* and *Phytophthora* species in forest soils in the southeastern United States. Plant Dis. Reptr. 51:929-932.

7. Cohen, Y. and Coffey, M.D. 1986. Systemic fungicides and the control of Oomycetes. Annu. Rev. Phytopathol. 24: 311-338.

8. Craft, C.M., and E.B. Nelson. 1996. Microbial properties of composts that suppress damping-off and root rot of creeping bentgrass caused by *Pythium graminicola*. Appl. Environ. Microbiol. 62: 1550-1557.

9. Chamswarng, C., and Cook, R.J. 1985. Identification and comparative pathogenicity of species from wheat roots and wheat-field soils in the Pacific Northwest. Phytopathology 75:821-827.

10. Dernoeden, P.H. Creeping Bentgrass Management: Summer Stresses, Weeds and Selected Maladies. John Wiley & Sons, INC. Hoboken, NJ. 2002.

11. Feng, Y. and Dernoeden, P.H. 1999. *Pythium* species associated with root dysfunction of creeping bentgrass in Maryland. Plant Dis. 83:516-520.

12. Fortnum, B.A., Rideout, J., Martin, S. B., and Gooden, D. 2000. Nutrient solution temperature affects *Pythium* root rot of tobacco in greenhouse float systems. Plant Dis. 84:289-294.

13. Garzon, C.D., Geiser, D.M., and Moorman, G.W. 2005. Diagnosis and population analysis of *Pythium* species using AFLP fingerprinting. Plant Dis. 89:81-89.

14. Hendrix, F.F. and Campbell, W.A. 1973. *Pythiums* as plant pathogens. Annu. Rev. Phytopathol. 11:77-98.

15. Hendrix, F.F. and Campbell, W.A. 1968. Pythiaceous fungi isolated from southern forest nursery soils and their pathogenicity to pine seedlings. Forest Sci. 14: 292-297.

16. Hodges, C.F., and Coleman, L.W. 1985. Pythium-induced root dysfunction of secondary roots of *Agrostis palustris*. Plant Dis. 69:336-340.

17. Hodges, C.F., and Campbell, D.A. 1994. Infection of adventitious roots of *Agrostis palustris* by *Pythium* species at different temperature regimes. Can. J. Bot. 72:378-383.

18. Johnson, L.F. 1988. Effects of atmospheric gases and light on changes in thickness of oospore walls and on germinabililty of oospores of *Pythium ultimum*. Phytopathology 78: 435-439.

19. Kouyeas, V. 1964. An approach to the study of moisture relations of soil fungi. Plant Soil 20: 351-363.

20. Levesque, C.A., and DeCock, A. 2004. Molecular phylogeny and taxonomy of the genus *Pythium*. Mycol. Res. 108:1363-1383.

21. Martin, F.N. 1996. *Pythium.* Pages 17-36, in: Pathogenesis and Host Specificity in Plant Diseases Vol II. Kohmoto, K., Singh, U.S., and Singh, R.P. (eds.) Pergamon Press, UK.

22. Martin, F. N. and Loper, J.E. 1999. Soilborne plant diseases caused by *Pythium* spp: ecology, epidemiology, and prospects for biological control. Crit. Rev. Plant Sci. 18: 111-181.

23. Nelson, E.B., and Craft, C.M. 1991. Identification and comparative pathogenicity of *Pythium* spp. from roots and crowns of turfgrass exhibiting symptoms of root rot. Phytopathology 81:1529-1536.

24. Nesmith, W. 2003. Tobacco: *Pythium* diseases in the tobacco float system-understanding the problem. Kentucky Pest News Univeristy of Kentucky of Cooperative Extension Service. February 24 # 976.

25. Rossman, A.Y. and Palm, M.E. 2007. Why are Phytophthora and other Oomycota not true Fungi? APS Feature, APS Press, St. Paul, MN.

26. Smiley, R.W., Collins, H.P., and Rasmussen, P.E. 1996. Diseases of wheat in long-term agronomic experiments at Pendleton, Oregon. Plant Dis. 80:813-820.

27. Smiley, R.W., Deroeden, P.H., and Clarke, B.B. Compendium of Turfgrass Diseases. APS Press, St Paul, MN, 1992.

28. Turgeon, A.J. Turfgrass Management, 5th edition. Prentice Hall, Upper Saddle River, NJ. 1999.

29. Tredway, L.P. and E.L. Butler. 2004. Preventative control of an unknown root disease of creeping bentgrass putting greens. Online http://www.turffiles.ncsu.edu/Extension/tredwaytrials/2004/prd%20preventative .pdf

30. Uddin, W., Soika, M.D., Soika, E.L., and Francl, A. 2003. Pythium foliar blight control with the use of fungicides. Fungic. Nemat. Tests 59:T037.

31. White, T.J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-324 in: PCR Protocols: A Guide to Methods and Applications, M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, eds. Academic Press, San Diego, CA.

CHAPTER 2 – PATHOGENICITY OF *PYTHIUM* SPECIES ASSOCIATED WITH PYTHIUM ROOT DYSFUNCTION OF CREEPING BENTGRASS AND THEIR IMPACT ON ROOT GROWTH AND SURVIVAL

ABSTRACT

Symptoms resembling Pythium root dysfunction have been observed on golf course putting greens established with creeping bentgrass across the Southeastern U.S. since 2002. Root isolations from 14 golf courses yielded 58 isolates of *Pythium volutum* and 16 isolates of *Pythium torulosum*. Pathogenicity of five isolates of *P. volutum,* two isolates of *P. torulosum*, and a combination of the two species was determined by inoculating mature 'A-1' creeping bentgrass plants. Inoculated plants were incubated for 4 wks at 24/16°C (day/night) to permit root infection, then temperatures were increased to 32/26°C to induce foliar symptoms. No isolates impacted root depth, root mass, or foliar disease severity after 4 wks at 24/16°C. After increasing the temperature to 32/26°C, isolates of *P. volutum* induced foliar disease severity ranging from 60 to 84%, whereas isolates of *P. torulosum* induced only 14 to 35% disease. Isolates of *P. volutum* consistently reduced root mass and root depth after 4 wks at 32/26°C, but *P. torulosum* exhibited no effect. These results demonstrate that *P. volutum* is a pathogen of mature creeping bentgrass plants. Infections that occur during cool weather reduce the growth and survival of creeping bentgrass roots during hot weather and give rise to foliar symptoms.

INTRODUCTION

Creeping bentgrass (*Agrostis palustris* Huds.) is a cool-season grass species that is widely planted on golf courses in temperate and subtropical climates. This is the preferred

turfgrass species for golf course greens because it tolerates extremely close mowing and produces a fast, uniform putting surface (19). Creeping bentgrass is not well-adapted to the persistent hot and humid conditions experienced during summer months in the Southeastern United States, which often results in reduced plant vigor and increased susceptibility to disease. In addition, most putting greens are constructed with artificial root zones containing 85 to 100% sand and 0 to 15% organic matter by volume to facilitate rapid drainage and drying of the playing surface. These root zones have extremely low water- and nutrient-holding capacities (19). While these construction practices may reduce the development of fungal diseases that are encouraged by wet soil conditions, they may also enhance other diseases that are encouraged by physiological stress.

Despite the importance of creeping bentgrass to the golf course industry, few root diseases have been described and thoroughly researched on this host. Take-all patch, caused by *Gaeumannomyces graminis* var. *avenae*, is a common disease of creeping bentgrass in temperate climates but is not widely distributed in the subtropical climates of the Southeastern U.S. (15). Summer patch, caused by *Magnaporthe poae,* was originally observed in creeping bentgrass in Florida in 1987 (4) and was recently documented in North Carolina (16). Two Pythium-induced root diseases have been described in creeping bentgrass: Pythium root rot (1, 6, 12) and Pythium root dysfunction (5, 7). However, the etiology, epidemiology, and management of these *Pythium* diseases remain poorly understood.

Pythium root rot is characterized by a crown and root decline that results in chlorotic turfgrass plants with reduced vigor and density. Roots and stolons are necrotic and generally have water-soaked appearance. Stand symptoms are typically nondescript, yet small yellow

or necrotic patches may occur on creeping bentgrass after prolonged wet conditions (12). Abad et al. (1) isolated 33 *Pythium* species from roots and crowns of creeping bentgrass exhibiting symptoms of root and crown rot in North Carolina. Eight species were classified as highly virulent to creeping bentgrass seedlings at 28°C and 32°C, which included *P. arrehenomanes, P. aristosporum, P. aphanidermatum, P. graminicola, P.myriotylum, P. tardicrescens, P. vanterpoolii, and P. volutum.* However, the most frequently isolated species were *P. torulosum, P. catenulatum, P. arrehenomanes,* and *P. vanterpoolii.*

Pythium root dysfunction (PRD) of creeping bentgrass was first described by Hodges and Coleman in 1985 (7). They observed that newly established, high-sand content (>60% sand) putting greens were rapidly killed during the summer. *Pythium aristosporum* and *P. arrhenomanes* were consistently isolated from diseased roots, and were highly virulent towards the secondary roots of creeping bentgrass. Diseased roots exhibited a yellowish tan discoloration compared to uninfected roots. Root tips were described as bulbous and were ultimately killed.

Feng and Dernoeden (5) identified eight Pythium species in a collection of 28 isolates from 109 putting green samples exhibiting symptoms of PRD: *P. aristosporum*, *P. aphanidermatum*, *P. catenulatum, P. graminicola*, *P. torulosum*, *P. vanterpoolii*, *P. volutum*, and *P. ultimum* var. *ultimum*. Most of the isolates were obtained from putting greens that were less than 3 years old. *Pythium aristosporum* and *P. aphanidermatum* were highly aggressive towards creeping bentgrass seedlings at 18°C and 28°C, but *Pythium volutum* was only highly aggressive at 18°C. *P. aristosporum* was isolated three times whereas *P. aphanidermatum* and *P. volutum* were only isolated once from 109 putting green samples. The remaining species were either non-pathogenic or exhibited low to moderate

aggressiveness. Based on the frequency of isolation and pathogenicity experiments, it was concluded that *P. aristosporum* was the most important causal agent of PRD in the Mid-Atlantic region of the U.S..

Symptoms characteristic of Pythium root dysfunction (PRD) have been observed on creeping bentgrass putting greens throughout the Southeastern United States since 2002. The foliar symptoms of PRD initially appear as wilt, drought stress, or chlorosis in distinct, circular patches ranging from 6 to 30 cm in diameter. If the disease is left uncontrolled, foliar symptoms progress to a yellow-orange die-back and much of the turf may eventually die. Affected roots are a light-tan color with bulbous root tips and have few root hairs. The most severely affected greens are typically less than six years old and have root zones constructed of 85 to 100% sand and 0 to 15% organic matter by volume. Symptoms are most commonly observed during hot weather in summer, but may also be seen during warm, dry conditions in fall, winter, or spring. Root depth is often not negatively impacted during the fall and spring, yet root depth is significantly reduced in affected patches during the summer months. *Pythium volutum* was consistently isolated from diseased root tissue in North Carolina and was demonstrated to be highly aggressive toward mature creeping bentgrass roots in controlled environments (9).

Hodges and Coleman (7) recognized the importance of inoculating mature turfgrass plants and were able to reproduce PRD symptoms in the greenhouse by inoculating the secondary roots originating from creeping bentgrass stolons. Prior to our discovery of *P. volutum* as a causal agent of PRD in North Carolina (9), this species was isolated infrequently from turfgrasses exhibiting symptoms of root rot and root dysfunction (1, 5). Inoculations in both studies were performed on creeping bentgrass seedlings, thus the impact

of *P. volutum* on mature creeping bentgrass plants and secondary root growth remains unknown.

Although the expression of PRD symptoms is most common in summer, when creeping bentgrass is subjected to persistent heat stress, our observations indicate that *P. volutum* is not highly active under these conditions. Instead, the majority of hyphal growth, oospore production, and root infection are observed during the fall, winter, and spring, coinciding with the time when creeping bentgrass roots are most actively growing. These observations are consistent with the results of Feng and Dernoeden (5), who showed that *P. volutum* was highly virulent toward creeping bentgrass seedlings at 18°C but only slightly virulent at 28°C. It is unknown if *P. volutum* infections reduce the re-growth of creeping bentgrass roots during the fall and spring, reduce root survival during hot weather, or simply reduce the root's ability to absorb water and nutrients from the soil solution.

To further improve our ability to diagnose and manage root diseases in creeping bentgrass, additional research is needed to determine the distribution of *Pythium* species causing Pythium root dysfunction in sand-based root zones. The objectives of this study were to (i) determine the distribution of *Pythium* species causing PRD in North Carolina and neighboring states; (ii) determine the pathogenicity and virulence of *Pythium* species on mature creeping bentgrass plants; and (iii) assess the impact of *Pythium* species on the growth of creeping bentgrass roots during cool weather and their survival during hot weather.

MATERIALS AND METHODS

Isolation and identification of *Pythium* species. Isolates of *Pythium* (n=75) were obtained from creeping bentgrass putting greens exhibiting symptoms of Pythium root dysfunction between September 2003 and April 2005. Samples were obtained from 11

locations in North Carolina and one location in Virginia (Table 2.1). S.B. Martin (Clemson University) provided one isolate from Guilford Co. NC, four isolates from South Carolina, and three isolates from Georgia (Table 2.1).

Roots with a significant amount of *Pythium* hyphae and oospores (>10) were tan, lacked root hairs, and possessed bulbous root tips. Sections of roots (1 cm) exhibiting these symptoms were washed under continuously flowing tap water for at least 6 hr and rinsed in sterile deionzed water. Infected roots were blotted dry with sterile paper towels and placed on PARP medium (11), clarified V8 juice cholesterol agar (SV8) (11), or were baited with creeping bentgrass seedlings. Baiting was performed by placing washed root sections in the root zone of 'A-4' creeping bentgrass seedlings grown in calcined clay (Turface Allsport, Profile Products LLC, Buffalo Grove, IL), incubating in a saturated condition at room temperature for 5 to 7 days, washing the infected seedling roots as described above, and plating on SV8. After incubation for 3 days in the dark at 18°C, colonies resembling *Pythium* were transferred to a fresh plate of SV8. If bacterial contamination occurred, isolates were purified by placing mycelial plugs under a block of fresh PARP medium. Mycelium growing through the top of the medium was then transferred to a fresh plate of SV8. Working cultures were maintained on SV8. For long-term storage of isolates, 10 mycelial plugs cut from a 4-day old SV8 culture were placed in a test tube containing 10 ml of sterile deionized water and stored at 10°C (11).

Morphological identification of *Pythium* species. All isolates identified as a *Pythium* species were transferred to grass-leaf cultures to induce production of sporangia, antheridia, oogonia, and oospores. Four to six 1 cm^2 mycelial plugs were placed in a sterile Petri dish containing 20 ml of sterile deionized water and 10-15 pieces (~1.5 cm long) of

autoclaved (30 min) creeping bentgrass leaves (1,11). The grass-leaf cultures were incubated at room temperature under continuous fluorescent light for 3 to 4 days. All isolates produced sporangia, antheridia, oogonia, and oospores in the grass-leaf cultures. *Pythium* species were identified using the keys and descriptions of Van der Plaats-Neterink (20) and Dick (3). Identification was based on the morphology and dimensions of 20 arbitrarily selected reproductive structures and colony morphology on SV8, water agar, and corn meal agar. Ten isolates also were sent to the Plant Pathogen Identification Laboratory at NC State University to confirm identification.

Phylogenetic analysis using ribosomal DNA sequences. Each isolate was grown for 7 days at room temperature (23 to 25°C) in 2 ml of potato dextrose broth (Difco Lawrence, KS). The mycelial suspension was transferred to a 1.5-ml microcentrifuge tube and harvested by centrifugation at 13,200 rpm for 5 min. Genomic DNA was extracted using the Easy-DNA Kit (Invitrogen Corp., Carlsbad, CA), quantified by spectrophotometry at 260 nm, and then standardized to 50 ng/µl.

PCR amplification of the ITS1, 5.8S, and ITS2 regions of the ribosomal DNA (rDNA) was performed using primers ITS4 and ITS5 (21). Reactions were 50 µl in volume and consisted of 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTP, 200 nM each primer, 1.5 U of *Taq* polymerase (Invitrogen Corp., Carlsbad, CA), and 50 ng of genomic DNA. Thermal cycling conditions consisted of an initial denaturation step at 95°C for 3 min; followed by 33 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 45 s; and a final extension step at 72°C for 2 min. PCR products were purified with a Qiaquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Cycle sequencing reactions were performed using an ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing

Kit (Applied Biosystems Inc., Foster City, CA). Reactions were cleaned using Centrisep columns (Princeton Separations Inc., Adelphia, NJ), dried, and then submitted to the University of Iowa DNA Sequencing Facility (Iowa City, IA) for electrophoresis and fluorescence analysis.

A variety of *Pythium spp*. sequences were obtained from GenBank for comparison. All sequences were aligned using the ClustalW algorithm in MegAlign 5.05 (DNASTAR Inc., Madison, WS) and adjusted by visual examination. A phylogenetic tree was constructed in MEGA 2.1 (10) using the neighbor-joining algorithm from genetic distances calculated using the Kimura two-parameter model. Bootstrap values were calculated in MEGA 2.1 based on 1,000 random samples of the data set.

Impact of *Pythium* species on root growth and survival. Cone-tainers (3.8 cm x 20 cm) containing sand meeting USGA specifications (19) (BB 205, Golf Agronomics Inc., Rockingham, NC) were seeded with 'A-1' creeping bentgrass (9.7 g m^{-1}). The cone-tainers were placed in a greenhouse at 26°C/22°C (12-h day/night cycles) and misted twice daily to encourage rapid germination. Following germination, the turf was maintained in the greenhouse by irrigating twice daily with a complete nutrient solution containing 106.23 mol m^{-3} nitrogen, 10.41 mol m^{-3} phosphorus, and 111.03 mol m^{-3} potassium. The turf was trimmed weekly with scissors to a height of 1.27 cm.

Six weeks after seeding, each cone-tainer was inoculated with sterilized creeping bentgrass leaves infested with one of five *Pythium volutum* isolates (PRD48, PRD38, PRD39, OC2, and PRD11), one of two *Pythium torulosum* isolates (SV3 and PV3), or a co-inoculation of one *P. volutum* and one *P. torulosum* isolate (OC2 and SV3). The inoculum was prepared by placing three to four 3-mm mycelial plugs into sterile water containing five

to seven sterilized 1-cm long creeping bentgrass leaves. The inoculum was incubated under continuous fluorescent light at room temperature (23 to 25^{o}C) for 3 days. Inoculations were performed by cutting the root system at a 5-cm depth, the turf plug was removed from the cone-tainer, the sand in the cone-tainer was discarded and replaced with fresh sand, 5 to 7 *Pythium*-colonized grass blades were placed onto the surface of the fresh sand, and then the turf plug was placed on top of the colonized grass blades. An uninoculated control was included in each experiment by cutting the roots at 5 cm then repotting onto fresh, uninfested sand.

Inoculated cone-tainers were transferred to a growth chamber and arranged in a completely random design with 10 replications per isolate. Initial growth chamber conditions were 12-h day/night cycles at 24/16°C to mimic conditions during fall and spring. After four weeks, four of the 10 replications were destructively sampled to measure root necrosis, root depth, and root mass. Root necrosis was estimated visually on a scale of 0 to 10 (0=no necrosis 10=100% root necrosis). Root depth was assessed by removing the creeping bentgrass plug from the cone-tainer and measuring the distance from the soil surface to the deepest root tip. The sand was then thoroughly washed from the root system by gently massaging the roots in deionized water. *Pythium* isolates were obtained from a 30 mg sub-sample of freshly washed roots. All isolates were collected by washing the roots for 6 hr in continuously flowing tap water and four root sections were plated onto SV8 and were identified as described above. Dry root weights were recorded after drying at 60°C for 72 hr.

The remaining six replicates remained in the growth chamber, and the temperature was raised to 32/26°C (day/night) to encourage development of foliar symptoms. The

severity of foliar symptoms was assessed at 0, 15, 23, 30, and 38 days after heat exposure using a scale of 0 to 10, corresponding to the percentage of foliar tissue exhibiting chlorosis or die-back (0= 0%, 5=50%, and 10=100%). After 38 days, all cone-tainers were destructively sampled to measure root necrosis, root depth, and root mass as described above. Isolations after the 4 wk heat exposure were performed by plating four washed root sections each on PARP and SV8 as described above. Growth chamber experiments were conducted twice.

All statistical analyses were performed using SAS (version 8.02; SAS Inc., Cary, NC). Analysis of variance was conducted using PROC GLM to estimate the effects of experiment and isolate on disease severity, area under the disease progress curve (AUDPC) (14), root mass (before and after heat exposure), and root depth (before and after heat exposure). Dunnett's t-test was used to compare each isolate to the uninoculated control. The Waller-Duncan *k* ratio *t* test (*k*=100) was used to separate means for comparison among *P. volutum* and *P. torulosum* isolates.

RESULTS

Morphological identification of *Pythium* species. Most of the *Pythium* isolates were obtained during the spring and fall of 2003 and 2004, but isolates from Mecklenburg Co. (location 13) and Moore Co. were collected in April 2005. Of the 75 *Pythium* isolates obtained, 59 were identified as *P. volutum* and 16 were identified as *P. torulosum* (Table 2.1). Isolations were not successful during the summer months and only yielded saprophytes such as *Curvularia, Alternaria, Fusarium, and Colletotrichum* species. *Pythium volutum* was the dominant species isolated at all locations except Guilford Co. and New Hanover Co. (Table 2.1). *Pythium torulosum* was isolated from six of the 14 golf courses sampled and was

only obtained when PARP was utilized as the isolation media (Table 2.1). *Pythium volutum* isolates were characterized by five to seven diclinous antheridia, inflated lobate sporangia, large oospores (28 to 31 µm), and a cottony colony morphology on SV8 (Table 2.2, Fig. 2.1). An antheridium that coiled around the oogonial stalk was observed in all *P. volutum* isolates (Fig. 2.1). *Pythium torulosum* isolates were characterized by single monoclinous antheridia, inflated lobate sporangia, small oospores (15 to 18 µm), and rosette colony morphology on SV8 (Table 2.2, Fig. 2.1).

Phylogenetic analysis using ribosomal DNA sequences. According to ITS sequence data, six *P. volutum* haplotypes and four *P. torulosum* haplotypes were identified in the sample population (Table 2.2). The majority of *P. volutum* isolates belonged to the PV4 and PV6 haploptypes and the majority of the *P. torulosum* isolates belonged to the PT1 and PT2 haplotypes (Table 2.2). All isolates from this study identified as *P. volutum* were placed in the same clade as the *P. volutum* ITS sequences obtained from GenBank (Fig. 2.2). Fifteen isolates identified as *P. torulosum* were placed in the same clade as the *P. torulosum* sequences obtained from GenBank (Fig. 2.2). Isolate PRD20 was identified as *P. torulosum* based on morphological characters, but this isolate was placed outside of the *P. torulosum* clade.

Impact of *Pythium* species on root growth and survival. There were no significant differences in treatments among experiments, so data presented is the average of the two experiments. Root and foliar symptoms did not develop during the 4 week infection period at 24/16°C, and root mass and root depth were not negatively impacted by inoculation with *P. volutum* or *P. torulosum* during this period (Fig. 2.3). Isolations from infected root tissue

consistently yielded the inoculated species. Isolations from co-inoculations of *P. volutum* and *P. torulosum* yielded only *P. torulosum*.

After 15 days of heat exposure, foliar symptoms developed and quickly reached a plateau in the *P. volutum* infested cone-tainers (Fig. 2.4). Foliar symptoms initially appeared as wilt then progressed to chlorosis followed by foliar decline. Beyond 15 days of heat exposure, foliar symptoms declined slightly then remained constant for the duration of the experiment (Fig. 2.4). All *P. volutum* isolates were pathogenic towards creeping bentgrass according to Dunnett's t-test ($P<0.05$), and no significant differences were detected among isolates of this species (Fig. 2.5). In contrast, the two *P. torulosum* isolates and the co-inoculation treatment did not induce foliar symptoms that were significantly different from the uninoculated control according to Dunnett's t- test ($P<0.05$) (Fig. 2.5).

During heat exposure, root depth and mass continued to increase in uninoculated cone-tainers (Fig. 2.3). According to Dunnett's t-test ($P<0.05$) however, *P. volutum* infections prevented continued root growth, and also caused a slight reduction in root depth and substantial reductions in root mass relative to the measurements taken prior to heat exposure (Fig. 2.3). Roots from cone-tainers infested with *P. volutum* had a light tan color, lacked root hairs, and had bulbous root tips. However, significant differences were not detected when root necrosis was visually estimated (data not shown). Four of the five *P. volutum* isolates did not significantly differ in their impact on root depth or root mass (Fig. 2.3). Creeping bentgrass root growth was not significantly inhibited at high temperatures by *P. torulosum* infections according to Dunnett's t-test ($P<0.05$) (Fig. 2.3). *Pythium volutum* was not isolated from root tissue after the 4 wk exposure to heat.

DISCUSSION

The root diseases that attack creeping bentgrass grown in sand-based golf course putting greens remain poorly understood. Since 2002, patch-like symptoms typically indicative of a root disease have been observed in creeping bentgrass across the Southeastern United States. Based on these stand symptoms, and the presence of mild necrosis of roots and stolons many of these cases were initially diagnosed as take-all patch by the Plant Disease and Insect Clinic at NC State and other diagnostic laboratories. Upon further investigation, however, *G. graminis* var. *avenae* could not be isolated from the affected turf. Further investigation revealed that at least two diseases were present: summer patch caused by *Magnaporthe poae* (16, 17) and an uncharacterized Pythium disease (18). We later determined that this unknown disease was Pythium root dysfunction caused by *P. volutum* (9). The present study demonstrates that this pathogen is widespread in North Carolina and is also present in VA, SC, and GA. Moreover, this study provides important details on the epidemiology of Pythium root dysfunction.

Pythium volutum was previously isolated from creeping in NC and MD and demonstrated to be pathogenic towards creeping bentgrass seedlings (1, 5). Yet, it was not isolated more than twice in each study and was not thought to be a widespread, important pathogen. *Pythium volutum* was isolated 59 times in our study, comprising 79% of the *Pythium* isolates obatined. Moreover, *P. volutum* was isolated from all but two locations, indicating that this species is widely distributed throughout the sampling area. Prior surveys of *Pythium* species associated with creeping bentgrass roots showed that *P. torulosum* was the most frequently isolated species (1, 5, 6, 12, 13). In contrast, we only isolated *P. torulosum* 16 times from 6 locations. However previous researchers conducted isolations during the summer, whereas we performed isolations during the fall and spring only. Similar

to previous studies (1, 5, 12), *P. torulosum* was non-pathogenic to creeping bentgrass roots in our growth chamber experiments.

Previous surveys of *Pythium* populations in turfgrass roots utilized selective media (PARP) containing the antibiotics ampicillin, rifampicin, pimaricin, and PCNB (1, 5, 12). *Pythium volutum* is documented as a slow growing species (5) and we have observed that its growth can be further limited by these antibiotics. In this experiment, we were successful in isolating *P. volutum* when roots were washed in running tap water then plated on SV8 or when samples were baited with creeping bentgrass seedlings. Future surveys of *Pythium* species associated with turfgrass roots should employ these methods in addition to using a selective media to avoid inadvertently selecting for or against certain species.

Pythium volutum was highly aggressive on creeping bentgrass seedlings in previous experiments at temperatures between 12 and 32°C (1, 5). Yet, in our experiments, no symptoms occurred on mature creeping bentgrass plants at 24/16°C. Root mass and depth were not adversely affected at cooler temperatures, but once inoculated plants were exposed to extended periods of heat, root growth and survival were severely reduced and foliar symptoms developed. This supports our observations that *P. volutum* infects creeping bentgrass roots in the fall, winter, and spring, causing the plants to be more susceptible to heat or drought stress during the summer. In contrast, Hodges and Coleman (7) and Hodges and Campbell (8) demonstrated that *P. arrhenomanes* and *P. aristosporum* reduced root and shoot mass of creeping bentgrass at 26/18°C. The epidemiology of PRD may vary depending on the *Pythium* species involved and the prevailing environmental conditions.

