ABSTRACT

LEE, DAVID JACKSON. Population Dynamics of Rhizoctonia Species in Tall Fescue and Creeping Bentgrass in Response to Disease Control Programs.
(Under the Direction of Charles H. Peacock and H. David Shew)

Rhizoctonia species are important pathogens of all turfgrasses, yet little is known about the structure of populations of these pathogens or the effect of disease control programs on population dynamics. Isolates of *Rhizoctonia* were obtained from a single creeping bentgrass putting green in 2002 and 2003 and from multiple tall fescue landscapes in 2003. Creeping bentgrass isolates were obtained by placing 5 mm soil cores on alkaline water agar, incubated at ~24 C for 1 to 2 days and the actively growing hyphae were transferred to potato dextrose agar (PDA). Tall fescue isolates were obtained from foliar lesions using the same procedures. Isolates were characterized using morphological characteristics, anastomosis reactions, nuclear staining, and ribosomal DNA (rDNA) sequences. From creeping bentgrass, 21 distinct clones of *R. solani* AG 2-2 IIIB were present in the sample population in 2002. Seven of these clones were also recovered on multiple sampling dates. Fifteen of the 21 clones from 2002 were recovered in 2003. All seven clones that were isolated in multiple sampling dates in 2002 were also recovered in 2003. There were 29 and 21 isolates of *R. zeae* collected from the sample population in 2002 and 2003, respectively. All 21 clones exhibited similar EC_{50} values to mancozeb, chlorothanlonil and azoxystrobin. Of 224 isolates collected from tall fescue during 2003, 88 were *R. solani* AG 1, 105 were binucleate *Rhizoctonia* CAG 1, 14 were *R. zeae*, two were *R. cerealis*, and 15 were not identified to species or AG. One hundred and seventy four of the isolates were assayed for sensitivity to the fungicides

flutalonil, iprodione, tebuconazole, and trifloxystrobin. Mean EC_{50} values varied across fungicides and species, but generally were below 3.3 μg ml^{-1}. The diversity of *Rhizoctonia* populations associated with tall fescue, and the range in fungicide sensitivities among species indicates that response to management programs may vary with location and environment. The effects of phosphorous acid on fungal growth, disease incidence and turfgrass quality was evaluated *in vitro* and *in vivo*. Mean EC_{50} values of azoxystrobin (+SHAM) for *R. solani* 2-2 IIIB, and AG 1, *P. aphanidermatum*, and *R. zeae* were 1.27, 0.04, 0.012, and 0.145 mg L^{-1}, respectively. Applications of phosphorous acid to creeping bentgrass and tall fescue did not significantly reduce brown patch incidence or increase turfgrass quality in field or greenhouse experiments. The benefit of phosphorous acid as a fungicide or plant nutrient is questionable.

INDEX WORDS: Brown patch, *Rhizoctonia*, Population structure, Clonal diversity, anastomosis groupings, Phosphorous acid

POPULATION DYNAMICS OF RHIZOCTONIA SPECIES IN TALL FESCUE AND CREEPING BENTGRASS IN RESPONSE TO DISEASE CONTROL PROGRAMS

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in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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DEDICATION

This work is dedicated to my mother and father who always stressed the importance of education and made the necessary sacrifices for me to achieve my goals.

Thank you and I love you.

-David

BIOGRAPHY

The author of this text David J. Lee was born August 15, 1971, in Durham, North Carolina to Thomas H. and Virginia J. Lee. He graduated from C.E. Jordan High School in June of 1990. He married Wendy Holt in September, 1996, 3 days after Hurricane Fran. While a junior in high school he began work for a local landscape company and the following summer began working at a local golf course. It was during this time that the interest in turf management began.

Mr. Lee attended the University of South Carolina, Columbia, S.C., from 1990-1992. He then transferred to North Carolina State University in spring of 1993 to pursue an Agronomy degree. He graduated Cum Laude in December 1995 with a Bachelor's of Science. During his time at North Carolina State University he continued working at Hope Valley Country Club, Durham, N.C. and for Dr. Charles Peacock at the Turfgrass Field Laboratory. After graduating in 1995, he accepted a position as an Assistant Golf Course Superintendent at Hope Valley Country Club which he maintained from 1995 to 1998. In 1998, Mr. Lee was accepted in the master program in the Crop and Soil Science Department at North Carolina State University. His studies at NCSU were directed by Dr. Thomas W. Rufty Jr., Dr. Charles H. Peacock, and Dr. Arthur Wollum.

During the summer of 2000 he decided to continue his studies at NCSU at pursue a doctoral degree in Crop Science with an emphasis on turfgrass management and plant pathology. His degree requirements were completed in June 2004 with the approval and acceptance of this dissertation. He has accepted an Assistant Professor position in the Department of Horticulture at Louisiana State University.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Plant diseases induced by *Rhizoctonia* species and *Rhizoctonia*-like fungi (Hymenomycetes, Basidiomycota) have been a major focus of plant pathologists for years. The genus was described in 1815 by de Candolle (Parmeter and Whitney, 1970). This original characterization was further refined by Kuhn, who described *Rhizoctonia solani* [teleomorph: *Thanatephorus cucumeris* (Frank) Donk] as the most studied species. The study of *Rhizoctonia* and classification of the fungus has evolved significantly over the past century to further identify distinctive characteristics and subgroups of *R. solani* (Duggar, 1915; Parmeter and Whitney, 1970; Moore, 1987; Sneh et al., 1998). The morphological characteristics used to identify *R. solani* include: 1) brown pigmentation of hyphal cells, 2) branching near the distal septum in young vegetative hyphae, 3) constriction of hyphae and formation of septa near branch origins of hyphae, 4) dolipore septa, and 5) multinucleate cells in young vegetative hyphae (Parmeter et al., 1967). The sexual stage of these organisms are characterized by a web-like hymenium that contain basidia, which lack cross walls and have four prominent sterigmata.