Our results demonstrate that *P.volutum* is a widespread pathogen causing PRD of creeping bentgrass in North Carolina and is also present in SC, VA, and GA. Root infections

that occur during cool weather in the fall, winter, and spring induce symptom expression during periods of heat or other stresses. The mechanisms by which symptoms are induced remain unknown. *Pythium volutum* infections reduced the growth and survival of creeping bentgrass roots during heat exposure, which may lead directly to the expression of symptoms. As Hodges and Coleman (7) speculated, root infections may also hinder water and nutrient uptake. Further research is needed to define the temperatures at which root infection and symptom expression occur so that management practices can be optimized for this important root disease. More research also is needed to determine the distribution of this pathogen in the Southeastern U.S.

REFERENCES:

1. Abad, Z.G., Shew, H.D., and Lucas, L.T. 1994. Characterization and pathogenicity of *Pythium* species isolated from turfgrass with symptoms of root and crown rot in North Carolina. Phytopathology 84:913-921.
2. Chamswarng, C., and Cook, R.J. 1985. Identification and comparative pathogenicity of *Pythium* species from wheat roots and wheat-field soils in the Pacific Northwest. Phytopathology 75: 821-827.
3. Dick, M. W. 1990. Keys to *Pythium*. College of Estate Management, Whiteknights, Reading, UK.
4. Elliott, M. L. 1993. Association of *Magnaporthe poae* with a patch disease of creeping bentgrass in Florida. Plant Dis. 77:429.
5. Feng, Y., and Dernoeden, P.H. 1999. *Pythium* species associated with root dysfunction of creeping bentgrass in Maryland. Plant Dis. 83: 516-520.

6. Hendrix, F.F., Campbell, W.A., and Moncrief, J.B. 1970. *Pythium* species associated with golf turfgrasses in the South and Southeast. Plant Dis. Rep. 54:419-421.

7. Hodges, C.F., and Coleman, L.W. 1985. *Pythium*-induced root dysfunction of secondary roots of *Agrostis palustris*. Plant Dis 69:336-340.

8. Hodges, C.F., and Campbell, D.A. 1994. Infection of adventitious roots of *Agrostis palustris* by *Pythium* species at different temperature regimes. Can. J. Bot. 72:378-383.

9. Kerns, J.P., and Tredway, L.P. 2007. First report of Pythium root dysfunction of creeping bentgrass caused by *Pythium volutum* in North Carolina. Plant Dis. 91:632.

10. Kumar, S. Tamura, K., and Nei, M. 1993. MEGA: Molecular Evolutionary Genetics Analysis Version 2.1. The Pennsylvania State University, University Park.

11. Martin, F. N. 1992. *Pythium*. Pages 39-49 in: "Methods for Research on Soilborne Phytopathogenic Fungi". L. L. Singleton et al., eds. The American Phytopathological Society, St. Paul, MN.

12. Nelson, E.B., and Craft, C.M. 1991. Identification and comparative pathogenicity of *Pythium* spp. from roots and crowns of turfgrasses exhibiting symptoms of root rot. Phytopathology 81:1529-1536.

13. Saladini, J.L., Schmitthenner, A.F., and Larsen, P.O. 1983. Prevalence of *Pythium* species associated with cottony-blighted and healthy turfgrasses in Ohio. Plant Dis. 67:517-519.

14. Shaner G., and Finney, R.E. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67: 1051-1056.

15. Smiley, R.W. 1993. Historical perspectives of research on ectotrophic root-infecting pathogens of turfgrasses Pages 1-17 in: Turfgrass Patch Diseases Caused by Ectotrophic Root-Infecting Fungi. B.B. Clarke and A.B. Gould, eds. American Phytopathological Society, St. Paul, MN.

16. Tredway, L.P. 2005. First report of summer patch of creeping bentgrass caused by *Magnaporthe poae* in North Carolina. Plant Dis. 89:204.

17. Tredway, L.P. 2006. Genetic relationships among *Magnaporthe poae* isolates from turfgrass hosts and relative susceptibility of 'Pencross' and 'Penn A-4' creeping bentgrass. Plant Dis. 90: 1531-1538.

18. Tredway, L.P., and Martin, S.B. 2003. Stalking new disease in creeping bentgrass. Carolinas Green. Nov-Dec:22-26.

19. Turgeon, A.J. 1999. *Turfgrass Management*, 5th edition. Prentice Hall, Upper Saddle River, NJ. Pp 49-108.

20. Van der Plaats-Niterink, A.J. 1981. Monograph of the Genus *Pythium*. Studies in Mycology, vol 21. Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

21. White, T.J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-324 in: PCR Protocols: A Guide to Methods and Applications, M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, eds. Academic Press, San Diego, CA.

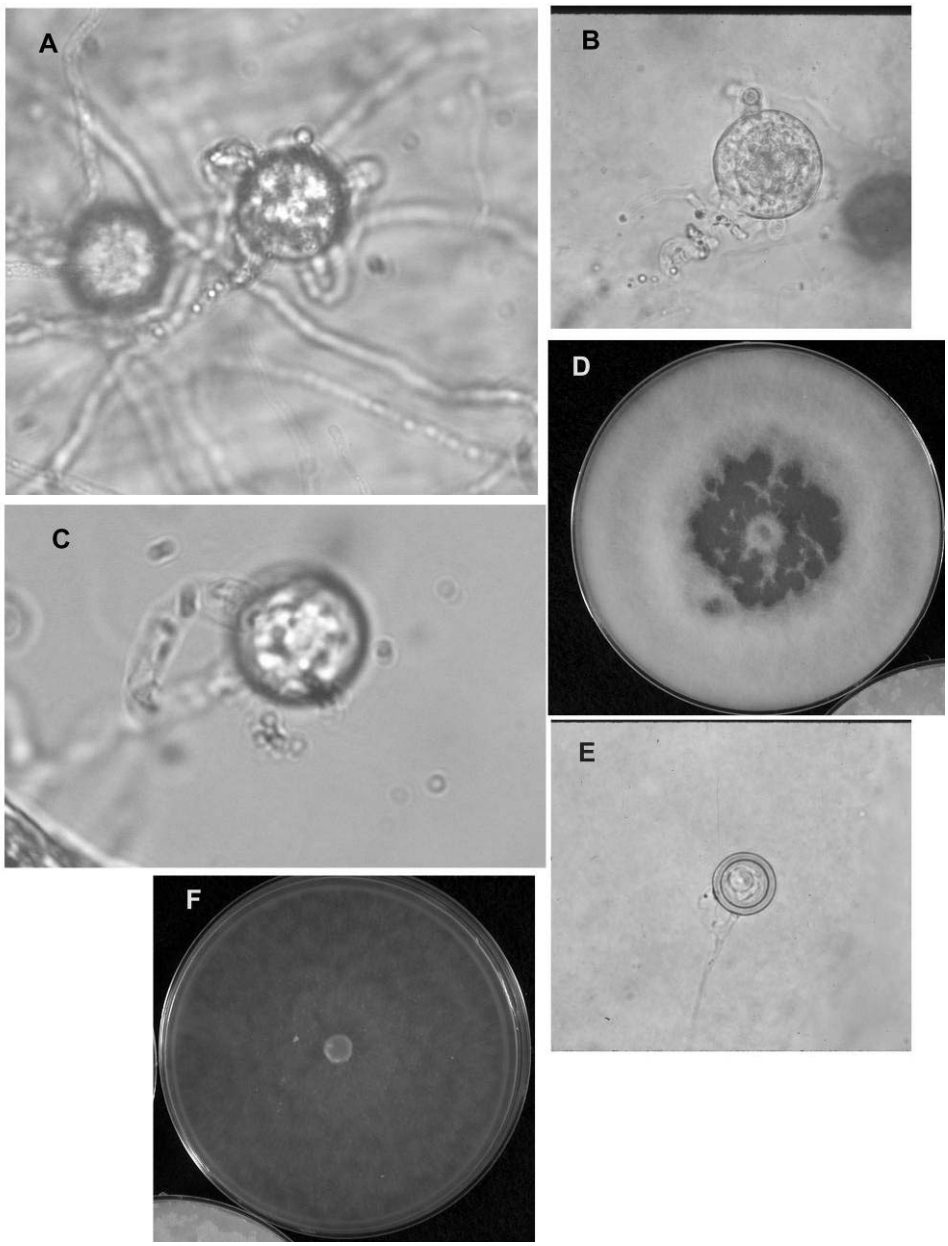

Fig. 2.1. Antheridial and oospore characteristics and colony morphology of *Pythium volutum* and *P. torulosum*. **A,** *P. volutum* oogonium with multiple diclinous antheridia. **B,** *P. volutum* oogonium with antheridium coiling around oogonial stalk. **C,** *P. torulosum* plerotic oospore. **D,** *P. volutum* colony morphology. **E,** *P. torulosum* oogonium with monoclinous antheridium. **F,** *P. torulosum* colony morphology.

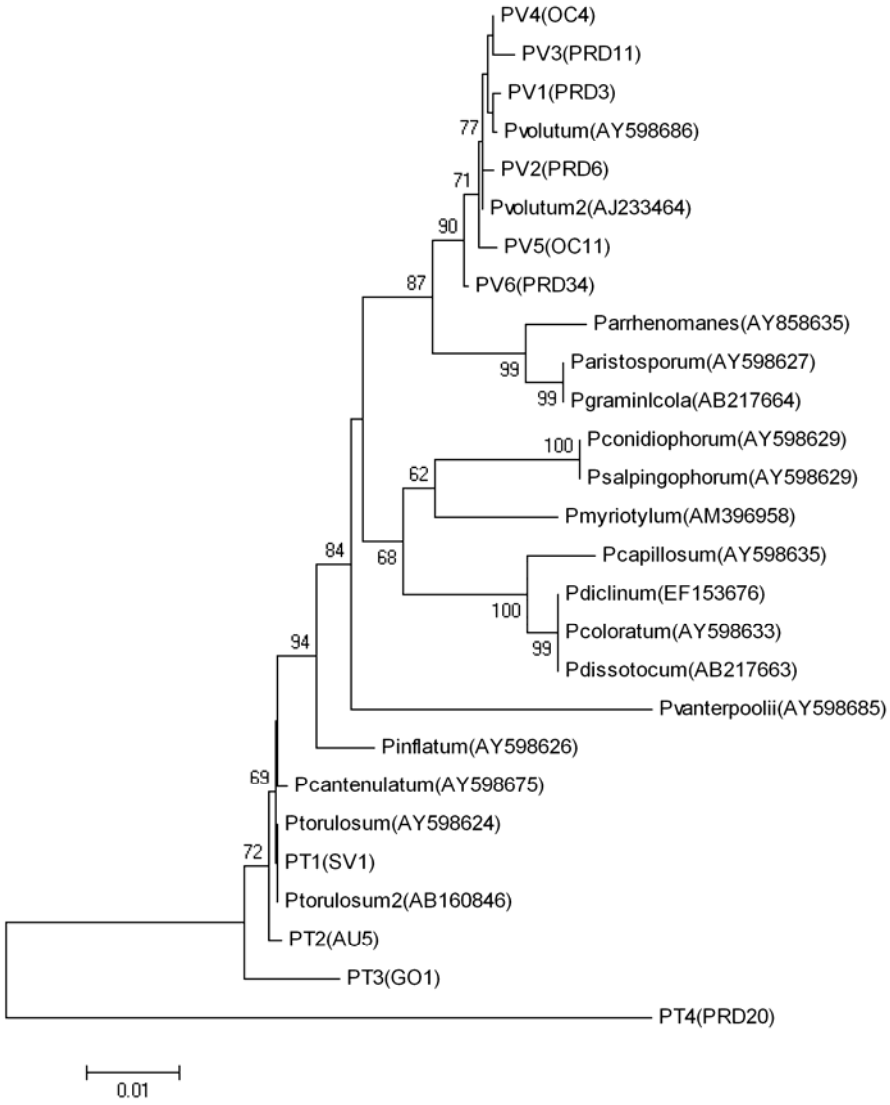

Fig. 2.2. Neighbor joining phylogram of *Pythium* species produced from sequences of rDNA regions ITS1, 5.8S, and ITS2. Scale bar indicates horizontal distance corresponding to genetic distance as calculated by the Kimura 2-parameter model. Bootstrap values are indicated adjacent to the nodes and are based on 1,000 resamplings of the data set. Species labeled PV1 to PV6 are *P. volutum*, while PT1 to PT4 are *P. torulosum*. GenBank species followed by two letters with six digit code.

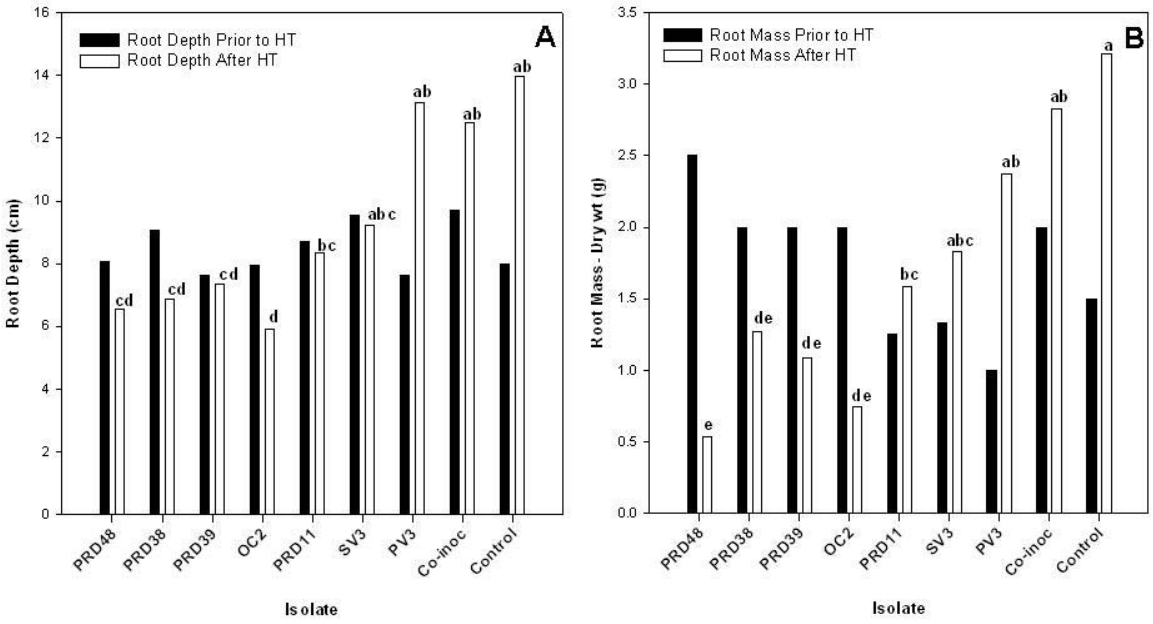

Fig. 2.3. Impact of *Pythium volutum* and *P. torulosum* on **A,** root depth and **B,** root mass of creeping bentgrass. White bars represent measurements prior to heat treatment and black bars represent measurements after 4 weeks of heat treatment. Isolates PRD38, PRD48, PRD39, OC2, and PRD11 are *P. volutum*, isolates SV3 and PV3 are *P. torulosum*, and 'co-inoc' represents a co-inoculation of OC2 and SV3. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio t-test (k=100).

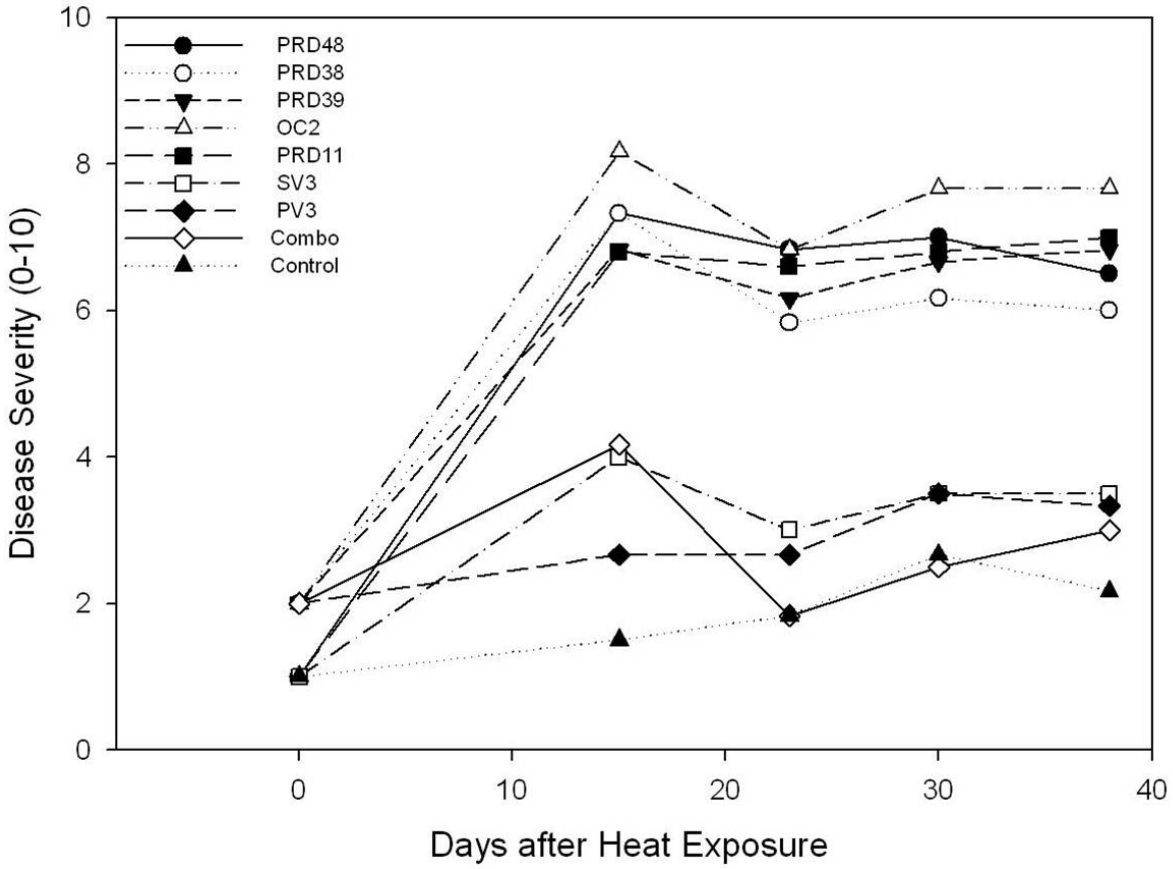

Fig. 2.4. Pathogenicity of five *Pythium volutum* isolates, two *P. torulosum isolates,* one co-inoculation, and an uninoculated control. Disease severity was evaluated on a scale of 0 to 100 (0= 0% and 10=100%) based on foliar tissue exhibiting decline. Isolates PRD38, PRD48, PRD39, OC2, and PRD11 are *P. volutum*, isolates SV3 and PV3 are *P. torulosum* and 'co-inoc' represents a co-inoculation of OC2 and SV3.

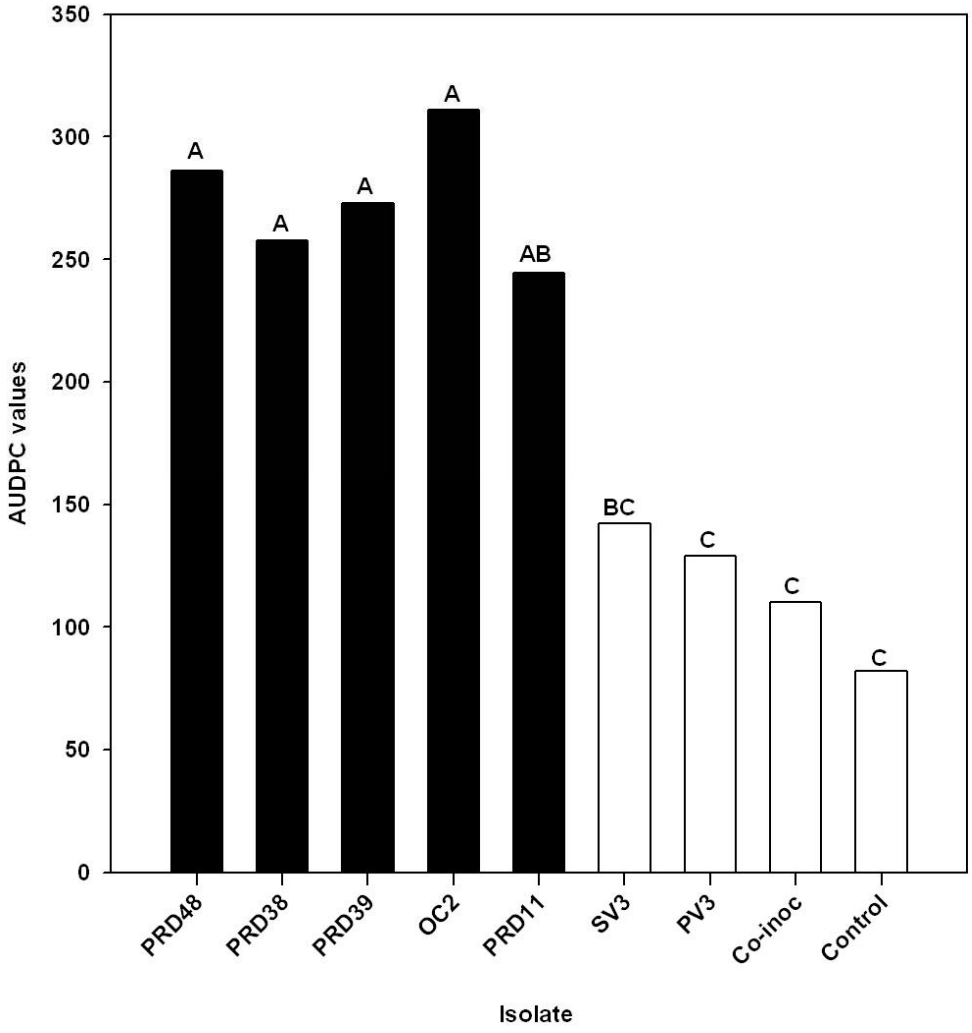

Fig. 2.5. Area under the disease progress curve (AUDPC) for five *Pythium volutum* isolates, two *P. torulosum isolates,* one co-inoculation, and an uninoculated control. AUDPC values were calculated from disease severity data collected weekly for 4 weeks. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio t-test (k=100). Isolates PRD38, PRD48, PRD39, OC2, and PRD11 are *P. volutum,* isolates SV3 and PV3 are *P. torulosum*, and 'co-inoc' represents a co-inoculation of OC2 and SV3.

Table 2.1. Origin of *Pythium* isolates, creeping bentgrass variety planted and year of putting green construction, isolation method and medium, number of isolates collected at each location, and the prevalence of *P. volutum* and *P. torulosum* at each location.

Golf Course Location (County, State)	Variety/Year Constructed	Isolation Method	Isolation Media	Isolates (no.)[z]	No. of *P. volutum*	No. of *P. torulosum*
1.Guilford Co, NC	Cato:Crenshaw / 1996	Direct plating	PARP	1	--	1
2. Orange Co, NC	A-1:A-4 / 2002	Direct plating	PARP	8	0	7
			SV8	9	9	0
3. Montgomery Co, NC	A-4 / 2002	Baiting	PARP w/ iprodione	8	4	3
4. New Hanover Co, NC	A-1:A-4 / 2003	Direct plating	PARP	1	--	1
5. Aiken Co, SC	A-1 / 2001	Direct plating	PARP	4	1	3
6. Chatham Co, NC	A-1:A-4 / 2001	Baiting	SV8	11	11	--
7. Mecklenburg Co, NC	A-1 / 2000	Direct plating	SV8	4	4	--
		Baiting	PARP	6	6	
8. Roanoke Co, VA	L-93 / 2000	Direct plating	PARP	8	8	--
9. Durham Co, NC	A-1:A-4 / 2001	Direct plating	PARP	3	2	--
10. Mecklenburg Co, NC	L-93 / 1996	Direct plating	PARP	1	1	--
11.Pender Co,NC	L-93 / 1999	Direct plating	PARP	1	1	--
12. Iredell Co, NC	L-93 / 2002	Direct plating	SV8	3	3	--
13. Mecklenburg Co, NC	G-2 / 2000	Direct plating	SV8	2	2	--
14. Moore Co, NC	G-2 / 1998	Direct plating	SV8	10	6	--
15. Richmond Co, GA	Unknown	Direct plating	PARP	3	1	1
			Totals	80	58	16

[z] Total number of *Pythium* isolates including isolates in addition to *P. volutum* and *P. torulosum.*

Table 2.2. Designation of ITS haplotype, type isolate, number of isolates per haplotype, and morphological characteristics of *Pythium volutum* and *P. torulosum*.

ITS Haplotype[x]	Type Isolate[y]	No. of Isolates in Haplotype	Arrangment of Antheridia	No. of Antheridia	Average diameter of oogonium (µm)[z]
PV1	PRD3	1	Diclinous	6	28.7 ± 2.1
PV2	PRD6	1	Diclinous	7	31.2 ± 1.7
PV3	PRD11	1	Diclinous	5	30.4 ± 1.2
PV4	OC4	40	Diclinous	7	28.1 ± 3.2
PV5	OC11	1	Diclinous	6	26.6 ± 1.1
PV6	PRD34	14	Diclinous	5	30.1 ± 2.6
PT1	SV1	8	Monoclinous	1	17.4 ± 1.1
PT2	AU5	6	Monoclinous	1	16.1 ± 1.7
PT3	GO1	1	Monoclinous	1	14.9 ± 1.9
PT4	PRD20	1	Monoclinous	1	18.2 ± 1.4

[x] PV= *Pythium volutum*; PT= *Pythium torulosum*

[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 haplotype.

CHAPTER 3 - INFLUENCE OF TEMPERATURE ON PATHOGENICITY OF *PYTHIUM VOLUTUM* TOWARD CREEPING BENTGRASS

ABSTRACT

Symptoms of Pythium root dysfunction (PRD) in creeping bentgrass are most common in the summer during periods of heat and drought stress. However, our observations indicate that *Pythium volutum*, a causal agent of Pythium root dysfunction, is most active during the fall and spring. Soil temperature thresholds for this pathogen must be determined so that preventative fungicide applications can be timed accurately. A mycelial growth assay was performed by incubating 11 *P. volutum* isolates at 10 °C, 12 °C, 14 °C, 18 °C, 22 °C, 24 °C, 26 °C, 28 °C, and 31 °C. To determine the optimal temperature range for infection by *P. volutum*, five isolates of *P. volutum* were used to inoculate mature 'A-1' creeping bentgrass plants. Inoculated plants were transferred to growth chambers at constant 12°C, 16°C, 20°C, 24°C, 28°C or 32°C (12 hr day/night cycles), then the temperature in all chambers was increased to 32°C/26°C day/night to induce foliar symptoms. Growth assays demonstrated that *P. volutum* grows best when temperatures are between 18°C and 26°C. Typical PRD foliar symptoms developed in the 12°C, 16 °C, 20 °C, and 24 °C treatments two weeks after the temperature in all growth chambers was elevated to 32°C/26°C day/night. Severity of PRD was greatest when *P. volutum* was incubated on creeping bentgrass roots at 16°C. Reductions in root depth and/or root mass were observed prior to raising the temperature to 32C/26C in the 12 °C,16°C, and 20 °C temperature treatments. Once temperature was elevated, extensive root dieback occurred in the 12°C, 16 °C, 20 °C, and 24 °C treatments. These results demonstrate that *P. volutum* is most active at temperatures prevalent during the

fall and spring in NC, supporting our hypothesis that the majority of root infection occurs during this time and that fungicides should be applied when soil temperatures are between 12°C and 24°C to achieve preventative control of PRD.

INTRODUCTION

Pythium root dysfunction was first described by Hodges and Coleman (5) in 1985. They observed that recently renovated creeping bentgrass putting greens with high sand content rootzones were rapidly dying during the summer. The affected roots were a light tan color, lacked root hairs, had bulbous root tips, and an abundance of coenocytic hyphae and oospores characteristic of a *Pythium* species was observed in the root cortex. The authors isolated *P. arrehenomanes* and *P. aristosporum* from the affected roots and showed that these species reduced shoot and root growth of mature creeping bentgrass plants. Yet, *P. arrehenomanes,* or *P. aristosporum* failed to kill creeping bentgrass or produce noticeable necrosis of roots. The authors named the disease Pythium root dysfunction (PRD) and hypothesized that infection by these two *Pythium* species impaired water and nutrient uptake. Feng and Dernoeden (4) isolated 8 *Pythium* species from roots displaying symptoms characteristic of PRD in 1999. They concluded that *P. aristosporum* was the most important species responsible for PRD in the Mid-Atlantic region of the U.S.

Symptoms characteristic of PRD have also been observed in North Carolina since 2002. From a collection of 75 *Pythium* isolates, 59 were identified as *P. volutum* and the remaining 16 were identified as *P. torulosum* (7). *Pythium volutum* was highly virulent towards roots of mature creeping bentgrass plants, whereas *P. torulosum* was non-pathogenic. In these growth chamber studies, *P. volutum* did not induce foliar symptoms or negatively impact root depth or root mass during an initial four week infection period when

temperatures cycled between 24°C during the day and 16°C at night. However, severe foliar symptoms developed after two weeks of heat exposure at 32°C/26°C (day/night). After 4 weeks of heat exposure, creeping bentgrass root depth and root mass was severely limited when compared to non-inoculated controls.

Prior to our discovery of *P. volutum* as a causal agent of PRD in North Carolina, this species was isolated infrequently from turfgrasses exhibiting root rot or root dysfunction (1, 4). Abad et al. (1) demonstrated that *P. volutum* was highly aggressive towards creeping bentgrass at 28°C or 32°C, whereas Feng and Dernoeden (4) observed that *P. volutum* was more virulent at 18°C than at 28°C. Feng and Dernoeden (4) also examined in vitro growth rates across a range of temperatures, and found that *P. volutum* growth was similar at 18°C and 28°C. It should be noted that both prior studies used seedlings of creeping bentgrass for inoculations. While this is informative pathogenicity information, the impact of temperature on root infection by *P. volutum's* remains unknown.

The epidemiology of PRD remains unclear, which hinders our ability to make sound management recommendations. In North Carolina, symptoms are most prevalent during the summer when creeping bentgrass is exposed to heat and drought stress. Nevertheless, the majority of pathogen growth and oospore production in creeping bentgrass roots occurs during the fall and spring when soil temperatures are much cooler. Hodges and Campbell (6) showed that species of *Pythium* induced significant reductions in root and shoot growth at temperatures of 24°C during the day and 13°C at night. However, no visible symptoms resulted and they speculated that exposure to heat for extended periods induced rapid root and foliar decline of creeping bentgrass. Based on our observations and those of Hodges and Campbell (6), we hypothesized that *P. volutum* infects creeping bentgrass roots during the

fall and spring, and that foliar symptoms develop only after creeping bentgrass is subjected to heat and drought stress. Determining soil temperature thresholds for pathogen activity will allow for better timing of preventative fungicide applications.

The objectives of this study were to (i) evaluate the influence of temperature on *P. volutum* mycelial growth *in vitro*; (ii) determine the influence of soil temperature on pathogenicity of *Pythium volutum* towards mature creeping bentgrass plants; (iii) evaluate the impact of *Pythium volutum* infections on creeping bentgrass root growth and survival in response to heat stress.

MATERIALS AND METHODS

Growth of *P. volutum* and *P. torulosum* in response to temperature. Radial growth rate of 10 *P. volutum* isolates and three *P. torulosum* isolates were determined using a mycelial growth assay. An agar plug (3 mm) was removed from the edge of a 3-day-old clarified V8 juice (SV8) (9) colony and placed in the center of a Petri dish (100 x 15 mm) containing SV8. Three replicate plates for each isolate were randomized in separate, dark incubators at 10, 12, 14, 18, 22, 24, 26, 28, or 31^{o}C. Colony diameters were measured in two opposing directions after two days of incubation using a digital caliper. Growth per day was calculated by averaging the two diameter measurements, subtracting the diameter of the agar plug from the colony diameter value, and then dividing the value by two. Each incubation temperature was replicated four times over time.

The data were analyzed as a randomized complete block, split-plot design with four replications. Incubators were considered blocks, temperatures were considered the main units, and the isolates were considered subunits. Growth rate data were analyzed using

PROC GLM in SAS, and means among *Pythium* isolates and incubation temperature were separated by the Waller-Duncan *k* ratio *t* test (*k*=100) (SAS Institute Inc. Cary, NC).

Infection of creeping bentgrass roots by *Pythium volutum* in response to soil temperature. Cone-tainers (3.8 cm x 20 cm) containing sand meeting USGA specifications (14) (BB 205, Golf Agronomics Inc., Rockingham, NC) were seeded with 'A-1' creeping bentgrass (9.7 g m^{-1}). The cone-tainers were placed in a greenhouse at 26°C/22°C (12-h day/night cycles) and misted twice daily to encourage rapid germination. Following germination, the turf was maintained in the greenhouse by irrigating twice daily with a complete nutrient solution containing 106.23 mol m^{-3} nitrogen, 10.41 mol m^{-3} phosphorus, and 111.03 mol m^{-3} potassium for four weeks. After four weeks, the turf was irrigated once daily with the complete nutrient solution mentioned above. The turf was trimmed weekly with scissors to a height of 1.27 cm.

Six weeks after seeding, each cone-tainer was infested with sterilized creeping bentgrass leaves infested with one of five *Pythium volutum* isolates (PRD48, PRD38, PRD39, OC2, and PRD11). The inoculum was prepared by placing three 3-mm *Pythium* colonized agar plugs into sterile water containing five to seven sterilized 1.5-cm long creeping bentgrass leaves. The inoculum was incubated under continuous fluorescent light at room temperature (23 to 25^{o}C) for 3 days (1, 9). Inoculations were performed by cutting the root system at a 5-cm depth, the turf plug was removed from the cone-tainer, the sand in the cone-tainer was discarded and replaced with fresh sand, 5 to 7 *Pythium*-colonized grass blades were placed onto the surface of the fresh sand, and then the turf plug was placed on top of the colonized grass blades. A non-inoculated control was included in each experiment by cutting the roots at 5 cm then repotting onto fresh, uninfested sand.

Infested cone-tainers were transferred to growth chambers and arranged in a completely random design with 10 replications per isolate. Initial growth chamber conditions were 12-h day/night cycles at 12, 16, 20, 24, 28, or 32^{o}C. Each infection temperature treatment (initial temp.) was replicated four times over time. After four weeks, four of the 10 replications were destructively sampled to measure root necrosis, root depth, and root mass. Root necrosis was estimated visually on a scale of 0 to 10 (0=no necrosis 10=100% root necrosis). Root depth was assessed by removing the creeping bentgrass plug from the cone-tainer and measuring the distance from the soil surface to the deepest root tip. The sand was then thoroughly washed from the root system by gently agitating the roots in deionized water. Dry root weights were recorded after drying at 60°C for 72 hr.

Impact of heat treatment on root growth and survival. The remaining six replicates remained in the growth chamber, in which the temperature was raised to 32°C/26°C (day/night) to induce foliar symptoms. Percentage of foliar tissue exhibiting chlorosis or die-back was assessed at 0, 12, and 24 days after heat exposure. After 24 days, all cone-tainers were destructively sampled to measure root necrosis, root depth, and root mass as described above.

All statistical analyses were performed using SAS (version 8.02; SAS Inc., Cary, NC). Analysis of variance was conducted using PROC GLM to estimate the effects of experiment and isolate on disease severity, area under the disease progress curve (AUDPC) (11), root necrosis, root mass (before and after heat exposure), and root depth (before and after heat exposure). Dunnett's t-test was used to compare infested pots to the non-infested pots for all the dependant variables mentioned above. The Waller-Duncan *k* ratio *t* test (*k*=100) was used

to separate means for comparison among *P. volutum* isolates and infection temperature treatments.

RESULTS

Growth of *P. volutum* and *P. torulosum* in response to temperature. Isolates of *P. volutum* and *P. torulosum* were analyzed separately due to inherent differences among growth rates. A significant interaction between temperature and *P. volutum* isolate was detected, therefore each temperature was analyzed separately. Growth among the *P. volutum* isolates were similar at 28°C and 32°C (Table 3.1). In general, OC4 grew the fastest among the *P. volutum* isolates tested and PRD48 had the slowest growth (Table 3.1). The average growth of all *P. volutum* isolates was highest at 18 °C, 22 °C, and 26°C. Mycelial growth was inhibited in all but one *P. volutum* isolate at 31°C (Table 3.1). Mycelial growth of *P. torulosum* isolates was highest 18 °C, 22 °C, 24 °C, and 26°C and lowest at 10°C (Table 3.1). *Pythium torulosum* isolates grew at similarly at 10 °C, 12 °C, and 26°C.

Infection of creeping bentgrass roots by *Pythium volutum* in response to soil temperature. Foliar symptoms were not observed during the four week infection period at any temperature regime. According to Dunnett's t-test ($P<0.05$), *P. volutum* infections negatively impacted root depth at the 16°C and 20°C infection temperatures four weeks after inoculation and prior to heat exposure (Table 3.2). Creeping bentgrass root mass was significantly less in inoculated cone-tainers at the 12°C and 16°C infection temperatures (Table 3.2). Significant differences in root necrosis were not detected prior to heat treatment (data not shown).

Impact of heat treatment on root growth and survival. After 12 days of heat treatment at 32 °C /26 °C, typical PRD foliar symptoms developed in the 12 °C, 16 °C, 20 °C,

and 24°C infection temperature treatments (Fig. 3.1). Foliar symptoms initially appeared as wilt or chlorosis, but then progressed to foliar decline 24 d after exposure to heat. Severity of PRD was greatest when infections occurred at 16°C, followed by 20°C, 24°C, and 12°C, respectively (Fig. 3.3). After 24 days of heat treatment, *P. volutum* did not induce significant foliar symptoms in the 28°C or 32°C infection temperature treatments. All *P. volutum* isolates were pathogenic towards creeping bentgrass according to Dunnett's t-test ($P<0.05$), and no significant differences were detected among the isolates (Fig. 3.2), with no significant interaction between isolates and infection temperatures.

After 24 days of heat treatment, root depth and root mass were significantly reduced in the 16°C, 20°C, and 24°C infection temperature treatments according to Dunnett's t-test (Table 3.2). Root depth was reduced in the 12°C infection temperature treatment, but root mass was unaffected. Root depth and mass measurements were similar among the non-inoculated and inoculated pots at 28°C and 32°C infection temperatures (Table 3.2). Roots from inoculated cone-tainers had a yellowish tint, were devoid of root hairs, and had inflated root tips. However, differences among *P. volutum* isolates or infection temperature treatments could not be detected from visual evaluations of root necrosis (data not shown). The greatest reductions in root depth were observed in the 16°C and 20°C treatments, followed by the 24°C and 12°C treatments (Fig. 3.4). Reductions in root mass were similar in the 12°C, 16°C, 20°C, and 24°C infection temperature treatments, yet root mass was significantly lower at these infection temperature treatments when compared to the 28°C and 32°C treatments (Fig. 3.4). On average, the non-inoculated controls exhibited 45 to 55 % reductions in root depth when exposed to the high exposure heat when compared to root

depths measured prior to heat exposure (Table 3.2). Conversely, inoculated plants displayed 60 to 73 % reductions in root depth on average when exposed to heat (Table 3.2).

DISCUSSION

Prior to this work, the epidemiology of PRD was poorly understood. In North Carolina, symptoms of PRD develop in the summer months when creeping bentgrass is subjected to heat and drought stress. However, oospores characteristic of *P. volutum* are rarely observed in affected root tissue during the summer. The majority of *P. volutum* hyphal and oospore production in creeping bentgrass roots is observed during the fall and spring when soil temperatures are cooler (7). This work presents evidence that *P. volutum* does indeed infect creeping bentgrass roots when soil temperatures are between 12^{o}C and 24^{o}C without inducing foliar symptoms. When cone-tainers inoculated with *P. volutum* were initially incubated at 28^{o}C or 32^{o}C, foliar symptoms did not develop after an additional 4 weeks of incubation at 32^{o}C, and root depth or mass were not negatively impacted. Therefore, we conclude that root infection does not occur when soils temperatures are 28^{o}C or greater. In order for preventative fungicide applications to be most effective, they should be applied when soil temperatures are between 12^{o}C and 24^{o}C.

In previous studies, inconsistencies are noted in the affect of temperature on aggressiveness of *P. volutum*. For example, Abad et al. (1) demonstrated that *P. volutum* was more virulent at 28^{o}C and 32^{o}C than at 16^{o}C. However, Feng and Dernoeden (4) showed that *P. volutum* was more virulent at 18^{o}C than 28^{o}C. In our work, *P. volutum* reduced root growth when soil temperatures were between 12^{o}C and 20^{o}C, which is similar to what Feng and Dernoeden (4) reported. Previous studies have demonstrated that pathogenicity responses among the same *Pythium* species can be isolate specific (10), which may explain the

differences observed among experiments. Differences in soil medium may also be responsible for these inconsistencies; Abad et al. (1) used a sand-peat mixture as a soil medium, Feng and Dernoeden (4) used a top-dressing mix (94% sand 6% clay), and we utilized a USGA specification sand. Furthermore, Abad et al. (1) and Feng and Dernoeden's (4) experiments used a postemergence seedling assay to evaluate the effects of temperature on virulence of *Pythium* species. While useful as an initial screen of pathogenicity, these assays are not representative of Pythium root rot and PRD as they are observed in the field. Therefore, the effects of temperature on the virulence of *Pythium* species may be dependant on the specific isolate collected, the age of plant material, and the type of soil media utilized for pathogenicity assays. Future pathogenicity assays with *Pythium* species associated with turfgrass should be conducted to mimic the disease as it is observed in nature.

Hodges and Campbell (6) recognized the importance of using plants of the appropriate age in order to make inferences on the epidemiology of *Pythium* diseases. They determined that various *Pythium* species reduced shoot and root growth under cooler temperatures (24^{o}C day/13^{o}C night), however, foliar decline was not observed. Similarly, our results showed that *P. volutum* infections reduced root mass and root depth when soil temperatures were between 12^{o}C and 20^{o}C. Yet, reductions in root growth did not induce foliar symptoms until creeping bentgrass was exposed to an extended heat treatment. Reductions in root mass and root depth as a result of *P. volutum* infection facilitates increased decline of creeping bentgrass roots during heat stress. This may be the mechanism that is responsible for the initiation of foliar symptoms during summer. However, more research is needed to determine if other physiological processes are impaired by infection of creeping bentgrass roots by *P. volutum*.

Our *P. volutum* isolates grew the best when incubated at 18°C, 22°C, and 26°C. Therefore the optimal temperature for growth of this pathogen is between 18°C and 26°C. This is more consistent with previous reports of *P. volutum* growth as influence by temperature (2, 3, 4, 8, 10, 13). The results of our *in vitro* growth assay corroborate our findings in the infection temperature experiment. Since *P. volutum* grows optimally between 18°C and 26°C, it is not surprising that this pathogen infects creeping bentgrass roots when soil temperatures are cooler. Furthermore, *P. volutum* growth was limited or inhibited at 28°C and 32°C, which also supports our conclusion that *P. volutum* is not actively growing or infecting creeping bentgrass roots during the summer months. Root infections during the fall, winter, or spring may reduce creeping bentgrass root depth and mass, which facilitates root dieback during the summer that lead to the production of PRD symptoms. The impact of *P. volutum* infection on creeping bentgrass nitrate and water uptake remains unknown however, and more research is needed to determine how *P. volutum* affects creeping bentgrass root growth and physiology.

REFERENCES:

1. Abad, Z.G., Shew, H.D., and Lucas, L.T. 1994. Characterization and pathogenicity of *Pythium* species isolated from turfgrass with symptoms of root and crown rot in North Carolina. Phytopathology 84:913-921.

2. Chamswarng, C., and Cook, R.J. 1985. Identification and comparative pathogenicity of *Pythium* species from wheat roots and wheat-field soils in the Pacific Northwest. Phytopathology 75: 821-827.

3. Dick, M. W. 1990. Keys to *Pythium*. College of Estate Management, Whiteknights, Reading, UK.

4. Feng, Y., and Dernoeden, P.H. 1999. *Pythium* species associated with root dysfunction of creeping bentgrass in Maryland. Plant Dis. 83: 516-520.

5. Hodges, C.F., and Coleman, L.W. 1985. *Pythium*-induced root dysfunction of secondary roots of *Agrostis palustris*. Plant Dis 69:336-340.

6. Hodges, C.F., and Campbell, D.A. 1994. Infection of adventitious roots of *Agrostis palustris* by *Pythium* species at different temperature regimes. Can. J. Bot. 72:378-383.

7. Kerns, J.P., and Tredway, L.P. 2008. Pathogenicity of *Pythium* species associated with Pythium root dysfunction of creeping bentgrass and their impact on root growth and survival. Plant Dis. *Accepted*.

8. Levesque, C.A., and De Cock, W.A.M. 2004. Molecular phylogeny and taxonomy of the genus Pythium. Mycol. Res. 108 (12): 1363–1383.

9. Martin, F. N. 1992. *Pythium*. Pages 39-49 in: "Methods for Research on Soilborne Phytopathogenic Fungi". L. L. Singleton et al., eds. The American Phytopathological Society, St. Paul, MN.

10. Martin, F. N. and Loper, J.E. 1999. Soilborne plant diseases caused by *Pythium* spp: ecology, epidemiology, and prospects for biological control. Critical Rev. Plant Sci. 18(2): 111-181.

11. Shaner G., and Finney, R.E. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67: 1051-1056.

12. Tredway, L.P. and Butler, E.L. 2004. Late curative control of an unknown root disease of creeping bentgrass putting greens. www.turffiles.com.

13. Tredway, L.P. and Butler E.L. 2004. Preventative control of an unknown root disease of creeping bentgrass putting greens. www.turffiles.com.

14. Turgeon, A.J. 1999. *Turfgrass Management*, 5th edition. Prentice Hall, Upper Saddle River, NJ. Pp 49-108.

15. Van der Plaats-Niterink, A.J. 1981. Monograph of the Genus *Pythium*. Studies in Mycology, vol 21. Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

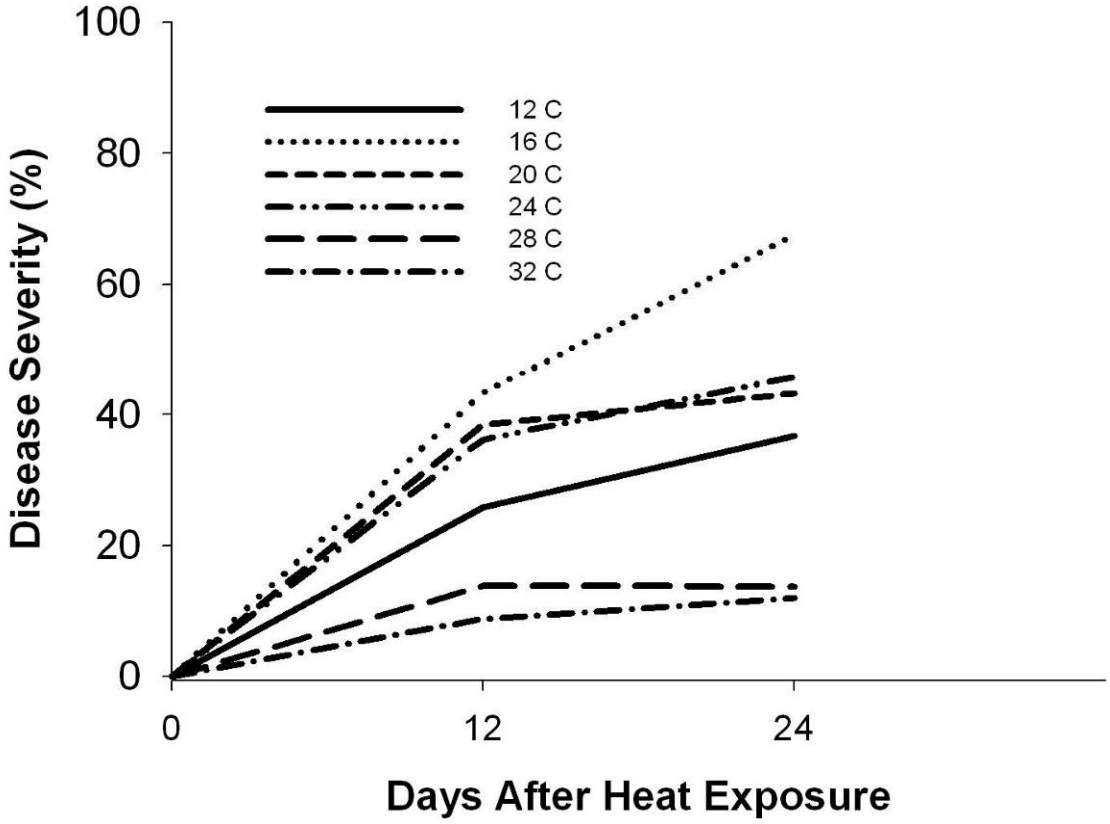

Fig. 3.1. Progression of PRD symptoms in response to infection temperature treatments ranging from 12^{o}C to 32^{o}C, after exposing plants to a high temperature treatment of 32^{o}C/26^{o}C (day/night). Disease severity was evaluated from 0 to 100 % based on foliar tissue exhibiting decline.

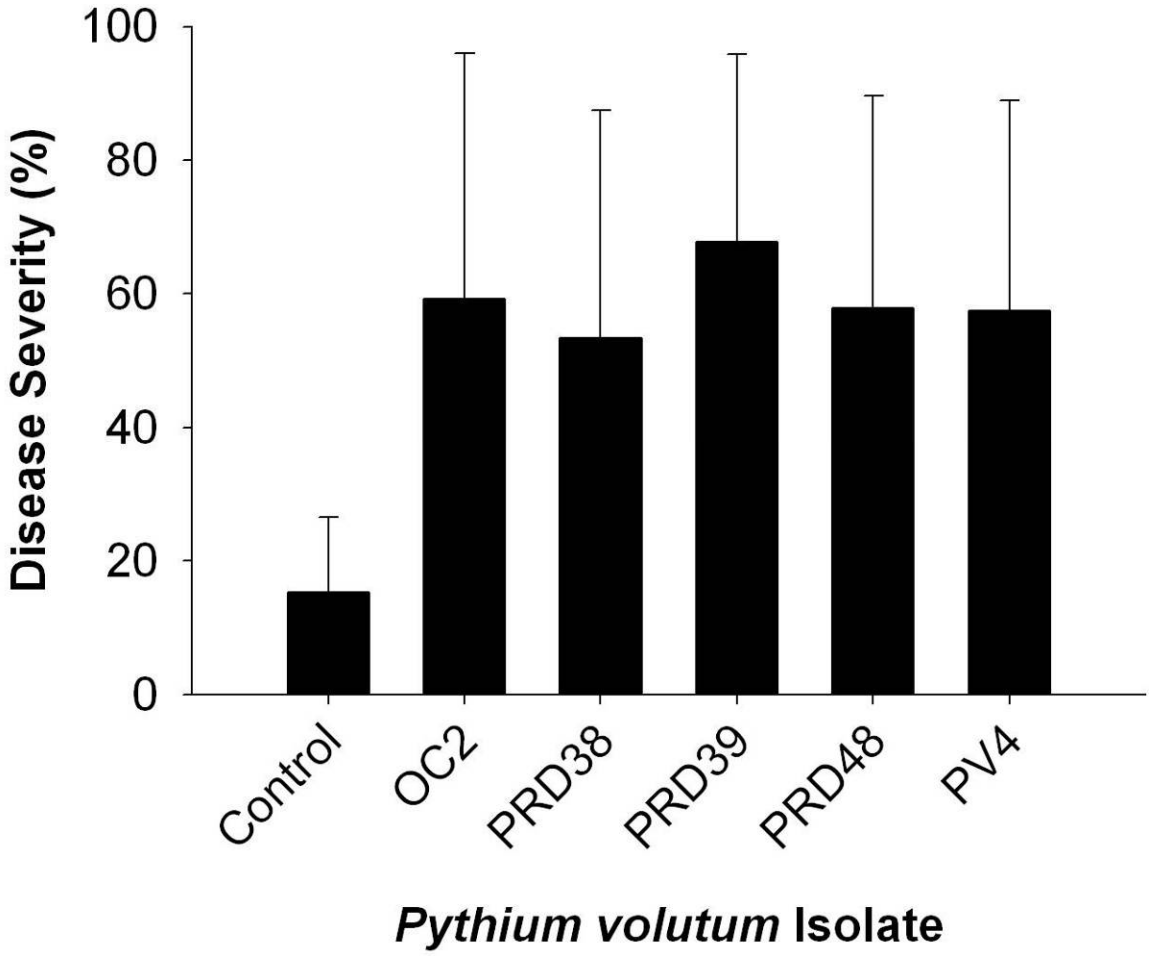

Fig. 3.2. Aggressiveness of five *Pythium volutum* isolates in response to heat treatment. Creeping bentgrass plants were initially exposed to temperatures ranging from 12°C to 32°C, then were exposed to four weeks of heat treatment at 32°C/26°C (day/night). Disease severity was evaluated from 0 to 100 % based on foliar tissue exhibiting decline. Aggressiveness of isolates did not differ among the isolates of *P. volutum* in response to temperature; therefore data represents virulence averages across all infection temperature treatments.

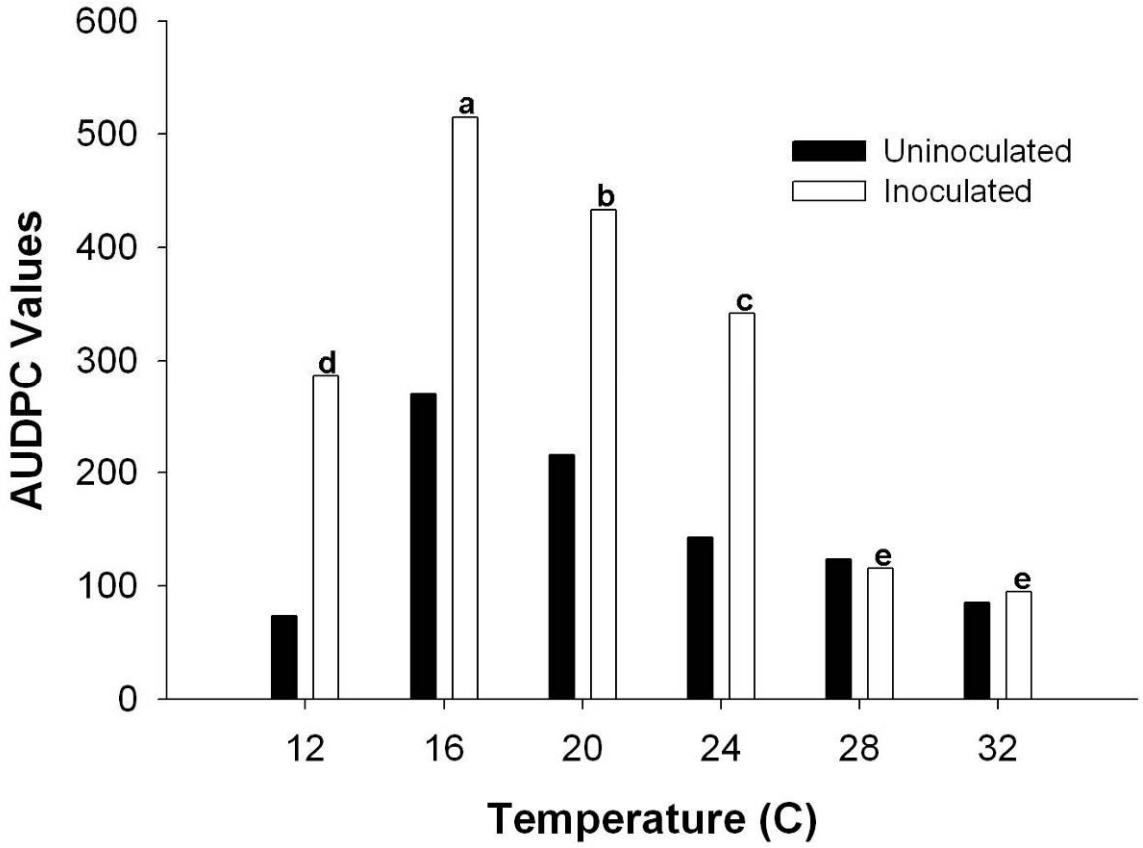

Fig. 3.3. Impact of temperature during infection on Pythium root dysfunction severity in response to heat treatment. Creeping bentgrass plants were initially exposed to temperatures ranging from 12°C to 32°C, then were exposed to four weeks of heat treatment at 32°C/26°C (day/night). Area under the disease progress curve (AUDPC) values were calculated from disease severity data collected weekly for 3 weeks. Black bars represent non-inoculated controls and white bars represent inoculated plants. Bars followed by the same letter are significantly different according to Waller-Duncan k-ratio t-test (k=100).