This classification scheme was sufficient to characterize *R. solani* but not *Rhizoctonia*-like organisms that induced diseases of many plants. These species differ from *R. solani* in morphological traits, such as altered pigmentation and the number of nuclei in the emerging young hyphal tissue. Ogoshi (1975) revised the characterization of the genus *Rhizoctonia* to better delineate between *R. solani*, *R. cerealis* van der Hoven (teleomorph: *Ceratobasidium cereale* Murray & Burpee), *R. zeae* Voorhees (teleomorph: *Waitea circinata* Warcup & Talbot), and *R. oryzae* Ryker & Gooch (teleomorph: *W. circinata*) (Ogashi, 1987). Identification of *Rhizoctonia* groups is based on

morphological characteristics of the isolate grown *in vitro*, observation of anastomosis reactions of hyphal fusion with known 'tester' isolates, and DNA sequences (Parmeter and Whitney, 1970; Ogoshi et al., 1985; Kuninaga and Yokosawa, 1980).

Rhizoctonia species induce a variety of diseases on fruits, vegetables, flowers, field crops, turfgrasses and ornamental trees and shrubs worldwide (Agrios, 1997). Symptom type varies widely according to the specific host-pathogen combination, stage of plant growth, plant parts infected, and environmental conditions. The pathogen causes many post-harvest diseases, damping off, stem cankers, foliar blights, sheath and culm blights, and tuber rots.

Rhizoctonia species are the causal agents for diseases of roots and shoots in managed turfgrasses. The major disease incited by *Rhizoctonia* in turfgrasses is brown patch, or *Rhizoctonia* blight, of foliar tissue. This disease has been associated with 12 species of turfgrass that include both warm and cool season grasses (Couch, 1985). The nomenclature for these diseases has not been very clear and there remains some confusion. *Rhizoctonia* diseases are typically diagnosed based on host species, time of year, and symptomology. Identification of the causal agent is rarely conducted to the species or anastomosis group level. *Rhizoctonia zeae*, *R. cerealis*, *R. oryzae*, binucleate *Ceratobasidium* spp., and 6 anastomosis groups (AG's) of *R. solani* have been associated with causing diseases of turfgrasses (Burpee and Martin, 1992; Zhang and Dernoeden, 1995). Further studies on the ecology, etiology, and population dynamics of these species and subgroups of *Rhizoctonia* in turfgrass are necessary to improve accuracy of diagnosis and facilitate the development of ecologically-based management programs.

Biology of Rhizoctonia

Species of *Rhizoctonia* are found worldwide. *Rhizoctonia* survives as a saprophyte and as a facultative parasite (Agrios, 1997). Survival during unfavorable periods occurs as sclerotia, sometimes referred to as 'bulbils' and/or monilioid cells in the soil, organic litter, infected plant tissue or on the bases of grass plants. These masses of cells (sclerotia) or chains of swollen hyphal cells (monilioid) remain dormant until environmental conditions favor germination or growth. Temperature optima for growth vary among species and anastamosis groups. Optimum temperatures for growth of *Rhizoctonia* species range from 21 to 32°C (Smiley et al., 1992). Under optimum environmental conditions, hyphae grow and rapidly cover leaf or sheath surfaces. Colonization of the leaf surface is followed by the formation of specialized infection structures that aid in the penetration of host tissue. The role of enzymes and toxins in the development of *Rhizoctonia* diseases is not clear. However, there have been approximently 20 host specific toxins that have been associated with specific host-pathogen interactions and are necessary for disease development (Markham and Hille, 2001). On individual leaf blades, irregular water-soaked lesions initially appear, which then turn brown to tan, with a dark brown border. Lesions expand over time and may span the entire width of the leaf blade, causing a foliar blight.

Dissemination occurs via growth of hyphae among leaves and turf plants. This leaf-to-leaf and plant-to-plant spread results in production of circular patches in the turf ranging from 0.1 to 1.0 m or more in diameter. Under ideal environmental conditions, these patches can coalesce and form large blighted areas in the turf. As environmental conditions shift towards less favorable conditions for pathogen growth, additional

sclerotia and monilioid cells are formed and remain in the soil until favorable conditions return. Therefore, during the course of several months (May to September in North Carolina) the mycelial growth from survival structures may be induced and ended multiple times; the number and severity of these cycles is directly dependent on environmental conditions.

The sexual stage of *R. solani* (*Thanatephorous cucumeris*) also has been observed in turfgrasses (Burpee and Martin, 1992). The basidiospores, usually 4 per basidium, develop on hymenia that are predominantly formed on the abaxial surface of the leaf. The hymenia are visible as a thin white or gray layer on the leaf surface. The relative importance of basidiospores in the disease cycle of brown patch is unknown, but could have a major impact on the genetic structure of populations if it occurs with significant frequency.

Hyphal Anastomosis

Of the *Rhizoctonia* species with a known teleomorph stage, *Thanatephorus, Waitea*, and *Ceratobasidium*, each is further subdivided based upon the ability of individuals to anastamose with other individuals belonging to the same group (Carling and Sumner, 2001). Anastomosis, or the fusion of hypha between different individuals, may result in the sharing of genetic material without sexual reproduction, but it also serves to isolate individuals from other members of the same species that do not share the same alleles for somatic compatibility (Agrios, 1997). This transfer of genetic information is an important mechanism of genetic recombination in fungi that remain in the imperfect state for long periods of time (Cubeta and Vilgalys, 1997).

Anastomosis reactions range from complete fusion of cell walls and membranes (self reactions), to an initial fusion and subsequent cell death (typical of reactions of isolates of the same anastomosis group called the necrotic reaction), to no reaction at all or massive cell death (Carling and Sumner, 2001). There are currently 13 anastomosis groups currently recognized in *R. solani* alone (Carling and Sumner, 2001; Sneh et al., 1998). These groups have been further divided into subgroups based on morphological and physiological characteristics, host range, and geographic distribution.