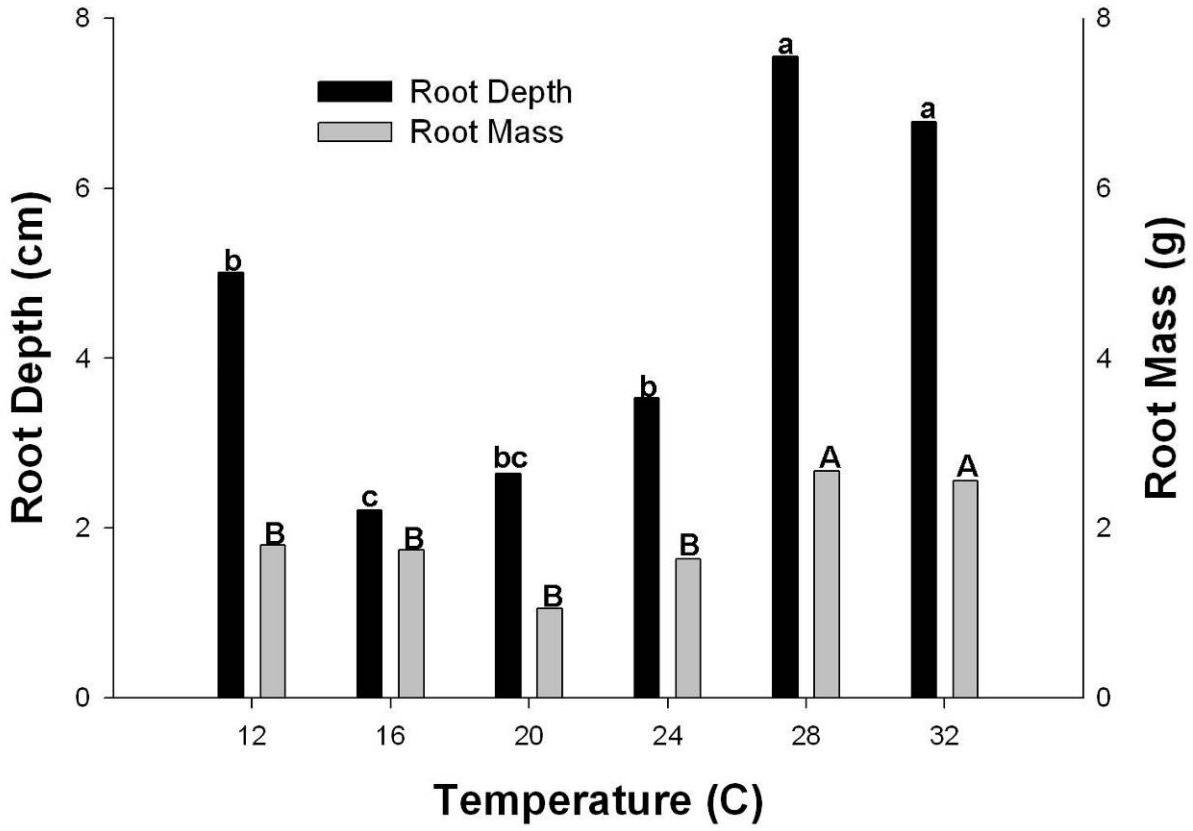

Fig. 3.4. Impact of *Pythium volutum* infections on root depth and root mass of creeping bentgrass in response to heat treatment. Black bars represent root depth measurements after 4 weeks of exposure to 32°C/26°C (day/night). Grey bars represent root mass measurements after 4 weeks of exposure to 32°C/26°C (day/night). Bars with the same letter are not significantly different according to Waller-Duncan k-ratio t-test (k=100). Lower case letter separate means for root depth and capital letters separate means for root mass.

Table 3.1. Mycelial growth for *Pythium volutum* and *P. torulosum* isolates in response to temperature.

	Mycelial Growth (mm d^{-1})[a]								
***P. volutum* Isolate**[b]	**10 °C**	**12 °C**	**14 °C**	**18 °C**	**22 °C**	**24 °C**	**26 °C**	**28 °C**	**31 °C**
PRD22	9.7 abc[c]	12.5 cde	17.3 c	25.3 d	24.5 d	16.5 c	21.2 d	9.3	0
PRD38	6.7 c	10.0 f	14.4 g	21.6 g	23.7 f	13.6 i	22.6 cd	10.4	0
PRD39	10.8 ab	15.6 a	16.2 e	23.6 e	23.2 g	13.9 g	27.1 a	12.0	0
PRD48	8.2 bc	12.1 de	12.2 i	20.4 j	21.8 j	13.4 j	21.0 d	12.0	0
OC1	12.2 a	15.2 ab	10.5 j	21.5 h	23.2 h	13.7 h	26.6 ab	9.8	0
OC2	10.9 ab	14.8 ab	19.0 b	27.2 b	26.1 b	16.8 b	26.8 a	14.9	3.1
OC4	10 abc	12.9 cde	19.5 a	28.2 a	27.8 a	17.3 a	24.6 bc	13.1	0
OC6	10.5 ab	13.9 bc	14.0 h	21.4 i	24.2 e	16.3 d	24.0 c	8.9	0
OC9	10.2 ab	13.7 bcd	16.6 d	25.0 c	25.7 c	15.0 e	24.2 c	13.1	0
SV8	8.7 abc	11.5 ef	14.4 f	21.9 f	22.9 i	14.5 f	26.1 cd	10.3	0
Average	9.9 F[d]	13.6 D	14.9 C	23.1 B	24.1 AB	14.9 C	24.8 A	11.5 E	0.3 G
***P. torulosum* Isolate**	**10 °C**	**12 °C**	**14 °C**	**18 °C**	**22 °C**	**24 °C**	**26 °C**	**28 °C**	**31 °C**
PRD11	10.0	13.0	19.8 a	29.0 b	31.3 a	34.8 b	29.5	23.7 b	17.3 b
PRD12	10.3	13.0	16.8 b	22.1 c	24.6 b	15.5 c	30.1	24.5 b	23.8 a
SV1	10.2	13.3	19.5 a	31.3 a	31.7 a	36.3 a	30.5	26.3 a	22.9 a
Average	10.2 G	13.1 F	18.7 E	27 B	29.2 AB	28.8 AB	30.0 A	24.9 C	21.3 D

[a] Growth per day was calculated by averaging the two diameter measurements, subtracting the diameter of the agar plug from the colony diameter value, and then dividing the value by two.
[b] Isolates were grown on clarified V8 juice agar
[c]Growth rate measurements followed by the same lowercase letter in the same column were not significantly different at P = 0.05 according to the Waller-Duncan k-ratio t-test (k=100).
[d]Growth rate measurements followed by the same capital letter in the same row were not significantly different at P = 0.05 according to the Waller-Duncan k-ratio t-test (k=100).

Table 3.2. Impact of infection temperature treatments on creeping bentgrass root depth and root mass pre- and post-heat treatment.

	12°C		16°C		20°C		24°C		28°C		32°C	
	Pre[b]	Post[c]	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
	----------Root Depth (cm)----------											
Non-inoculated	12.0	6.1	12.0	5.9	12.2	5.6	10.5	6.5	10.6	7.9	9.7	7.3
Inoculated[d]	13.4	5.0*	6.2*	2.2*	9.0*	2.6*	10.6	3.5*	11.9	7.6	10.4	6.8
	----------Root Mass (g)----------											
Non-inoculated	2.4	2.0	2.3	2.3	2.2	1.3	1.3	2.6	1.3	2.7	1.6	2.5
Inoculated	1.6*	1.8	1.5*	1.7*	2.0	1.1*	1.7	1.6*	1.8	2.3	1.6	2.2

[a] Treatments were initiated immediately after inoculation and lasted for four weeks.
[b] Root depth and root mass measured prior to exposure to 32°C /26°C heat treatment.
[c] Root depth and root mass measured four weeks after exposure to 32 °C /26°C heat treatment.
[d] Represents all five *P. volutum* isolates.
* Denotes a significant difference from the non-inoculated control within a column based on Dunnett's t-test.

CHAPTER 4 - PREVENTIVE CONTROL OF PYTHIUM ROOT DYSFUNCTION IN CREEPING BENTGRASS PUTTING GREENS AND SENSITIVITY OF *PYTHIUM VOLUTUM* TO FUNGICIDES

ABSTRACT

Pythium root dysfunction, caused by *Pythium volutum*, has been observed on golf course putting greens established with creeping bentgrass in the Southeastern U.S. since 2002. To evaluate preventative strategies for management of this disease, a three-year field experiment was conducted in Pinehurst, NC on a 'G-2' creeping bentgrass putting green. Fungicide treatments were applied twice in the fall (Sept and Oct) and three times in the spring (Mar, Apr, and May) in each of the three years. Applications of pyraclostrobin provided superior preventative control compared to other fungicides tested. Azoxystrobin and cyazofamid provided moderate control in two of three seasons. Experiments were conducted to determine the mechanism of disease suppression by pyraclostrobin. *In vitro* sensitivity to pyraclostrobin, azoxystrobin, and mefenoxam was determined for 11 *P. volutum* isolates, 3 *P. torulosum* isolates, and one *P. aphanidermatum* isolate. Isolates of *P. volutum* were most sensitive to pyraclostrobin (EC_{50} value = 0.0049) and cyazofamid (EC_{50} = 0.0041) followed by azoxystrobin (EC_{50} = 0.05) and mefenoxam (EC_{50} = 0.139). Applications of pyraclostrobin did not affect the foliar growth rate or visual quality of creeping bentgrass in growth chamber experiments in the absence of *P. volutum*. This work demonstrates that fall and spring applications of pyraclostrobin, azoxystrobin, and cyazofamid suppress the development of root dysfunction symptoms during summer, and that field efficacy is related to the sensitivity of *P. volutum* to these fungicides.

INTRODUCTION

Creeping bentgrass (*Agrostis palustris* Huds.) is widely planted in the Southeastern (SE) U.S. on golf course putting greens. This cool-season turfgrass is ideal for golf course putting greens because it tolerates extremely close mowing and produces a uniform playing surface (24). However,

most of the SE U.S. is in the transition zone for cool and warm season turfgrasses, and the hot, humid summers often result in reduced turf vigor and increased susceptibility to diseases. In addition, the United States Golf Association recommends a sand-based rootzone for putting green construction, which has low nutrient- and water-holding capacities (24). Although these construction practices may reduce the development of fungal diseases that are encouraged by wet soil conditions, they may also enhance diseases such as Pythium root dysfunction (PRD) that are encouraged by physiological stresses .

Pythium root dysfunction of creeping bentgrass was first described by Hodges and Coleman in 1985 (5). They observed that newly established bentgrass on high-sand content (>60% sand) putting greens wilted in irregular patterns, eventually progressing to foliar decline. *Pythium aristosporum* and *P. arrhenomanes* were consistently isolated from diseased roots, and were highly virulent towards the secondary roots of creeping bentgrass. Feng and Dernoeden (4) in 1999 identified eight *Pythium* species (*Pythium aristosporum*, *P. aphanidermatum*, *P. catenulatum, P. graminicola*, *P. torulosum*, *P. vanterpoolii*, *P. volutum*, and *P. ultimum* var. *ultimum*) in a collection of 28 isolates from 109 putting green samples exhibiting symptoms of PRD. Based on the frequency of isolation and pathogenicity experiments, it was concluded that *P. aristosporum* was the most important causal agent of PRD in the Mid-Atlantic region of the U.S. In 2007, Kerns and Tredway (9) identified two *Pythium* species, *P. volutum* and *P. torulosum*, from a collection of 75 *Pythium* isolates from symptomatic bentgrass. *Pythium volutum* was isolated from 59 samples and was shown to be highly virulent towards creeping bentgrass roots in controlled environments.

In North Carolina, symptoms of PRD typically develop during the summer months when creeping bentgrass is subjected to heat and drought stress. However, the majority of hyphal growth and oospore production is observed in infected roots in the fall, winter, and spring when creeping bentgrass roots are most actively growing (9). In growth chamber studies, *P. volutum* infected creeping bentgrass roots when soil temperatures were between 12 and 24^{o}C (10). Symptoms initially

developed as wilt or chlorosis then progressed to a foliar decline, which is likely due to the rapid loss of root function. These symptoms, however, are only induced when creeping bentgrass is exposed to temperatures in the range of 32°C/26°C (day/night) (9). Based on our current understanding of PRD epidemiology, fungicide applications during the time of pathogen activity may provide the most effective disease control.

Although PRD was first described in 1985, very little research has been conducted to determine an effective control strategy for this disease. Hodges (7) reported that contact and systemic fungicides specific for *Pythium* control were not effective, therefore golf course superintendents were forced to re-establish the diseased areas once air and soil temperatures became conducive for creeping bentgrass growth. Hodges (7) indicated that the primary problem was the inability to move fungicides into the rootzones. The only other research addressing PRD prior to 2004 was Feng and Dernoeden's (4), but no experiments on management were reported.

In North Carolina, Tredway and Butler (22, 23) tested fungicides for early and late curative control of PRD in an 'A-1' creeping bentgrass putting green. All treatments and programs that included pyraclostrobin were nearly free of disease symptoms during late June and early July. Suppression of disease symptoms was not observed with any of the standard *Pythium* fungicides, which included mefenoxam, fosetyl-Al, propamocarb, and ethazole. As heat and drought stress increased through late July and early August, no treatments or programs significantly suppressed PRD symptoms compared to the untreated controls. Curative control of PRD was demonstrated with these two studies, yet this work was conducted prior to determining the optimal infection conditions for *P. volutum*.

Pyraclostrobin belongs to the class of fungicides known as the quinone outside inhibitors (Q_oI) or the strobilurins. These fungicides inhibit mitochondrial respiration by binding to the Q_o center of cytochrome *b*, thereby blocking the electron transport chain, resulting in loss of ATP

production and inhibition of fungal growth(2). QoIs are most effective as a prophylactic treatments (1,8,12,17, 27).

The QoIs also affect plant growth (2,5,15). A significant increase in yield and grain quality resulting from applications of QoI's fungicides was documented in wheat and barley (2). Equivalent levels of disease control are provided by DMI-based programs when compared to Q_oI-based programs, yet Q_oI-based programs provided higher yields that could not be explained by enhanced disease control. This phenomenon was coined the "Q_oI greening effect" because it is associated with an increase in green leaf area late into the growing season, which in turn increases grain yield (2). QoI's are commonly used in turfgrass disease management programs, yet little is known about the effects of QoI applications on turfgrass growth and physiology.

Field resistance to the Q_oI fungicides has been documented with several turfgrass pathogens. California populations of *Collectotrichum cereale*, the causal agent of turfgrass anthracnose, demonstrated *in vitro* and *in vivo* resistance to azoxystrobin, trifloxystrobin, and pyraclostrobin (27). Populations of *Pyricularia grisea*, the gray leaf spot pathogen, displayed resistance to azoxystrobin and trifloxystrobin in field experiments and in *in vitro* assays (11, 26). Field resistance to azoxystrobin has been documented in populations of *Pythium aphanidermatum* as well (2). Determining the sensitivity of *P. volutum* to different fungicides may help support the results of previous and current field research. Fungicide sensitivity data would also provide a baseline for future monitoring if fungicide resistance develops in *P. volutum* populations.

To improve the management of PRD, additional research is needed to determine the basis of activity of pyraclostrobin and to evaluate fungicides for preventative control of *P. volutum*, the primary causal agent of PRD in NC. The objectives of this study were to (i) evaluate fungicides for preventative control of PRD in field experiments; (ii) determine the *in vitro* sensitivity of *P. volutum* to fungicides; and (iii) evaluate the effects of pyraclostrobin applications on creeping bentgrass foliar growth rate.

MATERIALS AND METHODS

Preventative control of PRD. A three year field experiment was conducted at the Pinehurst Golf Resort in Pinehurst, NC on a 'G-2' creeping bentgrass putting green that was severely affected with PRD. The putting green was constructed in 1993 according to USGA specifications (24) with a rootzone mix of 85% sand to 15% sphagnum peat moss. In 2005, the site was fertilized with 171 kg N ha^{-1} and was mowed daily at 3.2 mm throughout the year. In 2006 and 2007, the putting green received 195 kg N ha^{-1} yr^{-1} and was mowed daily at 3.2 mm during the fall and spring and at 3.5 mm during the summer months. The study site was irrigated as needed to prevent drought stress.

Fungicide treatments and application dates were initiated in September 2004 and continued through 2007 (Table 4.1). Fall applications were initiated when soil temperatures declined to 24^{o}C and spring applications were initiated when soil temperatures declined to 12^{o}C (Table 1). Treatments were applied with a CO_2-powered boom sprayer at 40 psi using flat fan nozzles (TeeJet 8004, R&D Sprayers, Opelousas, LA) calibrated to deliver 81.5 ml H_2O m^{-2}. All treatments except fosetyl-Al were watered in with 64 mm of H_20 immediately after application. Plots were 4.6 m^2 and were arranged in a randomized complete block design with four replications.

Disease severity was evaluated during the summer months on the following dates: 7-13-05, 7-22-05, 8-16-05, and 7-13-06 7-28-06, 8-18-06, 7-12-07, 8-9-07, and 9-4-07. Disease severity was accessed visually using a modified Horsfall-Barratt (HB) scale (11=100% 6=50 to 75% 0=0%) (3). The scale data was converted to the geometric mean of the percentage range corresponding to the scale number (3) in order to calculate area under the disease progress curve (AUDPC) values (19). Turf quality was assessed visually by estimating the overall uniformity, density, and color within each plot before every fungicide application and during each disease severity rating. Turfgrass quality was quantified using a 1 to 9 scale (9 = best, 5 = minimally acceptable 1= bare ground). 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.

In vitro sensitivity of *Pythium volutum* to fungicides. The sensitivity of 11 *P. volutum* isolates, three *P. torulosum* isolates, and one *P. aphanidermatum* isolate to pyraclostrobin, azoxystrobin, mefenoxam, and cyazofamid was determined in mycelial growth assays. Hyphal plugs (4 mm) from the edge of actively growing colonies on clarified V8 juice agar (13) were placed in the center of Petri dishes containing PDA amended with six concentrations (0, 0.0001, 0.001, 0.01, 0.1, 1, and 10 mg of active ingredient (a.i) kg^{-1} of commercially formulated pyraclostrobin, azxoystrobin, mefenoxam, or cyazofamid. Salicylhydroxamic acid (SHAM) was added to all pyraclostrobin and azoxystrobin concentrations at a concentration of 0.1% (v/v), and a PDA + SHAM control was included. Fungicide solutions were added to autoclaved PDA after cooling to 55°C.

Pythium volutum and *P. torulosum* cultures were incubated in the dark at 23°C for 3 d. Cultures of *P. aphanidermatum* were incubated for only 24 hr due its rapid growth rate. The diameter of each colony was measured in two perpendicular directions and the mean diameter was adjusted by 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 nonamended medium] was determined for each isolate, fungicide, and fungicide concentration. Each fungicide x isolate combination was replicated three times and the experiment was repeated twice.

The 50% effective concentration (EC_{50}) values were estimated by linear regression (PROC REG SAS Institute Inc., Cary, NC) of the probit-transformed relative inhibition value (RI = 1 - RG) on log_{10}-transformed fungicide concentration (15). The EC_{50} values for each *P. volutum* and *P. torulosum* isolate were subjected to an ANOVA (PROC GLM) and mean separations using the Waller-Duncan k-ratio t-test (k=100).

Impact of pyraclostrobin on creeping bentgrass growth. Cone-tainers (3.8 cm x 20 cm) containing sand meeting USGA specifications (24) (BB 205, Golf Agronomics Inc., Rockingham, NC) were seeded with 'A-1' creeping bentgrass (9.7 g m^{-1}). The cone-tainers were placed in a greenhouse at 26°C/22°C (12-h day/night cycles) and misted twice daily to encourage germination. Following

germination, the turf was maintained in the greenhouse by irrigating twice daily with a complete nutrient solution containing 106.23 mol m^{-3} nitrogen, 10.41 mol m^{-3} phosphorus, and 111.03 mol m^{-3} potassium. The turf was trimmed weekly with scissors to a height of 1.27 cm.

Six weeks after seeding, cone-tainers were transferred to a growth chamber with 12-h day/night cycles at 24/16°C. Treatments were arranged in a completely random factorial design with 6 replications. Nitrogen treatments (0, 50, 100, or 200 mol m^{-3}) were applied daily in a complete nutrient solution that contained 10.41 mol m^{-3} phosphorus and 111.03 mol m^{-3} potassium for four weeks. Four weeks after initiation of the nitrogen treatments, pyraclostrobin was applied at rates equivalent to 0, 140, 280, 560, 1120, 2240, or 4480 g ha^{-1}. Pyraclostrobin treatments were applied once in a spray chamber at 40 psi using a flat fan nozzle (Tee Jet 8004, R&D Sprayers, Opelousas, LA) calibrated to deliver 18.71 L ha^{-1}. The entire experiment was repeated twice.

Grass was cut with scissors to a height of 1.27 cm after being treated with pyraclostrobin and returned to the growth chambers for an additional 2 weeks, at which time foliar growth was assessed by removing tissue back to a height of 1.27 cm. Clippings were dried at 60°C for 3 days and weighed to assess foliar growth. A total of three clipping harvests were collected per experiment. Turf quality was assessed visually by estimating the overall uniformity, density, and color within each cone-tainer every two weeks as well. Turfgrass quality was quantified using a 1 to 9 scale (9=best, 5=minimally acceptable 1= bare ground). Statistical analyses were performed in SAS (version 8.02; SAS Inc., Cary, NC). An analysis of variance (ANOVA) was performed using PROC GLM to estimate the effects of experiment, nitrogen rate, pyraclostrobin rate, and interaction terms on clipping yield and turf quality. The Waller-Duncan k-ratio t test (k=100) was used to separate means to compare rates of nitrogen and rates of pyraclostrobin.

RESULTS

Preventative control of PRD. In 2005, 2006, and 2007 foliar symptoms did not develop in the experimental area during the fall and spring application periods. In addition, none of the treatments

impacted turfgrass quality during the fall and spring application periods. In 2005, PRD symptoms developed during the beginning of July immediately following a heavy sand topdressing event and the United States Golf Association U.S. Open tournament (Fig 4.1). During 2005, only applications of cyazofamid, trifloxystrobin, azxoystrobin, and pyraclostrobin significantly reduced PRD severity compared to the untreated control (Fig 4.2). These treatments also maintained acceptable turf quality throughout the summer (Fig. 4.3). Symptoms of PRD developed in the beginning of July of 2006 as well, but in general the symptoms were not severe as in 2005. In 2006, only pyraclostrobin provided suppression of disease symptoms when compared to the untreated control (Fig 4.4). No significant differences ($P<0.05$) in turf quality were detected in 2006 (Fig 4.3). Symptom development again occurred in early July in 2007, but severity of symptoms was minor at this time. However, symptoms became severe in the untreated controls due to extremely hot, dry conditions of late summer. Fosetyl-Al, ethazole, trifloxystrobin, cyazofamid, azxoystrobin, and pyraclostrobin treated plots provided significant suppression of PRD when compared to the untreated control (Fig 4.5). However, acceptable turf quality was only observed in plots treated with pyraclostrobin, azoxystrobin, and cyazofamid (Fig 4.5).

Sensitivity of *Pythium volutum* to fungicides. ANOVA detected no significant differences between experiments, therefore data presented is the average of the two experiments. *Pythium volutum* was highly sensitive to pyraclostrobin (EC_{50} = 0.0049) and cyazofamid (EC_{50} = 0.0041), moderately sensitive to azoxystrobin (ED_{50} = 0.05), and the least sensitive to mefenoxam (EC_{50} = 0.139) (Table 4.2). Growth of *P. torulosum* was not inhibited by pyraclostrobin, azxoystrobin, or menfenoxam (Table 4.2). *P. torulosum* mycelial growth was inhibited by cyazofamid to a greater degree than other fungicides in this experiment. The *P. torulosum* isolate PV3 was the most sensitive to cyazofamid followed by SV1 and GO1 respectively (Table 4.2). The single *P. aphanidermatum* isolate was the most sensitive to pyraclostrobin, cyazofamid, and menfenoxam and was the least sensitive to azoxystrobin (Table 4.2).

Impact of pyraclostrobin on creeping bentgrass growth. There were no significant differences between experiments, so data presented is the average of the two experiments. Regardless of rate, pyraclostrobin did not significantly ($p>0.05$) impact creeping bentgrass foliar growth (Table 4.3). Increasing nitrogen rates caused an expected increase in clipping yield, but a significant interaction between nitrogen and pyraclostrobin treatments was not detected (Table 4.3).

DISCUSSION

Pythium root dysfunction was first described in 1985 (6), yet very little is known about the management of this disease. Curative applications of pyraclostrobin made upon the onset of symptoms yielded reductions in disease severity, but only 28 to 30 days of disease suppression was observed (22, 23). Although symptom expression primarily occurs during the summer months, recent epidemiological studies demonstrated that the PRD pathogen, *P. volutum*, infects creeping bentgrass roots when soil temperatures are between 12 and 24^{o}C (10). This work demonstrates that applications of pyraclostrobin, azoxystrobin, and cyazofamid made when soil temperatures are conducive for infection of creeping bentgrass roots by *P. volutum* provide summer long suppression of PRD symptoms and maintain acceptable turf quality.

Hodges (7) reported in 1985 that contact and systemic fungicides specific for *Pythium* control were not effective for managing PRD. Similarly, standard *Pythium* fungicides such as mefenoxam, propamocarb, fosetyl-Al, and ethazole, did not suppress PRD symptoms when applied on a preventative basis in our study or on a curative basis in trials conducted by Tredway and Butler (21, 22). The relatively low sensitivity of *P. volutum* to mefenoxam may explain the ineffectiveness of this active ingredient in field studies. Sensitivities were not determined in this study for propamocarb, fosetyl-Al and ethazole, but this information may help to explain the lack of PRD control provided by these fungicides in our trial.

QoI's differ in their efficacy against diseases induced by oomycetes. Tredway and Butler (21, 22) showed that applications of pyraclostrobin were more effective on a curative basis than applications of azoxystrobin. However, applications of azoxystrobin have been shown to be more effective against Pythium foliar blight than pyraclostrobin (20, 25). Pyraclostrobin consistently provided the best suppression of PRD symptoms in field trials, and *P. volutum* isolates were very sensitive to this fungicide. *Pythium volutum* was also sensitive to cyazofamid and azoxystrobin, which provided good to moderate levels of PRD suppression in the field. Rebollar-Alviter (17, 18) reported that isolates of *Phytophthora cactorum*, the causal agent of leather rot of strawberries, were more sensitive to pyraclostrobin than to azoxystrobin. Additional investigations are needed to determine if oomycetes are more sensitive to pyraclostrobin than to azoxystrobin in general.

Creeping bentgrass growth was not impacted by applications of pyraclostrobin. In contrast, multiple studies have demonstrated enhanced growth and yield in cereal crops with applications of QoI fungicides, which was not attributed to enhanced disease control (2, 5, 12, 15). This indicates that the suppression of PRD symptoms from applications of pyraclostrobin is primarily due to fungicidal properties and not enhanced growth. However, we only examined growth rate under optimal conditions in this particular experiment. Applications of pyraclostrobin have been shown to improve stress management and delay leaf senescence when exposed to drought stress (12). An in depth examination of the effects of pyraclostrobin applications on creeping bentgrass physiology under drought conditions may help explain why pyraclostrobin was more effective against PRD than cyazofamid even though the *in vitro* sensitivities were similar.

Pythium root dysfunction has become an important disease of creeping bentgrass in the SE US (9). Prior to this work, golf course superintendents did not have an effective management strategy for PRD. Indeed, many superintendents have implemented a preventative control program based on this work and have observed success. As fungicides are increasingly used for control of PRD, fungicide resistance has the potential to develop. In order to combat this, we recommend that

growers rotate between applications of pyraclostrobin, cyazofamid, and a propamocarb + fosetyl-Al tank mix. If fungicide resistance does develop, then the results from our sensitivity assay will provide a baseline to monitor shifts in the *P. volutum* population towards fungicide resistance. Similar work should be completed with organisms associated with PRD development in other states.