Rhizoctonia and Turfgrasses

The genus *Rhizoctonia* has been associated with diseases of turfgrass since 1914 (Piper and Coe, 1919), when *R. solani* (Kuhn) was identified as a pathogen of creeping bentgrass (*Agrostis palustris* Hudson). This species was thought to be the causal agent of diseases in at least 12 different turfgrass species (Couch, 1995). In the last 25 years, turfgrass pathologists have identified additional *Rhizoctonia* species that are responsible for causing diseases in some of these hosts (Burpee, 1980; Burpee et al, 1980; Martin et al., 1984a; Martin et al., 1984b; Oniki et al., 1986a).

Many species of *Rhizoctonia*, both pathogenic and nonpathogenic, have been shown to coexist in the turf-soil ecosystem (Martin et al., 1984a). Induction and development of diseases caused by certain species of *Rhizoctonia* is often driven by temperature optimums for growth. *Rhizoctonia cerealis*, the cause of “yellow patch”, grows optimally at temperatures ranging from 10 to 20°C (Burpee, 1980). Martin et al. (1984a) identified *R. solani*, binucleate *Rhizoctonia*-like fungi, and *R. zeae* from tall fescue swards in North Carolina. *Rhizoctonia solani* often infect cool-season grasses

when temperatures range from 20 to 30°C, but certain anastomosis groups, e. g. AG 2-2 IV, infect warm-season grasses at temperatures below 20°C and are thought to be a 'cool-season' biotype of *R. solani* (Burpee and Martin, 1992; Oniki et al., 1986a). *Rhizoctonia zeae* has long been associated with growth and disease at high (>30 C) temperatures (Ryker and Gooch, 1938; Martin et al., 1984a). Recent research on a creeping bentgrass putting green inoculated with an isolate of *R. solani* AG 2-2 IIIB and *R. zeae* isolate indicated that *R. zeae* may become the dominant pathogen when temperatures consistently exceed 32°C (Royals, 2002).

Multinucleate Species

Rhizoctonia solani

Members of at least four anastomosis groups within *R. solani* (AG 1, AG 2, AG 4, and AG 5) are pathogenic to turfgrasses (Burpee and Martin, 1992). These subgroups have been further divided based primarily on morphological differences and symtomology (Table 1.1). Initially, *R. solani* isolates from tall fescue were assigned to AG 1 subgroup undesignated (Martin and Lucas, 1984a). However, these isolates are now most frequently placed in AG 1-IA. This subgroup is also associated with sheath and leaf blights of many crop and weed species (Sneh et al., 1998).

The anastamosis group AG 2 is divided into 4 subgroups [AG 2-1, AG 2-2 IIIB, AG 2-IV, and AG 2-2(unspecified)] based on pathogenicity and nutritional requirements (Sneh et al., 1998). Separation of AG 2-1 from AG 2-2 is based on the nutritional requirement for thiamine (Ogoshi, 1987; Rovira et al., 1986). All of the AG 2 subgroups induce disease in turfgrasses. Differentiation of subgroups of AG 2 are made based on

symptomology, physiology, and host range. AG 2-2 IV is associated with a rot of crown and leaf sheath tissue in warm-season grasses, called “large patch”. AG 2-2 IIIB mainly infect stems, sheaths, and leaves, causing ‘brown-patch’ of creeping bentgrass (Hurd and Grisham, 1983; Oniki et al., 1986a; Haygood and Martin, 1990).

Rhizoctonia solani AG 4 and AG 5 are also pathogenic to turfgrasses (Burpee and Martin, 1992; Martin and Lucas, 1984a), but the significance of these AGs in turfgrasses is not well understood. Anastamosis group 4 is a soilborne pathogen that incites seedling damping off and root rots of many hosts worldwide (Carling and Sumner, 2001). This group is subdivided into HG-I and HG-II based on DNA homology, but not by the frequency of hyphal anastamosis (Vilgalys, 1988). The AG 4 group is defined as the ‘praticola’ type according to some taxonomists and may represent a different species of *Rhizoctonia, R. praticola* (Parmeter and Whitney, 1970; Ogoshi, 1985). Martin and Lucas (1984a) isolated and identified AG 4 isolates as causal pathogens for damping off of red fescue (*Festuca rubra*), and crown rot of perennial ryegrass (*Lolium perenne*) and Tifton 419 bermudagrass [*Cynodon dactyon* (L.) Pers. X *Cynodon transvaalensis* (Burtt-Davy)].

<u>*Rhizoctonia zeae*</u>

Rhizoctonia zeae was first documented as a pathogen in 1934 when it was found to incite ‘sclerotial rot’ of corn (Voorhees, 1934). The organism also causes ‘sclerotial’ diseases of rice and Italian millet and has been associated with damping-off of pine seedlings and legumes (Saksena and Vaartaja, 1960; Wong and Sivasithamparam, 1985; Sneh et al., 1998).

Rhizoctonia zeae has been associated with diseases of centipedegrass (*Eremochloa ophiuroides*), St. Augustinegrass (*Stenotaphrum secundatum*), bermudagrass, tall fescue, and creeping bentgrass (Burpee and Martin, 1992). Symptom development is dependent on host species and cultural management practices. In higher mowed turfs (i.e. tall fescue, St. Augustinegrass, and centipedegrass), the organism has been isolated from foliar lesions that are similar in appearance to those caused by *R. solani*. Foliar lesions induced by *R. zeae* on warm-season grasses often appear as water soaked areas located at the lower basal portion of the leaf sheaths. *R. zeae* appears as grey to yellow arcs or circles on creeping bentgrass putting greens. Symptoms appear similar to 'brown patch' induced by *R. solani* and cannot reliably be distinguished based on symptoms; however, with *R. zeae* a dark grey 'smoke ring' may not form and the patches do not have the orange coloration commonly associated with brown patch caused by *R. solani* (Burpee and Martin, 1992).

Other than the requirement for high temperatures the epidemiology of diseases caused by *R. zeae* on turfgrasses is not well understood (Ryker and Gooch, 1938). A detailed study on the ecology of *R. zeae* in a creeping bentgrass putting green was conducted in South Carolina (Royals, 2002). The investigators monitored shifts in the populations of *R. zeae*, *R. oryzae*, and *R. solani* isolates from 1999 to 2001. In growth chamber experiments, *R. zeae* was more virulent to creeping bentgrass at 32 or 34 ^{o}C than *R. solani* and *R. oryzea*. Isolates of *R. solani* and *R. oryzae* were more aggressive at 28°C compared to *R. zeae*. The researchers also documented increased field populations of *R. zeae* in plots treated with thiophanate-methyl, further substantiating previous reports on

the lack of sensitivity of *R. zeae* to benzimidazole fungicides *in vitro* (Martin et al., 1984a).

Binucleate Species

<u>*Rhizoctonia cerealis*</u>

Binucleate *Rhizoctonia* species also have been associated with diseases of turfgrasses (Table 1.2) (Burpee et al., 1980; Dale 1978; Sanders et al., 1978). Many of the binucleate *Rhizoctonia* spp. have a moderate temperature optimum for growth (~24°C) and represent a single taxonomic species, *R. cerealis.* This species causes chlorosis of foliar tissue; hence, it was proposed that the disease name be changed from 'cool-weather brown patch' to 'yellow patch', which more accurately describes the symptoms incited by this organism (Burpee, 1980). Host turfgrasses for *R. cerealis* include creeping bentgrass, tall fescue, Kentucky bluegrass (*Poa pratensis* L.), zoysiagrass (*Zoysia japonica*), and bermudagrass.

Other binucleate *Rhizoctonia* spp. that differ morphologically from the isolates of *R. cerealis* and that exhibited higher temperature optima for growth have been isolated from turfgrasses (Martin and Lucas, 1983; Martin et al., 1984b; Sanders et al., 1978; Hurd and Grisham, 1983). There have also been other reports of binucleate species inciting diseases in turf, but the taxonomy of these isolates is largely unknown (Burpee and Martin, 1992).

Non-pathogenic binucleate *Rhizoctonia* spp. have biocontrol characteristics against pathogenic *R. solani* in turfgrasses and other plant systems (Burpee and Goulty, 1984; Cardoso and Echandi, 1987a; Cardoso and Echandi, 1987b; Hwang and Benson,

2003; Goodman and Burpee, 1991; Yuen et al., 1994). Creeping bentgrass cv. 'Pencross' inoculated with 3 different binucleate isolates in combination with a pathogenic *R. solani* isolate exhibited significantly less brown patch symptoms as compared to the plots that were inoculated with a virulent *R. solani* isolate alone (Burpee and Goulty, 1984). All 3 of the binucleate isolates in combination with the virulent *R. solani* isolate reduced disease severity; however, 2 of the 3 binucleate isolates tested in combination with the *R. solani* isolate reduced percent disease to that of the uninoculated control. The binucleate isolates alone did not cause 'brown patch' symptoms on creeping bentgrass.

Although it has been shown that some binucleate *Rhizoctonia* species may be effective as biological control agents, it remains unclear what mechanisms are responsible for disease suppression. There is some evidence that binucleate *Rhizoctonia* can induce host defense mechanisms in some host-pathogen interactions (Hwang and Benson, 2003). The potential of binucleates to suppress turfgrass diseases may repsent an important component of Integrated Pest Management (IPM) programs for *Rhizoctonia* diseases.

Population Biology of *Rhizoctonia solani*

The population biology of *R. solani* in turfgrasses is not well understood. The number of anastamosis groups and subgroups present in the turf ecosystem, and the traditional taxonomic traits used for identification, make studies of population structure in this pathogen difficult (Ogashi, 1987; Cubeta and Vilgalys, 1997; Sneh et al., 1998). The two primary methods for determining population structure in *R. solani* are somatic

compatability and selected genetic markers (Sneh et al., 1991; Ceresini et al. 2002a, Ceresini et al. 2002b, Ceresini et al., 2002c; Cubeta and Vilgalys, 1997).

Differentiation of anastomosis groups within *R. solani* has traditionally been conducted through the evaluation of somatic reactions. Somatic reactions are separated into 4 types as outlined by MacNish et al. (1993) and Cubeta and Vilgays (1997). The 4 reaction types are: 1) C0 (No reaction), paired hypha grow past each other with no recognition; this indicates that the paired isolates belong to different AG groups, 2) C1 (Hyphal contact only), opposing hyphae make contact and may result in cell death; this reaction indicates that the paired isolates are distantly related and may belong to the same AG group, 3) C2 (killing reaction), fusion of cell walls is evident and cell death occurs at the fused cells; this indicates that the paired isolates belong to the same AG group but are genetically distinct, 4) C3 (perfect fusion) the fusion of cell walls and membranes is evident; this reaction type indicates that the paired isolates are clones, or genetically identical to one another.

Somatic compatibility reactions have been used to characterize populations of *Rhizoctonia* in field crops (MacNish et al., 1993; Ogoshi and Ui, 1983). Anastomosis reactions are sometimes difficult to classify because of an apparent continuum of reactions due to the complexity of the mating systems (homothallic vs heterothallic), different genetic states of fusing hyphal cells (homokaryons, heterokaryons, or both), timing of somatic observations, and variability in isolate preparation and laboratory conditions (Adams, 1996; Hyakumachi and Ui, 1987; Julian et al. 1996; Yokoyama and Ogoshi, 1988). A methodology to allow improved efficiency, detection and characterization of mycelial interactions between isolates of *R. solani* using a selective

media has been developed Gutierrez et al. (2004). The basal medium consists of dilute potato dextrose agar (PDA, 30 g/L) (Difco, Detroit, MI) to which red McCormick® food-color dyes (McCormick & Co., Hunt Valley, MD 21030) was added to enhance visualization of somatic reactions.