Our results demonstrate that long lasting preventative control of PRD can be achieved when fungicides are applied during temperatures that are favorable for *P. volutum* infection. Isolates of *P. volutum* are very sensitive to pyraclostrobin and moderately sensitive to cyazofamid and azoxystrobin. Preventative fungicide programs should be based upon these active ingredients. Applications of pyraclostrobin did not impact creeping bentgrass growth rates, yet further research is needed to determine the impact of Q_oI applications on other physiological processes in turfgrasses under stress.

REFERENCES:

1. Anesiadis, T., Karaoglanidis, G.S., and Tzavella-Klonary, K. 2003. Protective, curative, and erradicant activity of the QoI fungicide azoxystrobin against *Cercospora beticola* and *Erysiphe betae*. J. Phytopathol. 151:647-651.

2. Bartlett, D.W., Clough, J.M., Godwin, J.R., Hall, A.A., Hamer, M., and Parr-Dobrzanski, B. 2002. The QoI fungicides. Pest Manage. Sci. 58:649-662.

3. Campbell, C.L. and Madden L.V. 1990. *Introduction to Plant Disease Epidemiology*, 3rd edition. John Wiley and Sons Hoboken, NJ. Pp 113-118.

4. Feng, Y., and Dernoeden, P.H. 1999. *Pythium* species associated with root dysfunction of creeping bentgrass in Maryland. Plant Dis. 83: 516-520.

5. Gerhard M., Habermeyer, J., and Zindernagel, V., 1999. *The impact of strobilurins on plant vitality on winter wheat under field conditions*. Pages 197-208. in: Modern Fungicides and Antifungal Compounds II. 12th International Reinhardsbrunn Symposium, Friedrichroda, Thuringia, Germany. Intercept Ltd, Andover, UK.

6. Hodges, C.F., and Coleman, L.W. 1985. *Pythium*-induced root dysfunction of secondary roots of *Agrostis palustris*. Plant Dis 69:336-340.

7. Hodges, C.F. 1985. Pythium-induced root dysfunction of creeping bentgrass on high sand content greens. GCM. Sept-Oct. 11-13.

8. Karadimos, D.A., Karaoglanidis, G.S., and Tzavella-Klonary, K. 2005. Biological activity and physical mode of action the Q_o inhibitor fungicides trifloxystrobin and pyraclostrobin against *Cercospora beticola*. Crop Prot. 24:23-29.

9. Kerns, J.P., and Tredway, L.P. 2008**.** Pathogenicity of *Pythium* species associated with Pythium root dysfunction of creeping bentgrass and their impact on root growth and survival. Plant Dis. *Accepted*.

10. Kerns, J.P., and L.P. Tredway. 2007. Determination of the optimal infection temperature range of creeping bentgrass by *Pythium volutum*, a causal agent of Pythium root dysfunction. Phytopathology 97:S56.

11. Kim, Y.S., Dixon, E.W., Vincelli, P., and Farman, M.L. 2003. Field resistance to QoI (Q_oI) fungicides in *Pyricularia grisea* caused by mutations in the cytochrome *b* gene. Phytopathology 93:891-900.

12. Kohle, H., Grossmann, K., Jabs, T., Gerhard, M., Kaiser, W., Glaab, J., Conrath, U., Seehaus, K., and Herms, S. 2002. Physiological effects of the strobilurin fungicide F 500 on Plants Pages 61-74 in: "Modern Fungicides and Antifungal Compounds III." H.W. Dehne et al. eds. AgroConcept, Bonn, Germany

13. Ma, Z., Felts, D., and Michailides, T.J. 2003. Resistance to azoxystrobin in *Alternaria* isolates from pistachio in California. Pestic. Biochem. Physiol. 77:66-74.

14. Martin, F. N. 1992. *Pythium*. Pages 39-49 in: "Methods for Research on Soilborne Phytopathogenic Fungi". L. L. Singleton et al., eds. The American Phytopathological Society, St. Paul, MN.

15. Miller, G.L., Stevenson, K.L., and Burpee, L.L. 2002. Sensitivity of *Sclerotinia homeocarpa* isolates to propiconazole and impact on control of dollar spot. Plant Dis. 86:1240-1246.

16. Oerke, E.C., Beck, C., and Retzlaff, G. 2001. Physiological effects of strobilurins on wheat yield. Phytopathology 91:S67.

17. Rebollar-Alviter, A., Madden, L.V., Jeffers, S.N., and Ellis, M.A. 2007. Baseline sensitivity to two Q_oI fungicides among isolates of *Phytophthora cactorum* that cause leather rot and crown rot on strawberry. Plant Dis. 91:1625-1637.

18. Rebollar-Alviter, A., Madden, L.V., and Ellis, M.A. 2007. Pre-and post-infection activity of azoxystrobin, pyraclostrobin, mefenoxam, and phosphite against leather rot of strawberry, caused by *Phytophthora cactorum*. Plant Dis. 91:559-564.

19. Shaner G., and Finney, R.E. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67: 1051-1056.

20. Stiles, C.M., Datnoff, L.E., and Cisar, J.L. 2005. Evaluation of fungicides for control of Pythium blight in overseeded turfgrasses using a simple field inoculation technique. Online. Plant Health Progress doi: 10.1094/PHP-2005-0330-01-RS.

21. Tredway, L.P. and Butler, E.L. 2004. Late curative control of an unknown root disease of creeping bentgrass putting greens. www.turffiles.ncsu.edu/extension/tredwaytrials/2004/prd%20late%20curative.pdf.

22. Tredway, L.P. and Butler E.L. 2004. Preventative control of an unknown root disease of creeping bentgrass putting greens. www.turffiles.ncsu.edu/extension/tredwaytrials/2004/prd%20early%20curative.pdf.

23. Tredway, L.P., and Martin, S.B. 2003. Stalking new disease in creeping bentgrass. Carolinas Green. Nov-Dec:22-26.

24. Turgeon, A.J. 1999. *Turfgrass Management*, 5th edition. Prentice Hall, Upper Saddle River, NJ. Pp 49-108.

25. Uddin, W., Soika, M.D., Soika, E.L., and Francl, A. 2003. Pythium foliar blight control with the use of fungicides. Fungic. Nemat. Tests 59:T037.

26. Vincelli, P., and Dixon, E. 2002. Resistance to Q_oI (QoI-like) fungicides in isolates of *Pyricularia grisea* from perennial ryegrass. Plant Dis. 86:235-240.

27. Wong, F. P., Midland, S. L., and de la Cerda, K. A. 2007. Occurrence and distribution of QoI resistant isolates of *Colletotrichum cereale* from annual bluegrass in California. Plant Dis. 91:1536-1546.

28. Wong, P.F., and Wilcox, F.W. 2001. Comparative physical modes of action of azoxystrobin, mancozeb, and metalaxyl against *Plasmopara viticola* (grapevine downy mildew). Plant Dis. 85:649-656.

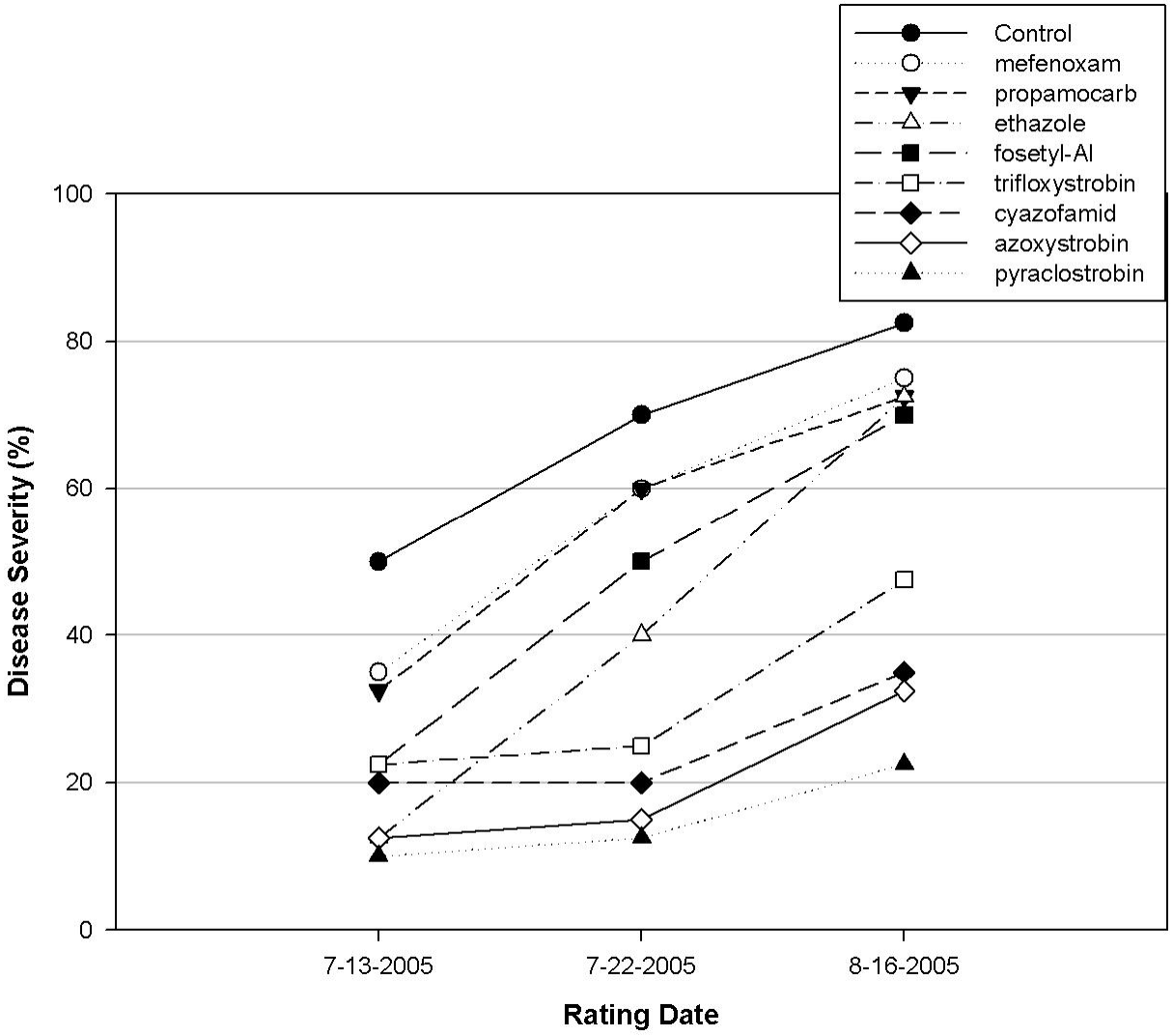

Fig. 4.1. Pythium root dysfunction severity in response to preventative fungicide applications in Pinehurst, NC. Disease severity was evaluated using a modified Horsfall-Barrett scale (0= 0%, 5= 50 to 75%, and 11=100%) based on foliar tissue exhibiting decline.

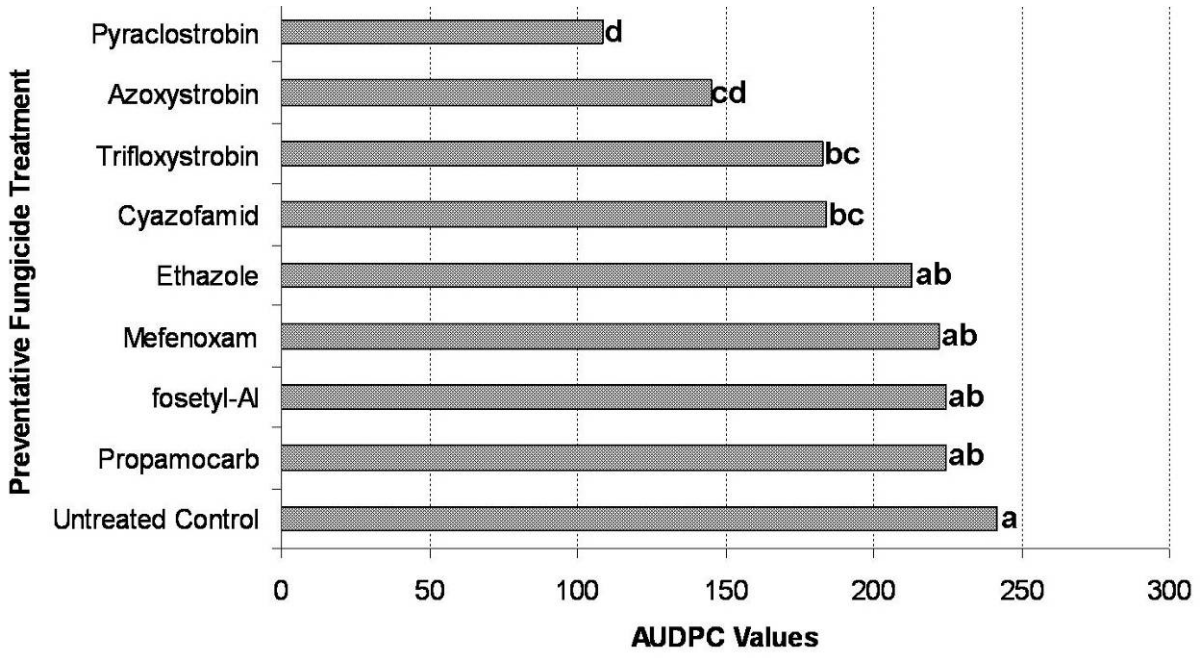

Fig. 4.2. Area under the disease progress curve values (AUDPC) in response to preventative fungicide applications in 2005. AUDPC values were calculated from disease severity data collected weekly for 3 rating dates. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio t-test (k=100).

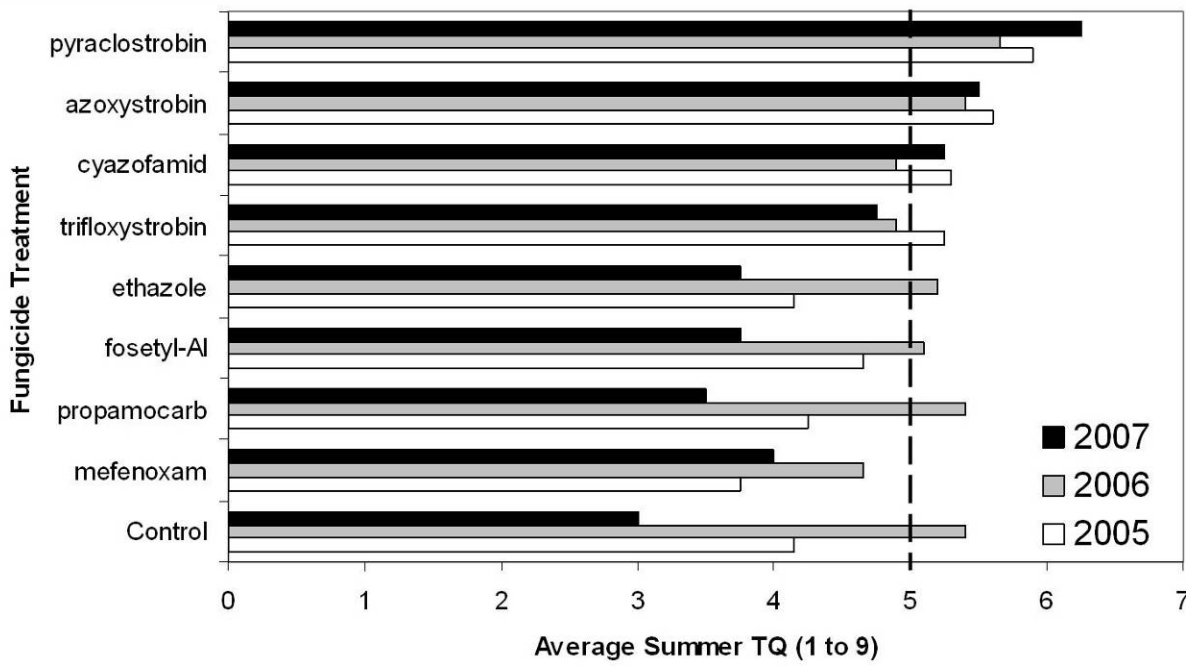

Fig. 4.3. Impact of preventative fungicide applications on turfgrass quality in 2005, 2006, and 2007. Turf quality was visually estimated on a scale of 1 to 9 (1=bare ground 5=minimally acceptable and 9=best). Bars represent the average of three turf quality rating dates.

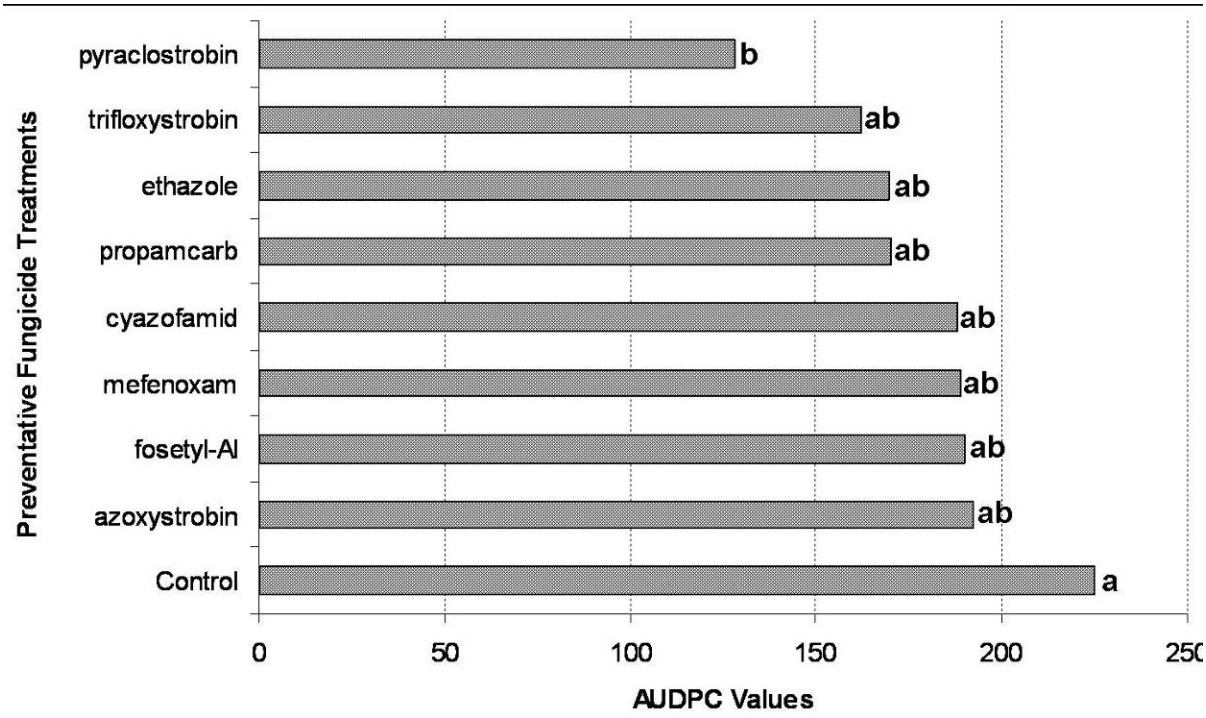

Fig. 4.4. Area under the disease progress curve values (AUDPC) in response to preventative fungicide applications in 2006. AUDPC values were calculated from disease severity data collected weekly for 3 rating dates. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio t-test (k=100).

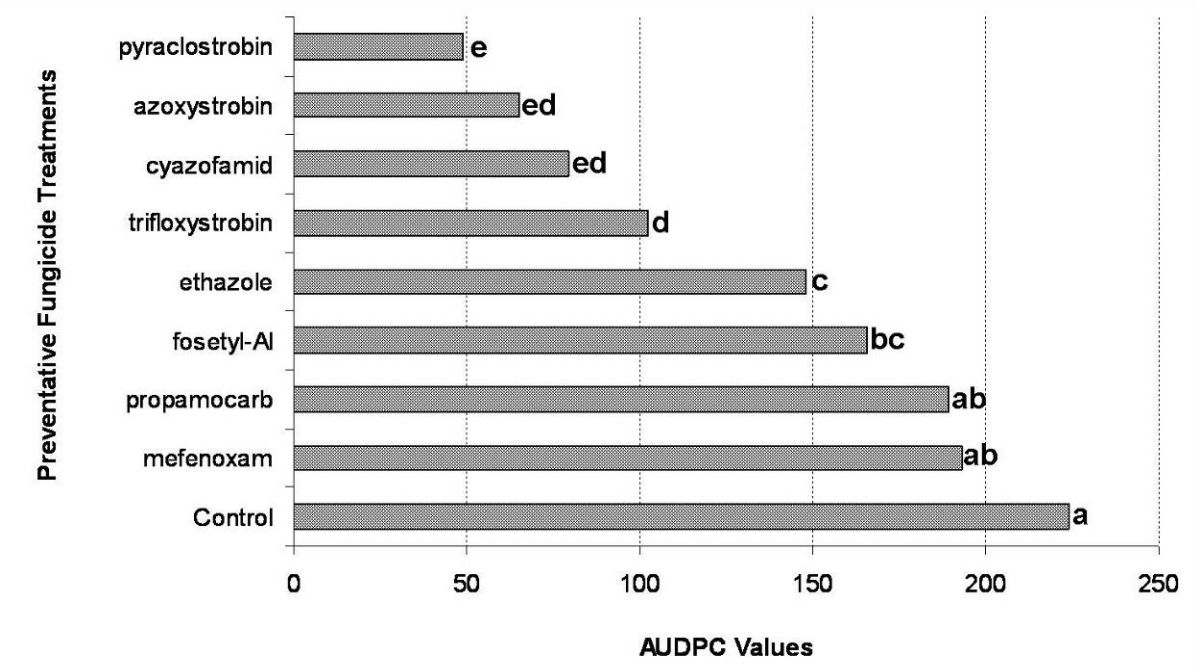

Fig. 4.5. Area under the disease progress curve values (AUDPC) in response to preventative fungicide applications in 2007. AUDPC values were calculated from disease severity data collected weekly for 3 rating dates. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio t-test (k=100).

Table 4.1. Treatments, rates, and application dates for preventative control of PRD in 2004 through 2007.

Fungicide	Rate[y] (g or ml m^{-2})	Watered In[z] (+ or -)
Control	-----	-
menfenoxam	0.31 ml	+
propamocarb	2.44 g	+
fosetyl-Al	1.3 ml	-
ethazole	1.22 g	+
azoxystrobin	0.12 g	+
pyraclostrobin	0.28 g	+
trifloxystrobin	0.076 g	+
cyazofamid	288 ml	+

[x] Applications were applied on 9-22-04, 10-20-04, 3-29-05, 4-19-05, 5-26-05, 9-4-05, 10-4-05, 3-15-06, 4-17-06, 5-17-06, 9-13-06, 10-12-06, 3-14-07, 4-12-07, 5-16-07.
[y] Treatments were applied in 81.5 ml H_2O m^{-2}.
[z] Treatments with a + received 6.4 ml of irrigation immediately after application.

Table 4.2. *In vitro* sensitivity of mycelium of *Pythium volutum, Pythium torulosum,* and *P. aphanidermatum* isolates to pyraclostrobin, azoxystrobin, cyazofamid, and mefenoxam.

	----------Fungicides[x]----------			
ISOLATE	pyraclostrobin	azoxystrobin	cyazofamid	mefenoxam
	----------EC_{50} Concentration µg ml^{-1}----------			
Pythium volutum				
OC 1	0.0035 a[y]	0.06 a	0.0027 a	0.094 b
OC 4	0.0041 a	0.04 a	0.0047 a	0.125 b
OC 6	0.0039 a	0.04 a	0.0023 a	0.074 b
OC 9	0.0053 a	0.04 a	0.0026 a	0.053 b
PRD 12	0.0049 a	0.05 a	0.0035 a	0.108 b
PRD 22	0.0048 a	0.05 a	0.0034 a	0.212 a
PRD 29	0.0083 a	0.05 a	0.0045 a	0.109 b
PRD 39	0.0059 a	0.06 a	0.0053 a	0.298 a
PRD 48	0.0046 a	0.04 a	0.0076 a	0.104 b
PRD 7	0.0036 a	0.04 a	0.0036 a	0.213 a
SV8	0.0054 a	0.05 a	0.0044 a	0.136 b
Average	0.0049	0.05	0.0041	0.139
Pythium torulosum				
GO 1	<10	<10	3.39 A[z]	<10
PV 3	<10	<10	1.72 C	<10
SV 1	<10	<10	2.29 B	<10
Pythium apanidermatum	0.270	0.48	0.210	0.230

[x] Commercial formulations of fungicides were used.
[y] Values followed by the same lower case letter within a column for the *P. volutum* isolates are not significantly different according to Waller-Duncan k-ratio t-test (k=100).
[z] Values followed by the same capital letter within a column for the *P. torulosum* isolates are not significantly different according to Waller-Duncan k-ratio t-test (k=100).

Table 4.3. Creeping bentgrass foliar growth as affected by nitrogen rate and pyraclostrobin rate.

	----------Pyraclostrobin Rate (g ha^{-}1)[v]----------					
	0	140	560	1120	2240	4480
Growth Rate[x] (mg d^{-1})	4.9[y]	5.1	4.7	4.2	5.8	5.1
	----------Nitrogen Rate (mol m^{-3})[w]----------					
		0	50	100	200	
Growth Rate (mg d^{-1})		1.7 a[z]	4.9 b	6.2 c	7.3 c	

[v] Pyraclostrobin treatments were applied once using a spray chamber at 40 psi using a flat fan nozzle (Tee Jet 8004, R&D Sprayers, Opelousas, LA) calibrated to deliver 18.71 L ha^{-1}.

[w] Nitrogen treatments were applied daily in a complete nutrient solution that contained 10.41 mol m^{-3} phosphorus and 111.03 mol m^{-3} potassium for four weeks.

[x] Growth rate was assessed by cutting the turf with scissors to a height of 1.27 cm after treated with pyraclostrobin and returned to the growth chambers for an additional 2 weeks, at which time foliar growth was removed to a height of 1.27 cm with scissors. Clippings were dried at 60^{o}C for 3 days and weighed to assess foliar growth rate.

[y] No significant differences were detected among pyraclostrobin rates with respect to creeping bentgrass foliar growth.

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

CHAPTER 5- IMPACT OF IRRIGATION FREQUENCY, ORGANIC MATTER CONTENT, AND CREEPING BENTGRASS CULTIVAR ON THE DEVELOPMENT OF PYTHIUM ROOT DYSFUNCTION

ABSTRACT

Symptoms of Pythium root dysfunction (PRD) have been observed on multiple golf courses in North Carolina and other southeastern states since 2002. Symptoms appear in irregular patches ranging in diameter from 12 to 40 cm. Initially, symptoms resemble wilt or drought stress then progress to an orange-yellow foliar decline. Our observations indicate that symptoms are most severe on high-sand content putting greens less than 3 years old. It is unknown if this distribution is the result of soil characteristics in newly constructed greens or due to increased susceptibility of modern creeping bentgrass cultivars. Three growth chamber experiments were established to determine the effects of cultivar, organic matter content, and irrigation frequency on PRD development. All creeping bentgrass cultivars were susceptible to *P. volutum*, but differed in their relative susceptibility. 'Crenshaw', 'Syn-96', and 'G-6' were the most resistant cultivars with area under disease progress curve (AUPDC) values of 78, 208, and 252, respectively. Cultivars 'A-1'and 'A-4' were moderately resistant with AUDPC values of 478 and 480, respectively, and 'LS-44', 'G-2', and 'Penncross' were susceptible with AUDPC values of 668, 911, and 1060, respectively. Symptoms of PRD were most severe when creeping bentgrass was irrigated 6 times a week, followed by 4, 3, 2, and 1 irrigation per week. In general, organic matter content at the time of establishment did not affect PRD severity. Our results indicate that cultivars of creeping bentgrass vary in their susceptibility to *P. volutum* and disease is less severe with reduced irrigation frequency

during the infection period. In combination with preventative fungicide programs, golf course superintendents should incorporate creeping bentgrass cultivars with improved resistance and practice deep and infrequent irrigation when conditions are conducive for *P. volutum* infection.