Molecular techniques and genetic markers represent an alternative to somatic compatibility for evaluation of population structure in *Rhizoctonia* (Ceresini et al. 2002a, Ceresini et al. 2002b, Ceresini et al., 2002c; Vilgalys and Cubeta, 1994). Genetic markers developed and used to identify *Rhizoctonia* species to the anastomosis group level include cellular fatty acids, DNA/DNA hybridization, electropheretic karyotyping, isozymes/zymograms, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), rDNA RFLP, and scn DNA RFLP. Of the previously mentioned techniques, DNA fingerprinting, electropheretic karyotyping, isozymes/zymograms, RAPD RFLP, and scn DNA RFLP have been used to identify genetic variation within anastomosis groups (Cubeta and Vilgalys, 1997).

The combination of somatic compatibility and molecular methods has emerged as one of the most effective ways to study the structure of *R. solani* populations on a local or regional scale. A study of *R. solani* AG 8 in West Australia using hyphal anastomosis and pectic zymograms showed that the isolates that demonstrated C3 (clonal) hyphal reactions belong to the same pectic zymogram group (MacNish et al., 1993). Using the same methods, this study showed that some of these clones were distributed over a broad geographical region; the same clones were found in Washington, Oregon and West Australia.

Ceresini et al. (2002a) studied the genetic structure of *R. solani* AG 3 populations in potato and tobacco from North Carolina using anastomosis pairings and genetic markers. All 32 isolates from potato had distinct AFLP phenotypes, and 28 somatic compatibility groups were identified by pairing *in vitro*. Of 36 AG 3 isolates from tobacco, 8 clones and 28 distinct AFLP phenotypes were identified. In isolates from potato compatible somatic interactions were only observed among isolates collected from the same field. In tobacco, clones were found at multiple sites and in different counties in North Carolina. The repeated recovery of clonal isolates from different locations was an indication that clones persist and are important in the population structure of *R. solani* AG 3 in a field and across a larger geographical area. Based on these criteria, the authors concluded that the *R. solani* AG 3 population structure has a clonal structure, with isolates from tobacco primarily clonal while potato isolates were introduced into fields on seed potatoes (Ceresini et al., 2002a; MacNish et al., 1997).

Ceresini et al. (2002b) also focused on the population structure of *R. solani* AG 3 within a single potato field in each of five counties in eastern North Carolina. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to identify genotypes in these populations. The study revealed a significant amount of gene flow among populations. Further evidence from pooled North Carolina populations indicated random association of alleles. However the analysis of all genotypes indicated alleles within and between loci that were not randomly associated. Thus, the results support a population model that includes both clonal individuals and some that evolve through recombination.

The perennial nature of managed turfgrasses distinguish them from other annual cropping systems. The perennial nature may impact the genetic structure of pathogen populations. There are a limited number of studies on the population structure of turfgrass pathogens. Tredway et al. (2003) developed a PCR-based assay to determine the mating type of *Magnaporthe grisea* isolates and determined that sexual recombination did not have a significant effect on population structure based on the presence of a single mating type in the majority of populations associated with tall fescue (*Festuca arundinacea*) and St. Augustinegrass (*Stenotaphrum secundatum*) in Georgia. A study using restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD) markers was done to evaluate the genetic variability of 181 *Sclerotinia homoeocarpa* isolates from Ontario, Canada and Japan. Isolates from Canada were highly similar to one another but distantly related to the Japan isolates. Of the Canadian populations, four were characterized by significant linkage disequilibrium, suggesting a clonal population structure (Hsiang and Mahuku, 1999).

Knowledge of population structure of *R. solani* isolates in managed turfgrass systems is very limited. Aside from the work done by Royals (2002), described above, no other studies have investigated how natural populations are constructed at any level. Understanding the structure of these populations will provide more information on the biology of the *R. solani* organism in managed turf. This information will be directly applied to developing more effective management strategies that may optimize fungicide application efficiency, reduce the total amount of fungicides used, and provide turfgrass breeders information to aid in the development of resistant cultivars.

Fungicides, Phosphorous Acid, and Disease Control

Maintaining plant health through fertilization to provide essential nutrients for plant growth is one of the most effective ways to minimize damage from any pest. Historically, research to evaluate the effect of plant nutrients on diseases in turf has focused on nitrogen (N) and potassium (K). Nutritional effects on turfgrass diseases were first reported in the literature as early as 1950, and studies on plant nutrients and disease interactions have continued through the present (Smith et al., 1988). Although the use of properly timed and applied nutrients can reduce disease development, these practices rarely achieve desired levels of control for high value turf areas, such as golf course putting greens. Therefore, fungicide applications have become a tool that many turf managers rely upon for disease control.

The use of chemicals for disease control in turfgrass began in 1917 with the use of Bordeaux mixture for control of brown patch, induced by *R. solani* (Monteith and Dahl, 1932). The emergence of inorganic and organic mercury compounds and other heavy metals alone, or in combination with thiram and cadmium, were the primary fungicides used from the 1930's to 1960's. These products provided broad spectrum control of many turf pathogens but often resulted in phytotoxicity to the turfgrass plant. These compounds also persist in the environment and have toxic effects on non-target organisms and for these reasons were eliminated from the US market in the 1960's. Removal of these broad spectrum materials from the market, and the development of resistance by *Sclerotinia homoeocarpa* to cadmium fungicides in the 1960's, provided the impetus for the development of multiple fungicide classes and has drastically changed the area of turfgrass pathology and turfgrass disease management.

Currently, there are 13 classes of fungicides used for control of turfgrass diseases. These fungicides include both contact and systemic groups that provide a wide array of control against many pathogens. There has been increased interest in the chemical class phosphonate. This group of fungicides includes aluminum (Al^{3+}), (K^{+}), and ammonium (NH_4^{+}) salts of phosphite (phosphorous acid) that are marketed as fungicides and a source of phosphorous (P) to the turf industry. The phosphonate fungicide, fosetyl-Al (Bayer Environmental Sciences, Montvale, NJ), is effective for control of oomycete fungi, such as *Pythium* species, which are common turfgrass pathogens (Sanders, 1984; Watkins, 1983). The biochemical mode of action of these materials remains largely unknown. The majority of the evidence collected to date indicates that these products induce host defense mechanisms and directly inhibit fungal growth inside the plant (Smillei et al., 1989; Fenn and Coffey, 1984; Jiang and Grossman, 1992).