INTRODUCTION

Creeping bentgrass (*Agrostis palustris* Huds.) is a cool-season grass species that is widely planted on golf courses throughout the U.S. This is the preferred turfgrass species for golf course putting greens because it tolerates extremely close mowing and produces a fast, uniform putting surface (Turgeon, 2004). Creeping bentgrass is not well-adapted to the persistent hot and humid conditions experienced during summer months in the Southeastern United States, which often results in reduced plant vigor and increased susceptibility to disease. In addition, most putting greens are constructed with artificial root zones containing 85 to 100% sand and 0 to 15% organic matter by volume to promote rapid drainage and drying of the playing surface. However, these root zones have extremely low water- and nutrient-holding capacities (Turgeon, 2004). While these construction practices can reduce the development of fungal diseases that are encouraged by wet soil conditions, they may also enhance other diseases that are encouraged by physiological stress.

Pythium root dysfunction is encouraged heat and drought stress (Kerns and Tredway, 2008). This disease was first described in Iowa by Hodges and Coleman (1985), who observed rapid foliar decline of newly established putting greens with high sand-content rootzones (>60% sand) during the summer months. From affected roots, the authors consistently isolated *P. arrehenomanes* and *P. aristosporum*, and both species were shown to reduce creeping bentgrass root and shoot growth. The authors described the affected roots as

buff-colored with devitalized and bulbous root tips. Feng and Dernoeden (1999) identified eight *Pythium* species out of a collection of 28 *Pythium* isolates isolated from 109 samples exhibiting symptoms of PRD. Based on the frequency of isolation and pathogenicity results, the authors concluded that *P. aristosporum* was the most important causal agent of PRD in the Mid-Atlantic States. In North Carolina, *P. volutum* was consistently isolated from creeping bentgrass roots exhibiting symptoms of PRD and was demonstrated to be highly virulent towards creeping bentgrass roots (Kerns and Tredway, 2008).

Symptoms of PRD typically appear in NC during the summer months as irregular patches ranging from 12 cm to 40 cm in diameter. The affected turf initially exhibits signs of drought stress, but progresses to a yellow to orange foliar decline. If the affected area is left untreated, the turf may die within two to three weeks after symptom development. Examination of infected roots using a soil probe reveals tan colored roots with reduced branching and bulbous root tips. Roots examined during the fall and spring are of normal length, but rapidly die back in response to heat stress. The pathogen, *P. volutum* apparently hinders the plant's ability tolerate heat stress, which results in an irregular patch resembling drought stress.

Pythium root dysfunction is most severe in newly constructed putting greens. The disease can appear as soon as 3 months after establishment and symptoms decline 6 to 8 years after establishment (Hodges and Coleman, 1985, Kerns and Tredway, 2008). However during the first 6 to 8 years after establishment, control of PRD can be difficult. Tredway and Butler (2004) found that curative suppression of PRD symptoms was achieved for approximately 28 d with only a few fungicides. Planting resistant creeping bentgrass

cultivars into rootzones that are less conducive for pathogen growth may help to maximize efficacy of preventative and curative fungicide applications.

Although the majority of symptom development occurs in the summer, *P. volutum* actively infects creeping bentgrass roots when soil temperatures are cooler (Kerns and Tredway 2008). Pythium root dysfunction severity was greatest when *P. volutum* was allowed to infect creeping bentgrass at 16^{o}C (Kerns and Tredway, 2008). Similarly, Hodges and Campbell (1994) presented evidence that *P. graminicola* and *P. arrehenomanes* reduced shoot and root growth at 25^{o}/13^{o}C (day/night), but not when temperatures were 32^{o}C/24^{o}C. In both studies, a prolonged exposure to heat was needed to induce foliar decline. Since PRD pathogens infect creeping bentgrass roots when temperatures are cooler, cultural control measures implemented during the infection process would be expected to be most effective.

Soil moisture has a profound effect on the behavior of *Pythium* species in soil. For instance at high soil moisture, *Pythium* species are the primary saprophytes, but as soil moisture decreases other fungi predominate (Kouyeas, 1964). Furthermore, studies have shown that infection of various plants by *Pythium* species was greater when soils were maintained at or above field capacity (Bratoloveau and Wallace, 1985, Lifshitz and Hancock, 1983, and Martin and Loper, 1999). Reducing the amount of free water in putting green rootzones during periods conducive for *P. volutum* infection may be an effective cultural control practice for PRD.

Hodges and Coleman (1985) reported that plants inoculated with various *Pythium* species grown in a 50:50 sand-soil (% v/v) mix exhibited smaller reductions in growth when compared to plants grown in a pure sand mixture, and they never observed PRD in putting greens with a high native soil content. Suppression of Pythium root rot of creeping bentgrass

was observed when certain composts were added to the potting mixture at the time of establishment (Craft and Nelson, 1996). Furthermore, studies have documented suppression of Pythium root rot of poinsettia when organic matter was added to the potting mixture (Boehm et al., 1993 and Inbar et al., 1991). Since, PRD is primarily a disease of high sand content putting greens, the effects of organic matter additions on PRD development warrant investigation.

New creeping bentgrass cultivars have been developed for use on golf course putting greens (Bruneau et al., 2001). Selection of these cultivars is based on a dense upright growth habit, very fine leaf texture, and tolerance to abiotic stresses such as close mowing, heat, and drought (Duich, 1999). Variability in resistance or susceptibility to certain turfgrass diseases has been observed with these new cultivars. For example, some of these cultivars are more resistant to take-all patch, a root disease incited by *Gaeumannommyces graminis* var. *avenae* than older cultivars such as Penncross, but are more susceptible to dollar spot (*Sclerotinia homeocarpa*) and summer patch (*Magnaporthe poae*) (Tredway 2006; Vincelli et al., 1997; Weibel et al., 2002). The relative susceptibility of creeping bentgrass cultivars to infection by *P. volutum* is unknown and should be examined based on the rapid increase of this disease in the SE US.

In order to improve PRD management, the factors that influence disease development must be examined. The objectives of this study were to (i) determine the relative susceptibility of eight creeping bentgrass cultivars (Penncross, LS-44, Syn-96, Crenshaw, A-1, A-4, G-2, and G-6) to *P. volutum*, (ii) evaluate the impact of irrigation frequency on infection of creeping bentgrass roots by *P. volutum*, and (iii) determine the impact of varying organic matter content on PRD severity.

MATERIALS AND METHODS

Susceptibility of Creeping Bentgrass Cultivars to *P. volutum*

Cone-tainers (3.8 cm x 20 cm) containing sand meeting USGA specifications (BB 205, Golf Agronomics Inc., Rockingham, NC) were seeded with eight different cultivars of creeping bentgrass (9.7 g m^{-1}) (Kerns and Tredway 2008). The cultivars used in this experiment included 'Penncross', 'G-6', 'G-2', 'A-1', 'A-4', 'Crenshaw', 'LS-44', and 'Syn-96'. After seeding, the cone-tainers were placed in a greenhouse at 26°C/22°C (12-h day/night cycles) and misted twice daily to encourage rapid germination. Following germination, the turf was maintained in the greenhouse by irrigating twice daily with a complete nutrient solution containing 106.23 mol m^{-3} nitrogen, 10.41 mol m^{-3} phosphorus, and 111.03 mol m^{-3} potassium for four weeks. After four weeks, the turf was irrigated once daily with the complete nutrient solution mentioned above. The turf was trimmed weekly with scissors to a height of 1.27 cm.

Six weeks after seeding, each cone-tainer was infested with sterilized creeping bentgrass leaves infested with a mixture of two highly aggressive isolates of *P. volutum* (OC2 and PRD38). The inoculum was prepared by placing three 3-mm mycelial plugs into sterile water containing five to seven sterilized 1.5-cm long creeping bentgrass leaves. The inoculum was incubated under continuous fluorescent light at room temperature (23 to 25^{o}C) for 3 days (Abad et al., 1994; Martin, 1992). Inoculations were performed by removing the turf plug from the cone-tainer and the root system was cut at a 5-cm depth. The sand in the cone-tainer was discarded and replaced with fresh sand, then 5 to 7 *Pythium*-colonized grass blades were placed onto the surface of the fresh sand, and finally the turf plug was placed on top of the

colonized grass blades. A non-inoculated control was included for each cultivar by cutting the roots at 5 cm then repotting the turf plug onto fresh, uninfested sand.

All cone-tainers were transferred to a growth chamber and arranged in a completely random design with 10 replications per cultivar. Initial growth chamber conditions were 12-h day/night cycles at 24°C/16°C. After four weeks, four of the 10 replications were destructively sampled to measure root necrosis, root depth, and root mass. Root necrosis was estimated visually on a scale of 0 to 10 (0=no necrosis 10=100% root necrosis). Root depth was assessed by removing the creeping bentgrass plug from the cone-tainer and measuring the distance from the soil surface to the deepest root tip. The sand was then thoroughly washed from the root system by gently agitating the roots in deionized water. Root weights were recorded after drying at 60°C for 72 hr.

The remaining six replicates of each treatment remained in the growth chamber, and temperature was raised to 32°C/26°C (day/night) to induce foliar symptoms. Percentage of foliar tissue exhibiting chlorosis or die-back was assessed at 0, 14, and 30 days after of incubation at the high temperature regime. After 30 days, all cone-tainers were destructively sampled to measure root necrosis, root depth, and root mass as described above. The entire experiment was conducted three times.

All statistical analyses were performed using SAS (version 8.02; SAS Inc., Cary, NC). Analysis of variance was conducted using PROC GLM to estimate the effects of experiment and cultivar on disease severity, AUDPC (Shaner and Finney, 1977), root necrosis, root mass (before and after heat exposure), and root depth (before and after heat exposure). Dunnett's t-test was used to compare inoculated plants to the non-inoculated plants for all the

dependant variables mentioned above. The Waller-Duncan *k* ratio *t* test (*k*=100) was used to separate means for comparison among creeping bentgrass cultivars.

Impact Irrigation Frequency on PRD Severity

Cylindrical pots (1,762 cm^3) containing sand meeting USGA specifications (BB 205, Golf Agronomics Inc., Rockingham, NC) were seeded with 'A-1' creeping bentgrass (9.7 g m^{-1}). The pots were established and managed as described above.

Eight weeks after seeding, pots were inoculated with sterilized creeping bentgrass leaves infested with *P. volutum* isolates OC2 and PRD38. Inoculations were performed as above, except that 60 to 70 colonized leaf blades were used per cylinder. Inoculated pots were transferred to a growth chamber and arranged in a randomized complete block design with 6 replications. Initial growth chamber conditions were 12-h day/night cycles at 24°C/16°C (day/night) for a 4 week inoculation period. During the infection period, irrigation treatments were applied by adding 400 ml of deionized water to each pot 6, 4, 3, 2, or 1 times per week. Pots were lightly sprinkled with the nutrient solution described above once daily during the inoculation period. Turf quality was visually estimated on a scale of 1 to 9 (1=bare ground, 5=minimally acceptable, 9=best) weekly during the infection period. After four weeks, two replications were randomly chosen and destructively sampled to measure root depth. Root depth was assessed by removing the creeping bentgrass plug from the pot and measuring the distance from the soil surface to the deepest root tip.

The remaining four replicates remained in the growth chamber, and temperature was raised to 32°C/26°C (day/night) to induce foliar symptoms. Plants were watered daily with the nutrient solution mentioned previously once the high temperature treatment was initiated.

Percentage of foliar tissue exhibiting chlorosis or die-back was assessed at 0, 14, 28, and 35 days after heat exposure. After 35 days, all pots were destructively sampled to measure root depth as described above. The entire experiment was repeated twice.

All statistical analyses were performed using SAS (version 8.02; SAS Inc., Cary, NC). Analysis of variance was conducted using PROC GLM to estimate the effects of experiment and irrigation frequency on disease severity, area under the disease progress curve (AUDPC), and root depth (before and after heat exposure). Dunnett's t-test was used to compare inoculated pots to the non-inoculated pots for all the dependant variables mentioned above. The Waller-Duncan *k* ratio *t* test (*k*=100) was used to separate means for comparison among irrigation treatments.

Impact of Organic Matter Content at Establishment on PRD Severity

The effects of organic matter content at the time of seeding was evaluated by seeding 'A-1' creeping bentgrass into the cylindrical pots (1762 cm^3) containing different mixtures of sand meeting USGA specifications and sphagnum peat moss. Treatments included a 100:0, 90:10, 80:20, 70:30, and a 50:50 sand:peat ratio (% v/v). Plants were established and inoculated according to the procedures outlined for the irrigation frequency experiment. During the infection period and heat treatment, pots were irrigated daily with the nutrient solution described above. Pots were transferred to the same growth chamber as the irrigation experiment and arranged in a randomized complete block design with six replications for each organic matter treatment. Root depth and disease severity data were collected according to the procedures outlined in the irrigation experiment as well. The entire experiment was repeated twice.

All statistical analyses were performed using SAS (version 8.02; SAS Inc., Cary, NC). Analysis of variance was conducted using PROC GLM to estimate the effects of experiment and organic matter content on disease severity, AUDPC, and root depth (before and after heat exposure). Dunnett's t-test was used to compare inoculated pots to the non-inoculated pots for all the dependant variables mentioned above. The Waller-Duncan *k* ratio *t* test (*k*=100) was used to separate means for comparison among organic matter treatments.

RESULTS

Susceptibility of Creeping Bentgrass Cultivars to *P. volutum*

An outbreak of Pythium blight in experiment three led to the loss of three of the 10 replications; therefore the data from this experiment were not used in the analysis. Significant differences were not present among experiments one and two, thus data from these experiments were combined for analysis. Additionally, trends observed in the root mass data were very similar to those observed in the root depth data, therefore only root depth is presented. According to Dunnett's t-test root depth of 'Penncross', 'G-2', 'LS-44', and 'A-4' was significantly reduced as a result of *P. volutum* infections prior to heat exposure (Fig. 5.1). However, no foliar symptoms were observed in any cultivar during the infection period. In addition, no significant differences in visual ratings of root necrosis were present.

The typical foliar symptoms of PRD developed in all cultivars 14 days after exposure to 32^{o}C/26^{o}C (day/night). At first, foliar symptoms appeared as wilt and chlorosis, then after 30 days in the high temperature regime, symptoms progressed to foliar decline. AUDPC values based on foliar disease severity were highest in 'Penncross' and lowest in 'Crenshaw'. (Fig. 5.2). However, susceptibility of 'Penncross', 'G-2', 'LS-44', 'A-1', and 'A-4' were not

statistically different from one another (Fig. 5.2). Susceptibility of 'LS-44', 'A-4', 'A-1', 'G-6', 'Syn-96', and 'Crenshaw' were statistically similar as well (Fig. 5.2). Root depth of inoculated 'G-6' and 'Crenshaw' was not negatively impacted at high temperature, and no significant differences were detected among cultivars when root necrosis was visually estimated. However, roots collected from inoculated cone-tainers were a light tan color, lacked root hairs, and had swollen root tips.

Impact Irrigation Frequency on PRD Severity

Pre-heat treatment:

Significant differences occured among experiments because foliar symptoms did not develop in the first experiment. As a result, only data from the second experiment is presented. Foliar symptoms of PRD did not develop prior to heat exposure. According to Dunnett's t-test *P. volutum* infections only reduced root depth when pots were irrigated 6 times per week (Table 5.1). Turf quality was minimally acceptable or better during the infection period when creeping bentgrass was irrigated 3, 4, or 6 times per week (Fig. 5.3).

Post-heat treatment:

Wilt and chlorosis typical of initial PRD symptoms developed 14 days after heat exposure, then by 35 days foliar decline was observed. Pythium root dysfunction severity was greatest when creeping bentgrass was irrigated 6 times per week during the infection period (Fig. 5.4). Significant differences between inoculated and non-inoculated pots were not detected when creeping bentgrass was irrigated 1 or 2 times per week (Fig. 5.4). After four weeks of heat treatment, reductions in root depth increased as the irrigation frequency increased (Table 5.1). *Pythium volutum* infections did not significantly reduce root depth

when compared to their respective non-inoculated controls at the two lowest irrigation treatments (Table 5.1).

Impact of Organic Matter Content at Establishment on PRD Severity

Only data from the second experiment is presented because no foliar symptoms developed in the first experiment. Foliar symptoms were not induced by *P. volutum* infections during the four week infection period. Prior to heat exposure, root depth was significantly reduced in inoculated pots containing 100% sand as compared to non-inoculated controls (Table 5.1). Wilt or chlorosis occurred after 14 days of heat exposure in inoculated pots. After 35 days of heat treatment, foliar decline was observed in all organic matter treatments. Pythium root dysfunction symptoms were most severe in pots containing 100% sand although the AUDPC values calculated for 100:0, 90:10, 80:20 and 50:50 treatments were not statistically different (Fig 5.5). Reductions in root depth were greatest in pots with 100 % sand followed by the 90:10, 50:50, 80:20, and 70:30 treatments respectively (Table 5.1).

DISCUSSION

All of the creeping bentgrass cultivars tested were susceptible to *P. volutum*, but they differed in their level of resistance. Mean separations from our cultivar experiment make it difficult to infer which cultivar was the most resistant, but there were clear trends in the data. 'Crenshaw', 'Syn-96', and 'G-6' were the most resistant cultivars based on AUDPC values. The creeping bentgrass cvs 'A-1' and 'A-4' were moderately resistant, and 'LS-44', 'G-2', and 'Penncross' were the least resistant cultivars studies. Pythium root dysfunction is observed most frequently on putting greens established with 'A-1' and 'A-4', however these cultivars are widely planted on newly constructed putting greens. Our results indicate that

'A-1' and 'A-4' are not the least resistant cultivars available to turfgrass managers. Although 'Crenshaw' appeared to be the least susceptible cultivar to PRD, it is one of the most susceptible creeping bentgrass cultivars to *Sclerotinia homoeocarpa* and is no longer recommended in the humid SE US.

In previous experiments, *P. volutum* infections predisposed creeping bentgrass (cv. 'A-1') roots to dieback following exposure to heat stress, and significant losses in root mass and root depth were observed (Kerns and Tredway, 2008). The A- and G-series bentgrasses were selected for their upright dense growth habit and their ability to perform under the intense heat that occurs in the SE US (Duich, 1999 and Stier, 2007). In the SE US, the A and G series bentgrass typically out perform 'Crenshaw', a variety bred exclusively to withstand heat stress, with respect to summer turf quality (Bruneau et al., 2001). Our hypothesis was cultivars of creeping bentgrass that were the most heat tolerant would have the greatest resistance to PRD. However, our results demonstrate that creeping bentgrass cultivars with increased heat tolerance are not necessarily more resistant to *P. volutum*. A similar phenomenon was observed with summer patch resistance (Tredway, 2006). Additional research is needed to determine the mechanisms for PRD resistance in 'Crenshaw', 'Syn-96', and 'G-6'.

Reductions in PRD severity were observed in our study when creeping bentgrass was irrigated 3 or 4 times a week during the infection period. Although turf quality was highest when creeping bentgrass was irrigated 6 times per week, turf quality ratings remained above five when pots were irrigated 3 and 4 times a week. These results are not surprising, as *Pythium* spp. are most virulent when soils are moist and behave primarily as saprophytes when soil moisture is low (Martin, 1999 and Lifshitz and Hancock, 1983). Therefore, golf

course superintendents should employ deep, infrequent irrigation events during the fall and spring in order to limit *P. volutum* infection and activity. Our observations indicate that frequent aerification reduces disease activity, thus cultural practices that improve soil drainage may also limit PRD severity.

Traditionally, Pythium root dysfunction was thought to be a problem of putting greens with high-sand content rootzones (Hodges and Coleman, 1985). As a result, we hypothesized that additions of more organic matter during putting green construction or renovation would minimize PRD symptoms. The results of our study indicate that adding more organic matter at the time of establishment in the form of sphagnum peat moss did not reduce the severity of PRD. Although PRD severity was similar among the non-inoculated and inoculated pots containing 70% sand and 30% sphagnum peat moss, a previous experiment showed that incorporation of peat moss at levels higher than 20% did not meet the USGA guidelines for physical properties (Chong et al., 2004). Yet suppression of Pythium root rot or creeping bentgrass was achieved when animal manures, rather than sphagnum peat moss, were added to the rootzone mixture (Craft and Nelson 1996). More research needs to be conducted to determine if organic matter source similarly influences PRD development. Furthermore we did not examine infection nor did we quantify *P. volutum* populations, which may have been slightly suppressed in response to higher concentrations of sphagnum peat moss. Additional research should be conducted to determine if sphagnum peat moss limits *P. volutum* infectivity or affects inoculum density of the pathogen.

In summary, the results from this experiment show that all creeping bentgrass cultivars are susceptible to *P. volutum*, but they vary in level of resistance. Irrigating 3 or 4

times a week as opposed to 6 times a week significantly reduced PRD severity, and more organic matter at the time of establishment did not affect PRD development. Water was applied every day in our organic matter experiment, which may have eliminated any potential effects of increasing organic matter. Future experiments should examine the interaction between irrigation frequency and organic matter content. Golf course superintendents should select creeping bentgrass cultivars that have lower levels of susceptibility and irrigate less frequently during the fall and spring when *P. volutum* is actively infecting creeping bentgrass roots. Furthermore, cultural practices that improve soil drainage may also help to limit infection of creeping bentgrass roots by *P. volutum*. These cultural practices should be coupled with a preventative fungicide program to achieve season-long control of PRD.

REFERENCES

Abad, Z.G., Shew, H.D., and Lucas, L.T. 1994. Characterization and pathogenicity of *Pythium* species isolated from turfgrass with symptoms of root and crown rot in North Carolina. Phytopathology 84:913-921.

Boehm, M.J., L.V. Madden, and H.J. Hoitink. 1993. Effect of organic matter decomposition level on bacterial species diversity and composition in relationship to Pythium damping-off severity. Appl. Environm. Micro. 59: 4174-4179.

Bratoloveanu, J., and H.R. Wallace. 1985. The influence of *Pythium* on the growth of barley seedlings as affected by soil water and inoculum density. Plant and Soil 85: 305-311.

Bruneau, A.H., C.A. Bigelow, R.J. Cooper, and D.C. Bowman. 2001. Performance of creeping bentgrass cultivars maintained at two mowing heights and under two fungicide regimes in North Carolina. Intern. Turf. Soc. Res. Journ. 9(2): 835-842.

Chong, S.-.K., R., Boniak, A., Zhang, H. Yanhe, and C.-.H, Ok. 2004. Physical properties of green sand mixes amended with sphagnum peat moss. Acta Hort. (ISHS) 661:59-64 http://www.actahort.org/books/661/661_5.htm.

Craft, C.M., and E.B. Nelson. 1996. Microbial properties of composts that suppress damping-off and root rot of creeping bentgrass caused by *Pythium graminicola*. Appl. Environ. Microbiol. 62: 1550-1557.

Duich, J.M. 1999. Creeping bentgrass *Agrostis palustris* (*stolonifera*) variety named 'Penn G-1'. US Patent # 5,969,216.

Feng, Y., and Dernoeden, P.H. 1999. *Pythium* species associated with root dysfunction of creeping bentgrass in Maryland. Plant Dis. 83: 516-520.

Hodges, C.F. 1985. Pythium-induced root dysfunction of creeping bentgrass on high sand content greens. USGA Green Sec. Record Sept/Oct: 11-13.

Hodges, C.F., and Coleman, L.W. 1985. *Pythium*-induced root dysfunction of secondary roots of *Agrostis palustris*. Plant Dis 69:336-340.

Hodges, C.F., and Campbell, D.A. 1994. Infection of adventitious roots of *Agrostis palustris* by *Pythium* species at different temperature regimes. Can. J. Bot. 72:378-383.

Inbar, Y., M.J. Boehm, and H.J. Hoitink. 1991. Hydrolysis of fluorescein diacetate in sphagnum peat container media for predicting suppressiveness to damping-off caused by *Pythium ultimum*. Soil Biol. Biochem. 23: 1183-1189.

Kerns, J.P., and Tredway, L.P. 2008. Pathogenicity of *Pythium* species associated with Pythium root dysfunction of creeping bentgrass and their impact on root growth and survival. Plant Dis. *Accepted*.

Kouyeas, V. 1964. An approach to the study of moisture relations of soil fungi. Plant Soil 20: 351-363.

Lifshitz, R. and J.G. Hancock. 1983. Saprophytic development of *Pyhtium ultimum* in soil as a function of water matric potential and temperature. Phytopathology 73:257-261.

Lifshitz, R. B. Sneh, and R. Baker. 1984. Soil suppressiveness to plant pathogenic *Pythium* species. Phytopathology 74: 1054-1061.

Martin, F. N. 1992. *Pythium*. Pages 39-49 in: "Methods for Research on Soilborne Phytopathogenic Fungi". L. L. Singleton et al., eds. The American Phytopathological Society, St. Paul, MN.

Martin, F. N. and Loper, J.E. 1999. Soilborne plant diseases caused by *Pythium* spp: ecology, epidemiology, and prospects for biological control. Crit. Rev. Plant Sci. 18(2): 111-181.

Shaner G., and Finney, R.E. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67: 1051-1056.

Stier, J.C. 2007. Understanding and managing environmental stresses of turfgrass. Acta Hort. (ISHS) 762:63-80. http://www.actahort.org/books/762/762_6.htm.

Tredway, L.P. 2006. Genetic relationship among *Magnaporthe poae* isolates from turfgrass hosts and relative susceptibility of 'Penncross' and 'Penn A-4' creeping bentgrass. Plant Dis. 90:1531-1538.

Tredway, L.P. and E.L. Butler. 2004. Preventative control of an unknown root disease of creeping bentgrass putting greens. Online

http://www.turffiles.ncsu.edu/Extension/tredwaytrials/2004/prd%20preventative .pdf

Turgeon, A.J. 2004. *Turfgrass Management*, 8th edition. Prentice Hall, Upper Saddle River, NJ. Pp 49-108.

Vincelli, P., J.C. Doney, and A.J. Powell. 1997. Variation among creeping bentgrass cultivars in recovery from epidemics of dollar spot. Plant Dis. 81: 99-102.

Weibel, E.N., L.P. Tredway, and B.B. Clarke. 2002. Impact of temperature and fungal isolate on the susceptibility of bentgrass cultivars to take-all patch. (Abstr.) Phytopathology 92: S146.

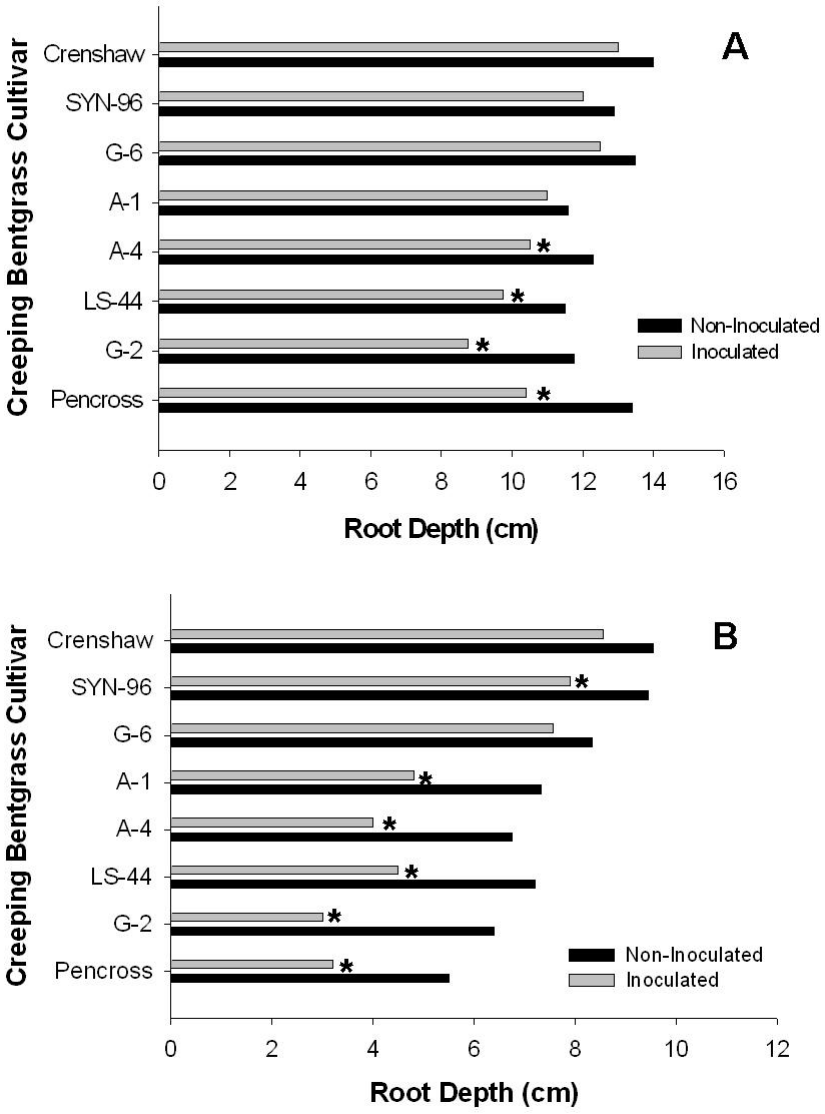

Fig 5.1. Impact of *P. volutum* infections on root depth of eight creeping bentgrass cultivars. **A.** Impact of *P. volutum* infections on root depth after four weeks at 24°C/16°C (day/night), **B.** Impact of *P. volutum* infections on root depth after four weeks incubation at 32°C/26°C (day/night). Bars with an asterisk are significantly different from non-inoculated controls according to Dunnett's t-test (P = 0.05).

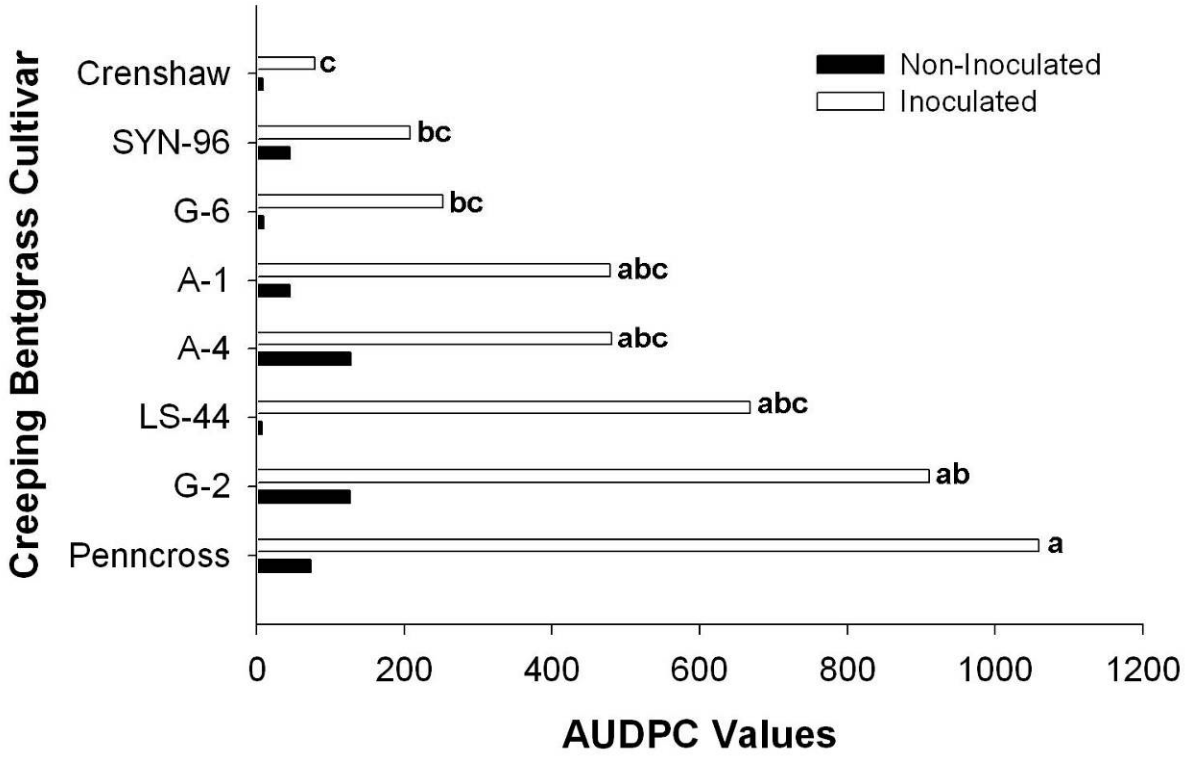

Fig. 5.2. Area under the disease progress curve (AUDPC) values for eight creeping bentgrass cultivars inoculated with *P. volutum*. AUDPC values were calculated from disease severity data collected weekly for 4 weeks. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio t-test (k=100). White bars represent average AUDPC value for inoculated pots for each cultivar and black bars represent average AUDPC values for non-inoculated pots for each cultivar.

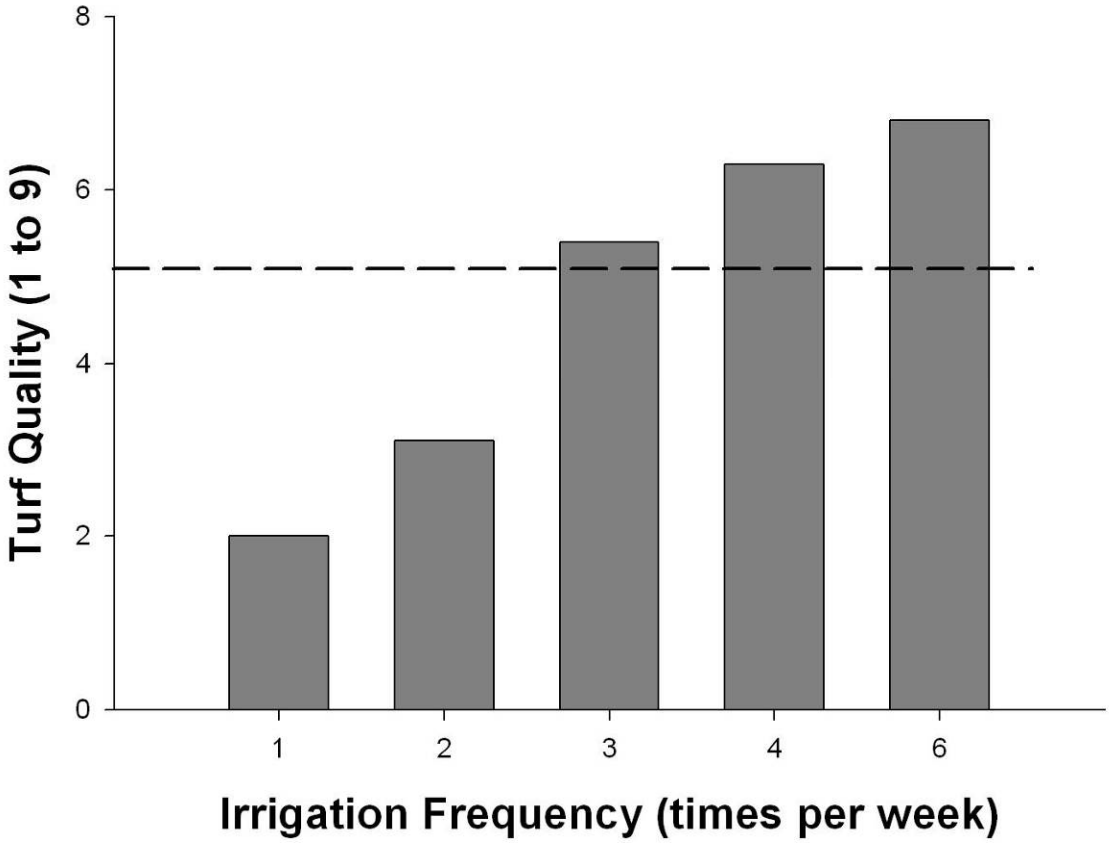

Fig 5.3. Impact of irrigation frequency during inoculation period on turf quality. Turf quality was visually estimated on a scale of 1 to 9 (1= bare ground, 5 = minimally acceptable, and 9 = best quality) based on color, uniformity, texture, and density. Dotted line represent level needed for minimally acceptable turf quality.

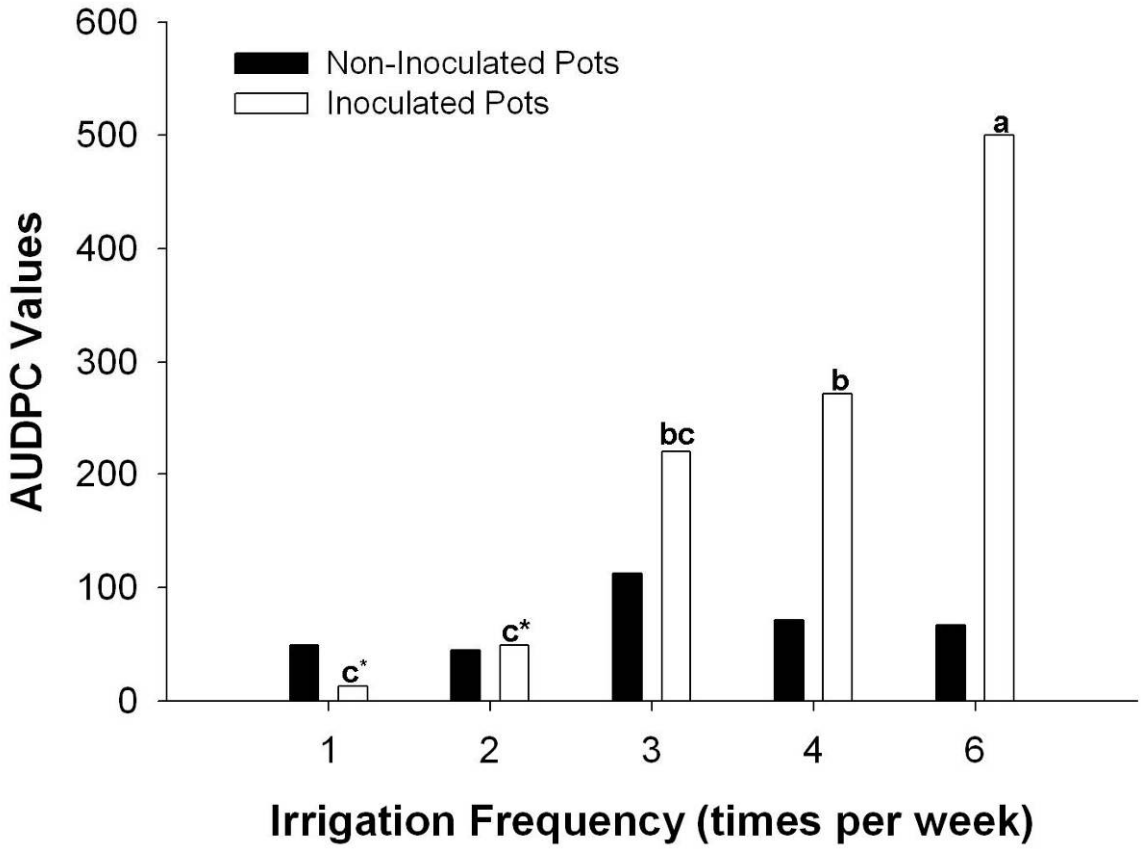

Fig 5.4. Influence of irrigation frequency during the inoculated period on the development of PRD in creeping bentgrass. Area under disease progress curve (AUDPC) values were calculated from disease severity data collected weekly for 4 weeks. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio t-test (k=100). Bars with asterisk are not significantly different from non-inoculated controls according to Dunnett's t-test (P = 0.05). White bars represent average AUDPC value for inoculated pots for each irrigation frequency and black bars represent average AUDPC values for non-inoculated pots of each irrigation frequency.

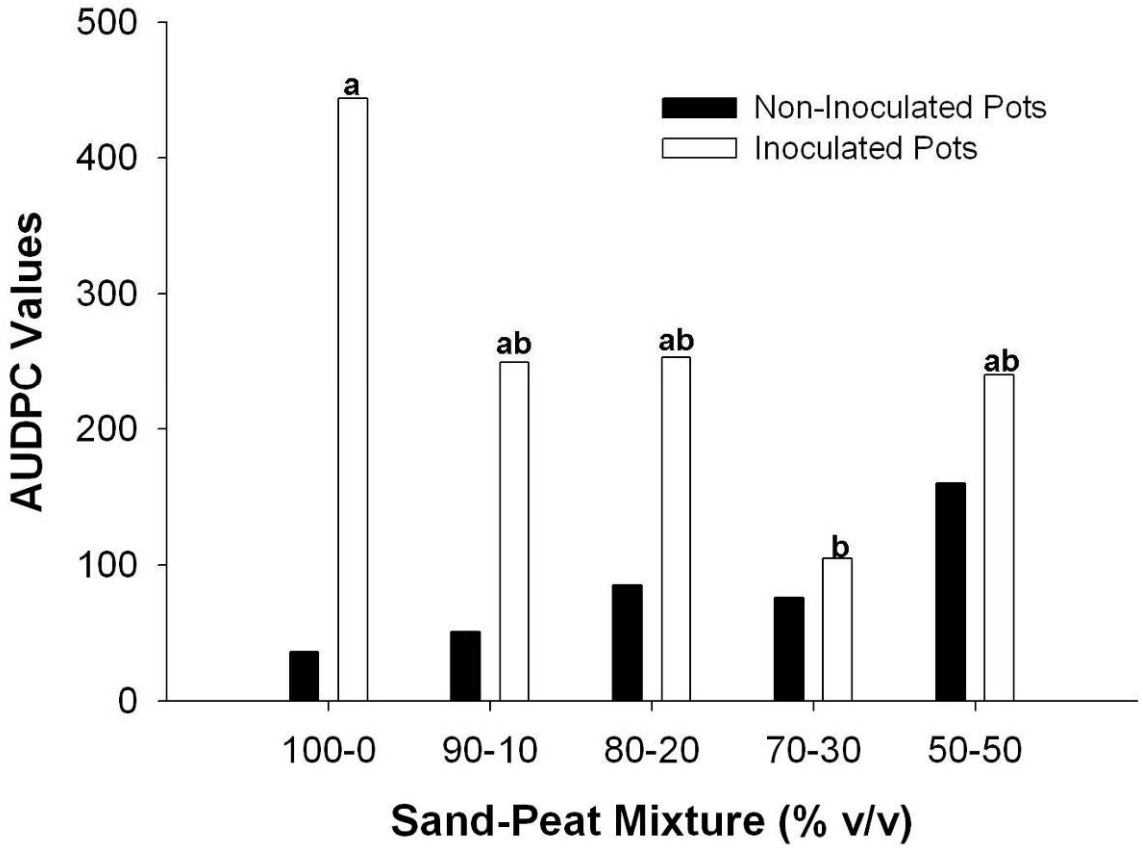

Fig. 5.5. Influence of organic matter content added during establishment on the development of PRD in creeping bentgrass. Area under disease progress curve (AUDPC) values were calculated from disease severity data collected weekly for 4 weeks. Bars followed by the same letter are not significantly different according to Waller-Duncan k-ratio t-test (k=100). White bars represent average AUDPC value for inoculated pots for each organic matter treatment and black bars represent average AUDPC values for non-inoculated pots of each organic matter treatment.

Table 5.1. Root depth of inoculated and non-inoculated 'A-1' creeping bentgrass plants as affected by irrigation frequency and organic matter content.

	Irrigation Frequency[a] (times per week)									
	6		**4**		**3**		**2**		**1**	
	Pre[b]	Post[c]	Pre	Post	Pre	Post	Pre	Post	Pre	Post
	----------Root Depth (cm)----------									
Non-inoculated	13.5	9.9	12.0	10.0	12.2	8.6	7.8	4.5	6.8	4.3
Inoculated	10.2*	5.0*	11.7	8.1*	11.1	7.2*	7.6	3.5	7.0	3.8
	Sand to Peat Mixtures[d] (% v/v)									
	100:0		**90:10**		**80:20**		**70:30**		**50:50**	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
	----------Root Depth (cm)----------									
Non-inoculated	12.5	8.7	11.7	9.9	13.5	8.5	12.6	10.2	11.1	7.8
Inoculated	9.9*	6.8*	10.6	7.1*	11.1	6.7*	11.1	9.1	9.9	5.8*

[a] Treatments were established during four week infection period at 24°C/16°C day/night by adding 400 ml of deionized water.
[b] Root depth and root mass measured prior to exposure to 32/26°C heat treatment.
[c] Root depth and root mass measured four weeks after exposure to 32/26°C heat treatment.
[d] Treatments consisted of sand meeting USGA specifications and sphagnum peat moss.
* Denotes a significant difference from the non-inoculated control within a column.

CHAPTER 6- SUMMARY, CONCLUSIONS, AND FUTURE RESEARCH

Although Pythium root dysfunction was first described in 1985 (3) and discovered again in Maryland in 1999 (2), this disease has largely been a mystery. When symptoms similar to PRD started to develop in the SE US, turfgrass pathologists were unfamiliar with the disease. As a result, the disease was mis-diagnosed as take-all patch for a number of years. Largely due to the fact that symptoms were patch-like and affected plants had necrotic crowns. In the fall of 2003 and the spring of 2004, symptoms appeared as areas of wilt or drought stress, and then progressed to irregular patches of yellow-orange foliar decline. From plants within these patches we consistently isolated *P. volutum* from creeping bentgrass roots that exhibited a tan color, lacked root hairs, and exhibited bulbous root tips. Foliar symptoms did not develop during a four week inoculation period with a diurnal temperature of 24^{o}C/16^{o}C. Our pathogenicity assays demonstrated that *P. volutum* induced foliar decline in 'A-1' creeping bentgrass after 2 to 4 weeks of exposure to a high temperature regime. Creeping bentgrass root mass and root depth were not adversely affected by *P. volutum* infections during the inoculation period. However once infected roots were exposed to the high temperature regime, root mass and root depth were severely limited. From this work we concluded that *P. volutum* infects creeping bentgrass roots during cooler temperatures, but symptoms are not expressed until creeping bentgrass plants are subjected to heat and drought stress. We also concluded that *P. volutum* infections made creeping bentgrass roots more susceptible to dieback when subjected to heat. *Pythium volutum* was found to be the most important causal agent of PRD in NC, which was different from the findings of other researchers (1, 2, 3). This is likely due to the time of sampling in our work. Most of our isolates were

collected during the spring and fall, whereas previous studies dealing with Pythium induced diseases of turfgrass sampled during the summer months. Future researchers examining PRD causal agents should perform isolations during the fall, spring and summer, in order to avoid inadvertently selecting for or against certain *Pythium* species.

In order to improve preventative fungicide timing, we evaluated the effect of temperature during the inoculation period on PRD development. *Pythium volutum* infects creeping bentgrass roots when soil temperatures were between 12 and 24^{o}C. Root mass and root depth of inoculated plants were significantly less than root mass and root depth of non-inoculated plants for the 16^{o}C, 20^{o}C, and 24^{o}C treatments prior to heat exposure. After four weeks of heat exposure, root depth and root mass were severely limited in the 12^{o}C, 16^{o}C, 20^{o}C, and 24^{o}C treatments. Foliar symptoms were most severe for the 16^{o}C temperature treatment followed by the 20^{o}C and 24^{o}C temperature treatments. Foliar symptoms developed in the 12^{o}C treatment, but they were not as severe when compared to the previously mentioned treatments. Foliar symptoms did not develop in the 28^{o}C and 32^{o}C treatments, so we concluded that *P. volutum* did not infect creeping bentgrass roots at these temperatures. From a mycelial growth assay, we concluded that *P. volutum* grows optimally between 18^{o}C and 26^{o}C, which supports the results from our infection temperature study.

There are differences in the root depth and root mass results from our pathogenicity experiment and our infection temperature experiment. During the infection temperature experiment, the inoculation temperature remained constant for four weeks, which could have enhanced infection that may have lead to reductions in root growth. In our pathogenicity assays, we utilized a diurnal inoculation temperature regime, which is

characteristic of the natural environment. Many questions were raised from these differences, for instance (i) do *P. volutum* infections make creeping bentgrass roots prone to die-back during heat?;(ii) or do *P. volutum* infections facilitate a more rapid decline in root growth in response to high temperatures?; (iii) or do *P. volutum* infections limit root depth in the spring and fall enough that creeping bentgrass is more sensitive to heat and drought stress? Future research should address these questions and a potential experiment will be briefly discussed later in this section.

Prior to this work almost nothing was known about the management of PRD. Hodges and Coleman (3) indicated that selective fungicides specific for oomycetes were ineffective against PRD. Their recommendation was to re-seed damaged areas during the fall. Our preventative control trial demonstrated that applications of pyraclostrobin in the fall and spring provided the best and longest-lasting suppression of PRD symptoms. Azoxystrobin and cyazofamid provided excellent levels of suppression, which only lasted until mid-July. The standard Pythium fungicides, mefenoxam, propamocarb, fosetyl-Al, and ethazole, were not effective against PRD. *In vitro* results demonstrated that *P. volutum* was very sensitive to pyraclostrobin and cyazofamid, moderately sensitive to azoxystrobin, and least sensitive to mefenoxam. Currently we are expanding our *in vitro* assay to include propamocarb, fluopicolide, and fluoxystrobin. We also examined the effects of pyraclostrobin applications on creeping bentgrass growth and found that creeping bentgrass growth was unaffected when pyraclostrobin was applied during conditions that are optimal for creeping bentgrass growth. Future research should examine the effects of pyraclostrobin applications on creeping bentgrass growth when subjected to various stresses like heat and drought. In order to limit fungicide resistance

in *P. volutum*, we currently are recommending an application of pyraclostrobin when soil temperatures reach 10 to 12^{o}C in the spring. Then an application of cyazofamid should follow approximately a month later followed by an application of a tank mixture of propamocarb and fosetyl-Al. When PRD symptoms are extremely severe, an application of pyraclostrobin should be applied when soil temperatures approach 24^{o}C in the fall. This was the first work to demonstrate control of PRD symptoms and will provide a basis for fungicide resistance screening if it develops in populations of *P. volutum*.

We have observed that PRD is most severe in putting greens with high-sand content rootzones established with 'A-1' or 'A-4'. We have also observed that PRD symptoms develop first on upland areas that drain very well. Therefore we wanted to examine how organic matter additions, creeping bentgrass cultivar, and irrigation frequency affect PRD development. We examined the relative resistance of eight creeping bentgrass cultivars to *P. volutum*. Crenshaw, Syn-96, and G-6 were the most resistant cultivars tested. A-1 and A-4 were moderately resistant and Penncross, LS-44, and G-2 were the least resistant cultivars tested. Although Crenshaw showed the highest level of resistance, it is not recommended as a primary putting green cultivar in the SE US due to its extreme susceptibility to dollar spot and other fungal disease. However, planting Syn-96 or G-6 could help to limit PRD development, thereby maximizing fungicide efficacy.

Pythium root dysfunction is most severe on newly established high-sand content putting greens. Yet, after six to eight years, as organic matter accumulates in the soil profile, the severity of the disease greatly declines. We hypothesized that more organic matter added at the time of establishment would limit *P. volutum* infection of creeping

bentgrass roots. Our results showed that additions of sphagnum peat moss did not limit PRD development. However, sphagnum peat moss is a "dark" organic matter source that has very little microbial activity. Incorporation of "lighter" organic matter sources, like animal manures, would have higher microbial populations that could compete with *P. volutum*.

Soil moisture has a profound influence on *Pythium* species. When soil moisture is around field capacity or slightly higher in a sandy loam, *Pythium* species release zoospores, the primary infective propagule (4). Therefore, limiting the amount of free-water in the rootzone during the optimal infection temperature regime of *P. volutum* may limit the development of PRD symptoms. Indeed this is what our results showed. When creeping bentgrass was irrigated six times a week during the infection period for *P. volutum*, PRD symptoms were the most severe once exposed to a high temperature regime. When creeping bentgrass was irrigated three or four times a week, PRD severity was reduced by 36 % and 26 % respectively while maintaining acceptable levels of turfgrass quality. Thus golf courses that experience PRD should irrigate deeply and infrequently during the fall and spring to minimize infection of creeping bentgrass roots by *P. volutum*.

Future Research

Although this work is the first comprehensive examination of PRD biology and management, there are many questions remaining. Some of the most important questions that should be addressed in future research are:

1. How do *P. volutum* infections affect the cytochemical and ultrastructure of creeping bentgrass root cells? This could be accomplished by inoculating small populations of creeping bentgrass plants with *P. volutum* and make thin slices of root tissue for examination with electron microscopy and conventional microscopy. Subsamples of additional infected roots could be digested for analysis of cytochemical changes in infected root cells. As the technology develops and the understanding of turfgrass genetics advances, gene regulation in response to infection by *P. volutum* also warrants investigation. Future researchers should consult Rey, P., Benhamou, N., and Tirilly, Y. 1998. Ultrastructural and cytochemical investigation of asymptomatic infection by *Pythium* spp. Phytopathology 88:234-244.

2. Do *P. volutum* infections cause a more rapid die-back of roots in response to heat or does infection limit root growth during the infection period? Future experiments should inoculate field plots and sample to measure root depth and root mass periodically during the infection period of *P. volutum* and during the summer to answer this question. A rhizotron would be beneficial to examine rooting characteristics, as well as the point-intersect method for root growth. A simple growth chamber experiment would consist of inoculated and non-inoculated plants that were destructively sampled weekly during the infection period and during the high temperature regime.

3. What are the mechanisms that drive symptom development? The appendix of this thesis outlines an experiment to examine the effects of *P. volutum* infection on creeping

bentgrass nitrate uptake, evapotranspiration, and photosynthesis. Preliminary results indicate that these processes are not adversely affected prior to exposure to heat stress. However, since the symptoms initially appear as areas of wilt or drought stress, the osmotic potential of infected roots should be compared to that of non-infected roots. Futhermore, the effect of *P. volutum* infections on creeping bentgrass root respiration should also be quantified. This could be done using a LI COR machine or by measuring CO_2 evolution similar to the methods used to determine microbial respiration.

4. Will additions of other sources of organic matter limit development of PRD symptoms? Growth chamber experiments could be utilized to answer this question. Various lighter organic matter sources, such as animal manures should be incorporated into sand meeting USGA specifications. Evaluation of PRD symptom development and inoculum density of *P. volutum* would provide evidence for or against organic matter sources. If an organic matter source does limit PRD development then researchers should test the mixture at different concentrations similar to those used in putting green construction.

5. How do microbial populations influence PRD development? This question could be answered by sampling microbial populations and diversity of multiple golf courses with PRD and comparing that to those not experiencing PRD. In addition, microbial sampling of new greens verses old greens would help to explain the decline in PRD symptoms over time.

6. What other cultural practices influence PRD development? Field experiment should be employed to answer this question. Multiple experiments could be designed to

examine the influence of nitrogen, aerification, and topdressing on PRD development. We have observed that nitrogen and aerification seem to alleviate PRD symptoms, but have not conducted research that demonstrates this observation. Knowing how these practices affect PRD development would expand the integrated management program for PRD.

REFERENCES:

1. Abad, Z.G., Shew, H.D., and Lucas, L.T. 1994. Characterization and pathogenicity of *Pythium* species isolated from turfgrass with symptoms of root and crown rot in North Carolina. Phytopathology 84:913-921.
2. Feng, Y., and Dernoeden, P.H. 1999. *Pythium* species associated with root dysfunction of creeping bentgrass in Maryland. Plant Dis. 83: 516-520.
3. Hodges, C.F., and Coleman, L.W. 1985. *Pythium*-induced root dysfunction of secondary roots of *Agrostis palustris*. Plant Dis 69:336-340.
4. Martin, F. N. and Loper, J.E. 1999. Soilborne plant diseases caused by *Pythium* spp: ecology, epidemiology, and prospects for biological control. Crit. Rev. Plant Sci. 18(2): 111-181.

APPENDIX

IMPACT OF *P. VOLUTUM* INFECTIONS ON ROOT FUNCTION AND PHOTOSYNTHETIC RATE OF CREEPING BENTGRASS

INTRODUCTION

Creeping bentgrass (*Agrostis palustris* Huds.) is a cool-season grass species that is widely planted on golf courses throughout the U.S. This is the preferred turfgrass species for golf course putting greens because it tolerates extremely close mowing and produces a fast, uniform putting surface (9). Creeping bentgrass is not well-adapted to the persistent hot, humid conditions experienced during summer months in the Southeastern United States, which often results in reduced plant vigor and increased susceptibility to disease. In addition, most putting greens are constructed with artificial root zones containing 85 to 100% sand and 0 to 15% organic matter by volume to promote rapid drainage and drying of the playing surface. However, these root zones have extremely low water- and nutrient-holding capacities (9). While these construction practices can reduce the development of fungal diseases in bentgrass that are encouraged by wet soil conditions, they may also enhance other diseases that are encouraged by bentgrass physiological stress.