Phosphonate fungicides (Signature, Bayer Environmental Sciences, Montvale, NJ), applied in combination with mancozeb (Fore, DowAgro Sciences, Indianapolis, IN), was shown to produce a synergistic effect that provided excellent control of brown-patch and increased overall turf quality on creeping bentgrass (Mudge, 1997). However, it remains unclear which component of this recommended tank mix (fosetyl-Al, mancozeb, or malachite pigment in mancozeb) has the most efficacy against pathogens.

Mancozeb and related ethylene dithiocarbamate (EBDC) fungicides provide effective control of leaf spots, red thread, rusts, and brown patch (Gould, 1967). The use of malachite pigment for control of turf diseases has been documented as early as 1933 in Britain (Smith et al., 1988). Further work by Smith (1953; 1957) found a solution concentration (100 $\mu g\ ml^{-1}$) of the green malachite pigment was effective in reducing

microdochium patch, red thread disease, and the mycelial stage of some *Pythium* diseases. Aside from the fungicidal effects reported, this material also has been associated with stimulated growth of grass, and has been used as a tracer for other pesticides or as a dye to increase the aesthetic quality of the turf (Smith, 1957; Karnok, 1984).

More recently, interest has focused on the efficacy of phosphonate fungicides, with phosphorous acid as the active ingredient, and fertilizers that contain phosphorous acid (also referred to as phosphite) on disease suppression and overall plant health. In tomato and pepper systems, phosphorous acid was effective in reducing the incidence of Phytopthora root and crown rot caused by *Phytophthora capsici* (Förster et al., 1998). Additional studies were conducted in North Carolina in 1995 and 1996 on the efficacy of foliar applications of phosphorus acid, fosetyl-Al, and traditional fungicides on the overall turf quality of creeping bentgrass. There was a positive correlation between phosphorous acid applications and turf quality throughout the summers of 1995 and 1996, and these treatments resulted in turf quality that was comparable to fungicide applications of fosetyl-Al alone and in combination with mancozeb (Dorer, 1996).

Research Objectives

Formulating Integrated Pest Management (IPM) programs that do not compromise the integrity of the environment or the quality of the turf is essential. Components of successful IPM programs employ both cultural and biological control methods to reduce total chemical inputs while maintaining high quality turfgrass (Bruneau et. al., 1992). Proper cultural practices include cultivar selection, proper

mowing practices, irrigation timing and frequency, and providing adequate nutrients for plant growth. Another approach to optimize disease management strategies is to develop a better understanding of pathogen biology. Much research has been conducted on the impact of nutrients on the incidence of *R. solani* in turf systems (Burpee, 1995; Fidanza and Dernoeden, 1996; Smiley et al., 1992; Bloom and Couch, 1960; Dorer, 1996; Englehard, 1989; Green et al., 1999; Gross et al., 1998). Researchers in other agronomic crops have been working on understanding the population biology of *R. solani* from a regional and localized geographic scale (Cardoso and Echandi 1987; Ceresini et al. 2002, Ceresini et al. 2002, Ceresini et al., 2002; Vilgalys and Cubeta, 1994). This research was initiated to:

1. Determine the effect of phosphorous acid on the incidence and development of brown patch in tall fescue and creeping bentgrass *in vivo*.
2. Measure the sensitivity of *R. solani* AG 1, *R. solani* AG 2-2 IIIB, *R. zeae*, and *Pythium aphanidermatum* to phophonate fungicides, phosphorous acid, and azoxystrobin.
3. Characterize the clonal population structure of *R. solani* 2-2 IIIB in a creeping bentgrass putting green, using anastomosis pairings and molecular techniques, over a 2-yr period.
4. Determine if shifts in populations of *R. solani* 2-2 IIIB clones are induced by disease management programs.
5. Identify the composition of *Rhizoctonia*-like isolates collected from foliar lesions in tall fescue landscapes.

6. Measure the sensitivity of *R. solani*, CAG-1, *R. zeae*, and *R. cerealis* isolates collected from tall fescue turf swards to iprodione, trifloxystrobin, tebuconozole, and flutalonil.

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Table 1.1. Anastamosis groups, subgroups and distinguishing characteristics of *R. solani* subgroups associated with turfgrasses (Adapted from Sneh et al., 1997; Zhang and Dernoeden, 1995).

Anastomosis Group	Subgroup	Characteristics and Hosts
AG 1	IA	Colonies light brown to brown; large (2-5mm) sclerotia; rapid growth rate (30mm/d); most commonly associated with brown patch of *Festuca arundinacea*, *Lolium perenne*, *Poa annua*, *Poa pratensis*, and *Festuca rubra*.
	IB	Mycellium darker brown when compared to AG 1 IA; smaller irregular shaped sclerotia that appear compounded into a single sclerotia. Most commonly associated with brown patch of *Festuca arundinacea*, *Lolium perenne*, *Poa annua*, *Poa pratensis*, and *Festuca rubra*.
AG 2	2-2IIIB	Colonies dark brown; higher temp optimum (~30C); small sclerotia (1-2mm); concentric color zones. Associated most commonly with brown patch of *Agrostis* spp.
	2-2 IV	Causes large patch on warm season grasses. Most commonly associated with crown and leaf sheath rots of warm season turfgrasses.
AG 4		Considered *R. practicola* type; Sclerotia may appear dark brown on PDA (HG-1 subgroup) or whitish gray/brown (HG-II). Associated with seedling damping offs and root rots of many hosts
AG 5		Considered weak pathogens on turfgrasses; Associated with damping off of *Festuca rubra*, and Crown rot of *Lolium perenne* and *Cynodon dactyon* (L.) Pers. X *C. transvaalensis* (Burtt-Davy).

Table 1.2. Species, subgroups and distinguishing characteristics of other multinucleate *Rhizoctonia* species and binucleate *Ceratobasidium* species in turfgrasses (Adapted from Sneh et al., 1997; Zhang and Dernoeden, 1995; Burpee and Martin, 1992).