Pythium root dysfunction is one of the diseases of creeping bentgrass that is encouraged by physiological stress (6). This disease was first described in Iowa by Hodges and Coleman (4), who observed rapid foliar decline of newly established putting greens with high sand-content root-zones (>60% sand) during the summer months. From affected roots, the authors consistently isolated *P. arrehenomanes* and *P. aristosporum*, and both species were shown to reduce creeping bentgrass root and shoot growth. The authors described the affected roots as buff-colored with devitalized and bulbous root tips. Feng and Dernoeden

(2) identified eight *Pythium* species out of a collection of 28 *Pythium* isolates isolated from 109 samples exhibiting symptoms of PRD. Based on the frequency of isolation and pathogenicity results, the authors concluded that *P. aristosporum* was the most important causal agent of PRD in the Mid-Atlantic States. In North Carolina, *P. volutum* was consistently isolated from creeping bentgrass roots exhibiting symptoms of PRD and was demonstrated to be highly aggressive towards creeping bentgrass roots (6).

Symptoms of PRD typically appear in North Carolina during the summer months as irregular patches ranging from 12 cm to 40 cm in diameter. The affected turf initially exhibits signs of drought stress, but progresses to a yellow to orange foliar decline. If the affected area is left untreated, the turf may die within two to three weeks after symptom development. Examination of infected roots using a soil probe reveals tan colored roots with reduced branching and bulbous root tips. Roots examined during the fall and spring are of normal length, but rapidly die back in response to heat stress. The mechanisms by which these symptoms are induced are unknown.

Kerns and Tredway (6) demonstrated that *P. volutum* infections reduced growth and survival of creeping bentgrass roots when exposed to heat. The authors considered this as a mechanism for symptom expression, but acknowledged that more research was needed to determine the cause. Hodges and Coleman (4) speculated that infections by PRD pathogens impaired water and nutrient uptake by creeping bentgrass roots. As a result, infected plants are thought to be less vigorous and unable to survive the stressful summer months.

Typically nitrogen is the most limiting nutrient in turfgrass systems (9). Nitrate is the primary form of nitrogen acquired by plants, so examining nitrate uptake is a valid estimate

of total N uptake by plant roots (3, 9). Since, nitrate is absorbed by root cells, measurements of nitrate uptake also can be used as an indicator of disruptions in root function (3).

The intent of this study was to begin a detailed evaluation of the impact of *P. volutum* on growth and key physiological processes of creeping bentgrass. The objectives of these experiments were to (i) to investigate the influence of *P. volutum* infections on creeping bentgrass growth and photosynthetic rate; and (ii) examine the impact of *P. volutum* infections on creeping bentgrass nitrate uptake and evapotranspiration rate in hydroponics.

MATERIALS AND METHODS:

Impact of *P. volutum* on nitrate uptake and evapotranspiration. Creeping bentgrass (cv. 'A-1) was seeded (9.7 g m^{-2}) into polyethylene cups with a fine nylon mesh (2 to 5 μm) affixed to the bottom. The cups were placed in cone-tainers (3.8 cm x 20 cm) containing sand meeting USGA specifications (9) (BB 205, Golf Agronomics Inc., Rockingham, NC). The cone-tainers were placed in a greenhouse at 26°C/22°C (12-h day/night cycles) and misted twice daily to encourage rapid germination. Following germination, the turf supplied with a complete nutrient solution containing 106.23 mol m^{-3} nitrogen, 10.41 mol m^{-3} phosphorus, and 111.03 mol m^{-3} potassium, and after four weeks, the solution was supplied once daily. The turf was trimmed weekly with scissors to a height of 1.27 cm.

Six weeks after seeding, each cone-tainer was infested with sterilized creeping bentgrass leaves infested with two highly aggressive *P. volutum* isolates (OC2 and PRD38). The inoculum was prepared by placing three 3-mm agar plugs into sterile water containing five to seven sterilized 1.5-cm long creeping bentgrass leaves. The grass-leaf-blade cultures were incubated under continuous fluorescent light at room temperature (23 to 25^{o}C) for 3 days (1, 6). For inoculation the creeping bentgrass root system was cut at a 5-cm depth, the

turf plug was removed from the cone-tainer, the sand in the cone-tainer was discarded and replaced with fresh sand, 5 to 7 *Pythium*-colonized grass blades were placed onto the surface of the fresh sand, and then the turf plug was placed on top of the colonized grass blades. A non-inoculated control was included by cutting the roots at 5 cm then repotting onto fresh, uninfested sand (6).

Inoculated cone-tainers were transferred to growth chambers with initial growth chamber conditions of 12-h day/night cycles at 24°C/16°C (day/night). After 4 weeks, 12 cone-tainers (six infested and six non-infested) were used to measure nitrate uptake and evapotranspiration. The polyethylene cups were removed from the cone-tainers and sand was gently washed from the root system. Cups with creeping bentgrass plants were placed into 12 L continuous flow hydroponics units with a flow rate of 12 L min^{-1}. Solution temperatures were maintained at a constant 24°C and the canopy temperature was maintained at 32°C. The hydroponics units were housed in an enclosed environmentally controlled walk-in growth chamber. The solution in the hydroponics units contained 0.6 mM KNO_3, 0.5 mM KH_2PO_4, 2 mM $CaSO_4$, 1 mM $MgSO_4$, and 71.6µM Fe as 10% chelated iron, 0.61 µM H_3BO_3, 0.12 µM $MnCl_2$, 0.11 µM $ZnSO_4$, 0.13 µM $CuSO_4$, and 0.003 µM Na_2MoO_4. Solution pH was maintained at 6.5 ± 0.1.

After a 3 d acclimation period, the cups were placed in 350 mL tubes containing the same nutrient solution as before. The tubes fitted with an electronic control system that maintained water levels automatically. The tubes were placed in a water bath to maintain a solution temperature of 24°C. The electronic water control system was fed by individual graduated cylinders initially containing 250 ml deionized water. Six inoculated and non-inoculated creeping bentgrass populations were exposed to oxygenated conditions with air

continually bubbled into the tubes and six inoculated and non-inoculated cups were exposed to anaerobic conditions established by bubbling N_2 gas. The nitrogen gas dissipated all measurable dissolved oxygen (DO) confirmed with a DO meter. Solution samples (1 mL) were taken at 0, 6, 12, 24, 36 hr, and 48 hr after transferring cups to the 350 mL. Nitrate in the samples was quantified using ion chromatography (Dionex Corp. Sunnyville, CA). Evapotranspiration was calculated for each tube from the amount of water dispensed from the graduated cylinders after 48 hr.

After 48 hr, cups were removed from the 350 ml tubes and dipped in 1 mM $CaSO_4$. The nutrient solution in the tubes was quickly replaced with a fresh solution was added with the same concentrations of nutrients as before, with the exception that the solution contained 5% A% $^{15}NO_3^-$ was used. Plants were exposed the labeled ^{15}n for 6 hr. At the end of the exposure period, the creeping bentgrass was harvested with roots and shoots separted from just below the crown tissue. The tissue was dried for 72 hr at 60^{o}C. Dried plant material was pulverized using a Geno 2000 ball grinder (Spex Certiprep, Metuchen, NJ) and 2 to 4 mg of powdered tissue was placed into 8 x 5 mm tin capsules. The tissue was analyzed for total N and ^{15}N using ratio mass spectrometry.

Thirty-six cone-tainers with cups containing populations of creeping bentgrass grown in USGA sand were exposed to 32^{o}C/26^{o}C (day/night) in a separate growth chamber in the North Carolina State Phytotron. Twelve plants were removed after 8, 11, and 17 d of high temperature exposure and were subjected to the analyses, methods, and conditions described above except that the solutions were maintained at 31^{o}C. The entire experiment was conducted twice, except the anaerobic treatment was not included in experiment two.

Impact of *P. volutum* infections on photosynthetic rate of creeping bentgrass.

Deepots (6.4 cm x 25 cm Stuewe and Sons., Inc. Corvallis, OR) containing sand meeting USGA specifications (BB 205, Golf Agronomics Inc., Rockingham, NC) were seeded with 'A-1' creeping bentgrass (9.7 g m^{-1}). The Deepots were placed in a greenhouse at 26°C/22°C (12-h day/night cycles) and misted twice daily to encourage rapid germination. Following germination, the turf was established and maintained according to the procedures outlined above. Six weeks after seeding, each Deepot was infested with sterilized creeping bentgrass leaves infested with a mixture of two highly aggressive isolates of *P. volutum* (OC2 and PRD38), as described above.

Deepots were transferred to a growth chamber and arranged in a split-plot design with two nitrogen treatments and nine inoculated and non-inoculated pots. Initial growth chamber conditions were 12-h day/night cycles at 24°C/16°C. Nitrogen treatments were imposed immediately after deepots were inoculated and transferred to the growth chamber. The two treatments were high nitrogen, daily applications of the nutrient solution and low nitrogen, nutrient solution applied once a week. Deepots receiving the nutrient solution once a week were watered daily with deionized water.

Beginning two weeks after inoculation, photosynthetic rate was measured weekly for several weeks using a Li-COR 6400 (Li-COR Biosciences Lincoln, NE) portable photosynthesis system that had a modified CO_2 chamber to fit tightly onto the Deepots. Grass in the Deepots were cut with scissors to a height of 1.27 cm after inoculation and returned to the growth chambers for an additional week, at which time foliar growth was assessed by removing tissue back to a height of 1.27 cm with scissors. Clippings were dried at 60°C for 3 days and weighed to assess foliar growth rate. A total of two clipping harvests were collected during the inoculation period. Turf quality was assessed visually by

estimating the overall uniformity, density, and color within each cone-tainer every two weeks. Turfgrass quality was quantified using a 1 to 9 scale (9=best, 5=minimally acceptable 1= bare ground).

After four weeks, four of the nine inoculated and non-inoculated pots were destructively sampled to measure root necrosis, root depth, and root mass. Root necrosis was estimated visually on a scale of 0 to 10 (0=no necrosis 10=100% root necrosis). Root depth was assessed by removing the creeping bentgrass plug from the Deepot and measuring the distance from the soil surface to the deepest root tip. The sand was then thoroughly washed from the root system by gently massaging the roots in deionized water. Dry root weights were recorded after drying at 60°C for 72 hr.

Statistical analyses were performed in SAS (version 8.02; SAS Inc., Cary, NC). An analysis of variance (ANOVA) was performed using PROC GLM to estimate the effects of experiment, nitrogen rate, inoculated, and interaction terms on photosynthetic rate, clipping yield and turf quality. Dunnett's t-test was used to compare inoculated pots to the non-inoculated pots within nitrogen treatments.

Impact of *P. volutum* infections and heat on creeping bentgrass photosynthetic rate. The remaining five replicates remained in the growth chamber, and the temperature was raised to 32°C/26°C (day/night) to induce foliar symptom development. Percentage of foliar tissue exhibiting chlorosis or die-back was assessed at 0, 7, 14, 21, and 28 days after heat exposure. Photosynthetic rate and clipping harvests were conducted according to the methods outlined above at 7, 14, 21, and 28 d after the temperature was elevated. After 28 days, all deepots were destructively sampled to measure root necrosis, root depth, and root mass as described above.

All statistical analyses were performed using SAS (version 8.02; SAS Inc., Cary, NC). Analysis of variance was conducted using PROC GLM to estimate the effects of experiment, nitrogen treatment, and inoculation on area under the disease progress curve (AUDPC), root necrosis, root mass (before and after heat exposure), and root depth (before and after heat exposure). Dunnett's t-test was used to compare inoculated pots to the non-inoculated pots for all the dependant variables mentioned above.

RESULTS AND DISUSSION

Impact of *P. volutum* on nitrate uptake and evapotranspiration. In inoculated plants, ^{15}N-nitrate uptake was enhanced under oxygenated and anaerobic conditions prior to heat exposure and 10 d after heat exposure (Fig. 6.1). Foliar symptoms characteristic of PRD developed 11 d after heat exposure. Nitrate uptake dramatically declined 11 d after heat exposure in inoculated plants (Fig 6.1). Nitrate loss from solution mirrored ^{15}N-nitrate, uptake as more nitrate was lost from tubes with inoculated plants than those with non-inoculated plants (Fig. 6.2). Evapotranspiration rates were similar among non-inoculated plants exposed to aerobic and anaerobic conditions and inoculated plants exposed to aerobic conditions throughout the course of the experiment (Fig 6.3). When inoculated plants were exposed to anaerobic conditions, evapotranspiration rates were elevated (Fig 6.3).

The mechanism that results in PRD symptom expression remains unclear. Previous researchers demonstrated reductions in creeping bentgrass growth when plants were inoculated with PRD pathogens (4, 5, 6). Yet, the pathogens did not induce noticible necrosis of roots that would indicate a loss of root function. However, infections by PRD pathogens did result in foliar decline, as a result the researchers hypothesized that infections by PRD pathogens impair nutrient and water uptake (4). This study provides evidence that

infection of creeping bentgrass roots by *P. volutum* does not inhibit nitrate and water uptake during the fall and spring. Nitrate and water uptake was only inhibited after 10 d of exposure to a high temperature regime. On the contrary *P. volutum* infections enhanced nitrate uptake, with less root mass than the non-inoculated controls. It appears that infection by *P. volutum* stimulates a compensatory reaction of creeping bentgrass roots toward nitrate, in order to overcome slight reductions in root depth and root mass.

In our experiments, we did not examine individual roots or infected host cells. Infection of host cells also was not quantified or meticulously examined. These types of analyses may be necessary to determine the mechanisms of *P. volutum* pathogenicity toward creeping bentgrass. Ultrastructral analysis of *Pythium* infection of tomato roots revealed that infection was primarily limited to epidermal and outer cortical tissue with very few hyphae penetrating into the stele (8). This resulted in major cytological changes that included cell disorganization and breakdown, induction of host defense reactions, and alteration of invading hyphae. Although major cytological changes occurred in infected tissue, symptoms did not develop in tomato roots when plants were grown at 25^{o}C/16^{o}C (day/night cycles) (8). These results indicate that *Pythium* infections can be limited to specific areas in mature roots, which implies that individual plants should be utilized for examining root function. The ultrastructural and cytological effects of *P. volutum* infections on creeping bentgrass roots remain unknown and warrant investigation. In summary, *P. volutum* infections enhanced nitrate uptake and did not affect evapotranspiration rates, but additional studies are needed to confirm these observations.

REFERENCES

1. Abad, Z.G., Shew, H.D., and Lucas, L.T. 1994. Characterization and pathogenicity of *Pythium* species isolated from turfgrass with symptoms of root and crown rot in North Carolina. Phytopathology 84:913-921.

2. Feng, Y., and Dernoeden, P.H. 1999. *Pythium* species associated with root dysfunction of creeping bentgrass in Maryland. Plant Dis. 83: 516-520.

3. Glass, A.D. 2003. Nitrogen use efficiency of crop plants: physiological constraints upon nitrogen absorption. Critical Rev. Plant Sci 22(5): 453-470.

4. Hodges, C.F., and Coleman, L.W. 1985. *Pythium*-induced root dysfunction of secondary roots of *Agrostis palustris*. Plant Dis 69:336-340.

5. Hodges, C.F., and Campbell, D.A. 1994. Infection of adventitious roots of *Agrostis palustris* by *Pythium* species at different temperature regimes. Can. J. Bot. 72:378-383.

6. Kerns, J.P., and Tredway, L.P. 2008. Pathogenicity of *Pythium* species associated with Pythium root dysfunction of creeping bentgrass and their impact on root growth and survival. Plant Dis. *Accepted*.

7. Martin, F. N. 1992. *Pythium*. Pages 39-49 in: "Methods for Research on Soilborne Phytopathogenic Fungi". L. L. Singleton et al., eds. The American Phytopathological Society, St. Paul, MN.

8. Rey, P. Benhamou, N., and Tirilly, Y. 1998. Ultrastructural and cytochemical investigation of asymptomatic infection by *Pythium* spp. Phytopathology 88: 234-244.

9. Turgeon, A.J. 1999. *Turfgrass Management*, 5th edition. Prentice Hall, Upper Saddle River, NJ. Pp 49-108.

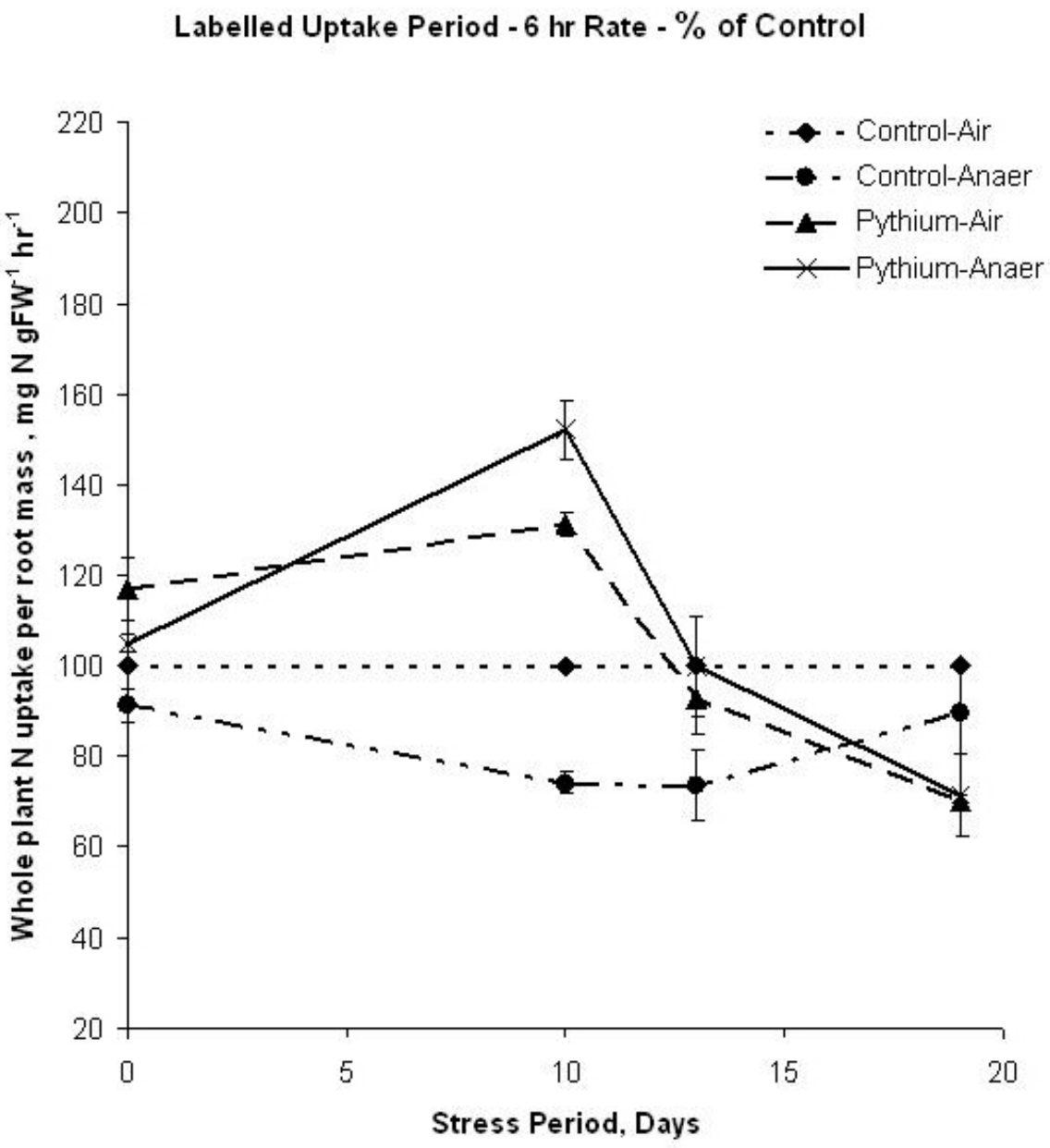

Fig 6.1. Influence of *Pythium volutum* infections on ^{15}N uptake of creeping bentgrass roots in response to aerobic and anaerobic conditions. Plants were exposed to 6 hr of ^{15}N solution. Stress period is the number of days creeping bentgrass plants were subjected to heat stress in sand matrix and in hydroponics.

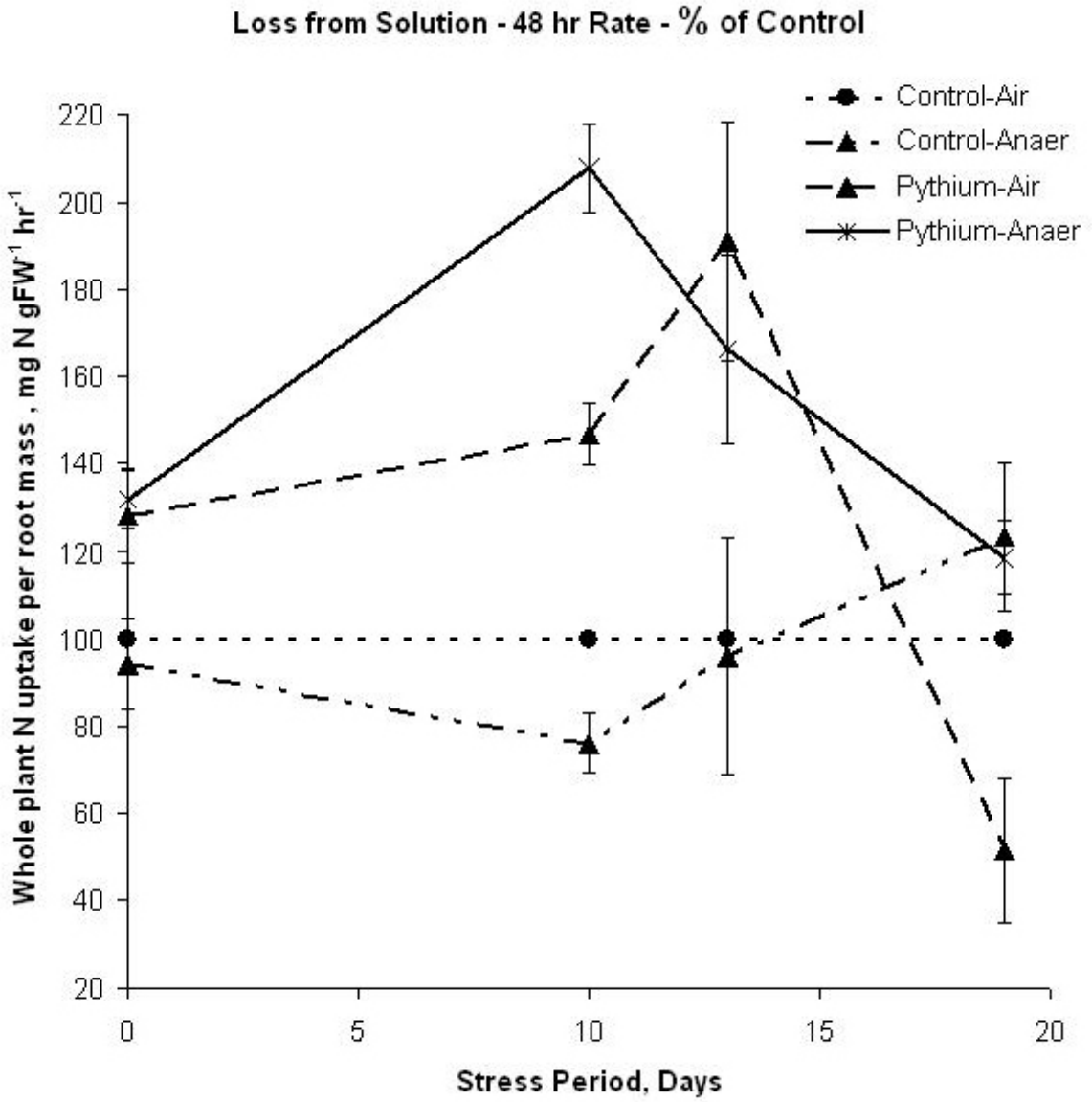

Fig 6.2. Effect of *Pythium volutum* infections on nitrate uptake of creeping bentgrass in response to aerobic and anaerobic conditions. Points on the graph represent total loss of nitrate from the solution over a course of 48 hr for each time period. Stress period represents the total number of days plants were exposed to heat in sand matrix and in hydroponics.

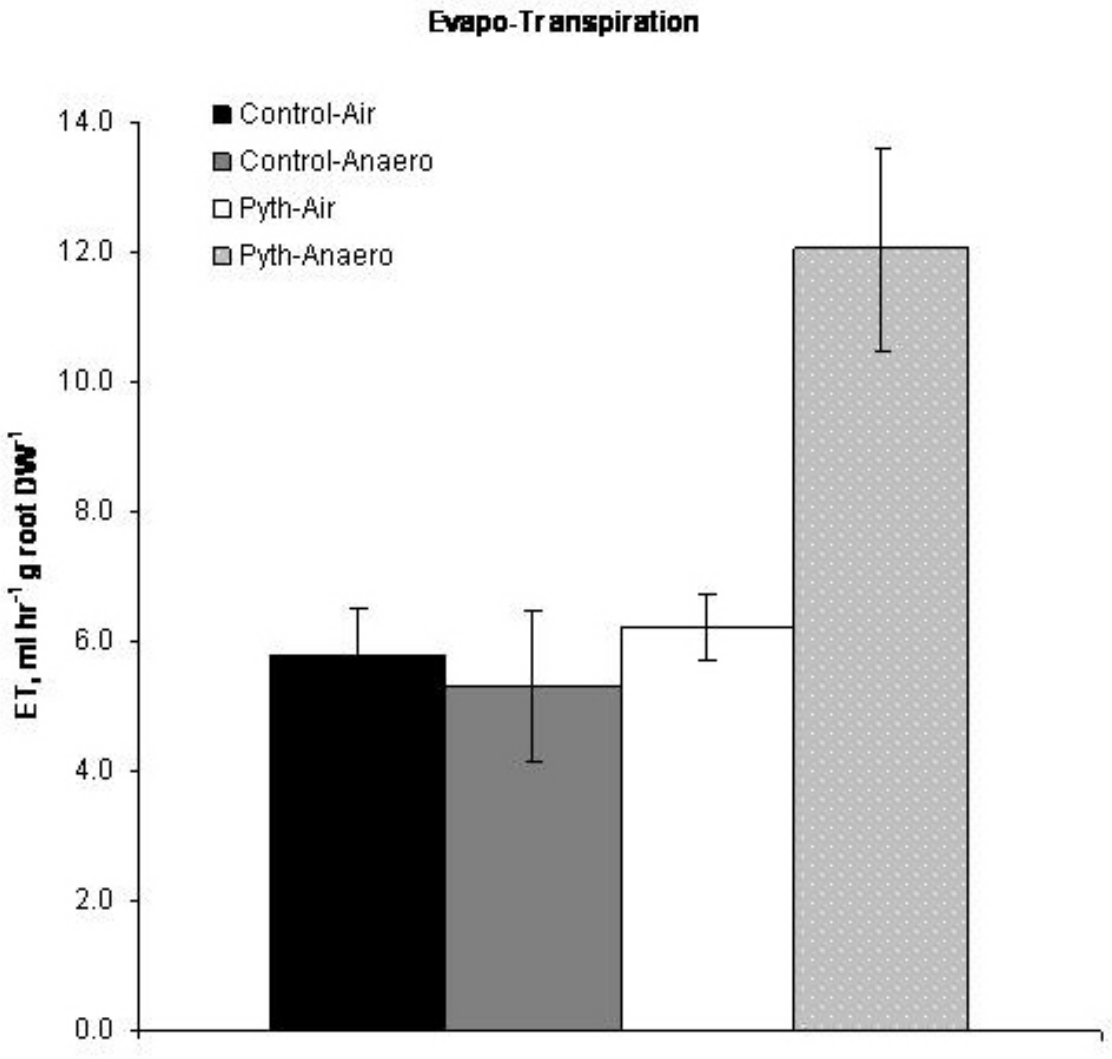

Fig 6.3. Impact of *Pythium volutum* infections on creeping bentgrass evapotranspiration rate in response to aerobic and anaerobic conditions. Evapotranspiration rates were based on the total loss of solution from the experimental apparatus over the course of 48 hr.