Species	Subgroup	Characteristics and Hosts
Multinucleates:		
R. zeae	WAG-Z	Colonies appear white to orange colored on PDA; Sclerotia small (0.5-1.0 mm) and dark reddish brown and form submerged in agar; rapid growth rate (30mm/d); most commonly associated with brown patch of *Festuca arundinacea*, *Agrostis palustris*, *Lolium perenne*, *Poa annua*, *Poa pratensis*, and *Festuca rubra*.
R. oryzae	WAG-O	Colonies appear white to salmon colored on PDA; Sclerotia vary between (<1.0 - >3.0 mm) and form on the agar surface. *Festuca arundinacea*, and *Agrostis palustris.*
Binucleates:		
C. gramineum	AG-D	Colonies are white to buff on PDA. Moderate temperature optimum (~24°C), some isolates fail to form sclerotia. causes a yellowing (chlorosis) of infected plant tissue; hosts include *Agrostis palustris*, *Festuca arundinacea*, *Poa pratensis, Zoysia japonica*, and *Cynodon* spp.
C. cornigerum	AG-Q	Colonies are white to buff on PDA. Sclerotia do not form. Slower growing (1.3 mm/d) than AG-D.

CHAPTER 2

INFLUENCE OF PHOSPHOROUS ACID ON THE INCIDENCE OF BROWN PATCH CAUSED BY *RHIZOCTINIA SOLANI* IN TALL FESCUE AND CREEPING BENTGRASS

ABSTRACT

Lee D. J., Tredway L. P., Shew H. D., and Peacock C. H.   2004. The effects of phosphorous acid compounds on the incidence of *Rhizoctonia solani* in tall fescue and creeping bentgrass. To be submitted to Plant Disease.

Phosphorous acid compounds are effective for control of certain fungal pathogens. The objective of this study was to evaluate the effect of phosphorous acid on *Rhizoctonia* species and *Pythium aphanidermatum in vitro* and *in vivo*. One isolate each of *Rhizoctonia solani* AG 1, *R. solani* AG 2-2 IIIB, *R. zeae*, and *Pythium aphanidermatum* were obtained from turfgrasses and assayed for sensitivity to phosphorous acid and the fungicides fosetyl-Al, azoxystrobin, and azoxystrobin amended with salicylhydroxamic acid (SHAM). EC_{50} values for each isolate was determined by measuring radial growth in potato dextrose agar amended with 0, 0.01, 0.1, 1.0, or 10 µg ml^{-1} of each fungicide. Mean EC_{50} values were observed for all four pathogens with the azoxystrobin (+SHAM) amended agar. Effective concentrations for the *R. solani* 2-2 IIIB, *R. solani* AG 1, *P. aphanidermatum*, and *R. zeae* were 1.27, 0.04, 0.012, and 0.145 mg L^{-1}, respectively. Azoxystrobin without SHAM did not reduce colony growth by 50 % in the 2-2 IIIB and *R. zeae* isolate. Phosphorous acid and fosetyl-Al did not inhibit mycelial growth below 10 µg ml^{-1}. Phosphorous acid applications did not reduce disease incidence of *R. solani* in tall fescue and creeping bentgrass turf. Phosphorous acid applications increased the elemental phosphorus composition of shoot tissue, but resulted in phytotoxic injury in field and greenhouse experiments. Phosphorous acid applications

should not be considered adequate substitutes for phosphonate fungicides or phosphate fertilizers.

INTRODUCTION

Brown-patch, induced by multiple anastomosis groups (AG) of *Rhizoctonia solani*, is a common disease of cool-season turfgrasses grown in temperate and sub-tropical climates. Brown patch is the most common and destructive disease of creeping bentgrass putting greens and tall fescue landscapes in the Southeast United States (8,20). *Rhizoctonia* species are also thought to be a component of "summer decline' syndromes observed in these grasses. Extended periods of high temperatures and relative humidity present in the Southeastern U.S. enhance disease development on these cool-season turfgrasses from May through September. Control measures for *Rhizoctonia* species vary widely and are often dependent on the primary use of the turf as well as the availability of resources. Traditionally, fungicide use is the most effective control method. However, increasing public concerns over the presence of pesticides in the environment may limit the number of options available for control of this group of pathogens.

Formulating Integrated Pest Management (IPM) strategies that do not compromise the integrity of the environment or turfgrass quality is essential. A primary goal of any IPM program is to minimize total chemical inputs (5). To accomplish this goal, multiple strategies and approaches have been investigated for management of *Rhizoctonia* species in turfgrasses. One approach involves fertilization prior to periods that are conducive to disease development. Nutrients are essential for normal plant growth, but also have been observed to suppress disease development (10,14,18). In managed turfgrass systems, high nitrogen rates are positively correlated with increased disease severity, while applications of phosphorus (P) and potassium (K) reduce brown-patch incidence (4,12).

Recently, much interest has focused on the effect of phosphonate fungicides (Al^{3+}, K^{+}, or NH_4^{+} salts of phosphorous acid) and fertilizers that contain or consist of phosphorous acid (also known as phosphites) on disease suppression and overall plant health (7, 9, 18). For example, in tomato and pepper systems, phosphorous acid reduced the incidence of *Phytopthora* root and crown rot caused by *Phytophthora capsici* compared to untreated controls (13). In turfgrasses, the phosphonate fungicide fosetyl-Al (Signature or Aliette, Bayer Environmental Sciences, Montvale, NJ) applied in combination with mancozeb (Fore, DowAgro Sciences, Indianapolis, IN) provided excellent control of brown patch in creeping bentgrass and was shown to increase turfgrass quality (17). Foliar applications of phosphorous acid have been shown to increase turfgrass quality (9). However, the summer decline of cool-season turfgrasses is attributed to multiple biotic and abiotic stresses and it remains unclear which component of the above treatments, the phosphonate fungicide, mancozeb, or malachite pigment are most effective in improving plant health.

The biochemical mode of action of phosphonate fungicides and phosphorous acid fertilizers on plant health and disease incidence is unclear. Studies have shown that these products induce host defense mechanisms and also directly inhibit fungal growth (11,16,21). These products are frequently used in turfgrass disease management plans and may prove to be important components in developing more environmentally friendly IPM programs. The objectives of this study were to (i) determine the effect of phosphorous acid applications on the incidence of brown patch in tall fescue and creeping bentgrass turf and (ii) measure the sensitivity of *R. solani* AG 1, *R. solani* AG 2-2 IIIB, *R. zeae*, and

Pythium aphanidermatum to phosphorous acid, phosphonate fungicides, and azoxystrobin.

MATERIAL AND METHODS

Isolate collection and fungicide sensitivity

Isolates of *R. solani* AG 1 (RS 1, G. Abad, North Carolina State University) and 2-2IIIB (RS 2, N. Tisserat, Kansas State University), *R. zeae* (RS 3, L. Tredway, North Carolina State University), and *P. aphanidermatum* (M. Benson, North Carolina State University) were isolated from diseased turfgrass for use in tests. Stock cultures were maintained on 2% water agar (Bacto Agar, Difco, Detroit, MI) or potato dextrose agar (PDA, Difco, Detroit, MI). Compounds tested included azoxystrobin (Heritage 50 WG, Syngenta Crop Protection, Greensboro, NC), fosetyl-Al (Aliette 80 WDG, Bayer Environmental Sciences, Montvale, NJ), and phosphorus acid (H_2PO_3, Sigma-Aldrich Co., St. Louis, MO). Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) were amended with each material at a concentration of 0.0, 0.01, 0.1, 1.0, or 10.0 mg L^{-1}. Azoxystrobin was added with and without salicylhydroxamic acid (SHAM), which was dissolved in acetone and added at a final concentration of 0.1 % (v/v).

Each isolate was grown for 7 days on 1.5 % water agar, and then transferred to the center of amended PDA plates. Cultures were incubated at 24 C for 48 h. The diameter of each colony was measured in two perpendicular directions and the mean diameter was determined. Effective concentration (EC_{50}) values, representing the fungicide concentration that reduced radial growth by 50%, were determined by nonlinear

regression of the percentage of growth inhibition on $\log_{10}$ transformed fungicide concentration (SAS Institute Inc., Cary, NC). Each fungicide x isolate combination was replicated three times and the experiment was repeated twice.

Greenhouse Study

A single *R. solani* AG 1, RS 1, isolate that was previously determined as virulent on tall fescue in a greenhouse pot study was selected. To prepare inoculum of the isolate, mycelial (~ 4 mm^2) plugs were taken from the actively growing margin of a colony on PDA and placed on rye grain inoculum (250 cm^3 ryegrain, 10 g $CaCO_3$, 220 ml dH_20, autoclaved @ 121 C for 45 min in 1L Erelenmeyer flasks) and allowed to grow at 25 C for 2 weeks. Tall fescue cultivar 'Wolfpack' was seeded at a rate of 29.3 g m^{-1} in 15 cm diameter pots containing 100 % sand. Seedlings were fertilized weekly with ½ strength Hoaglands solution (15) for eight weeks and irrigation was provided as needed from an overhead system. The turf was clipped to a height of 7.6 cm weekly using scissors. Phosphorus (P) deficiencies were induced in half of the pots by fertilizing with a ($-PO_4$) modified Hoagland's solution. Nutrient deficiencies were verified by collecting tissue samples and determining elemental P levels before and after the induction of phosphorous acid treatments. Treatments included 4 phosphorous acid rates (0.0, 0.5, 1.0, 1.5 g m^{-2}) and were applied using a CO_2 powered boom sprayer (R & D Sprayers, Opelousas, LA). Treatments were applied three times on 7 day intervals before the plants were inoculated and again 7 days after inoculation.

Each pot was inoculated with 3.0 g of infested rye grain by placing the inoculum within the turf canopy in the center of the pot. Immediately after inoculation, the turf foliage was misted with water to runoff. A mist chamber was constructed within the

greenhouse and the pots were arranged in a randomized complete block design. Disease incidence was determined by visually estimating the percentage of the symptomatic leaves every 3 days. Area under the disease progress curve (AUDPC) was calculated using the formula Σ_i^{n-1} [$(y_i + y_{i+1})/2$] $(t_{i+1} - t_i)$ where i = 1,2,3,4,5, y_i is disease incidence on the ith rating date and t_i is the day of the ith rating. (3,16). Individual evaluation dates and AUDPC were subjected to analysis of variance and mean separations using the Waller-Duncan k-ratio t-test (k=100). Each treatment was replicated 6 times and the entire experiment was repeated twice.

Field Studies

Tall fescue experiments were established on sod farms in areas with restricted air movement that had previously experienced brown patch epidemics. The 2002 tall fescue trials were located in Hendersonville, NC and Candor, NC. The tall fescue cultivars at the Hendersonville site were a blend of ‘Black Magic’, ‘Endeavor’ and ‘Onyx’ and at the Candor site ‘Rebel III’. In 2003, tall fescue trials were established on tall fescue ‘Coronado’ at one site in Rolesville, NC. The turf at each site was maintained at a height of ~7 cm, irrigated as needed, and received between 175 and 244 kg N ha^{-1} yr^{-1}.

Creeping bentgrass experiments were conducted in 2002 and 2003 at the North Carolina State University Turfgrass Field Laboratory in Raleigh, NC on a stand of 'A-4' creeping bentgrass maintained under golf course putting green conditions. The turf was mowed at ~ 4 mm, irrigated as needed, and received ~292 kg N ha^{-1} yr^{-1}.

Chemical treatments and rates are shown in Table 2.1 and were initiated prior to symptom development on May 28 at Hendersonville, May 24 at Candor, May 25 at Raleigh in 2002 and May 28 at Rolesville and Raleigh in 2003. Treatments were applied

four times to tall fescue sites on 28 day intervals and eight applications were made to the creeping bentgrass location on 14 day intervals through the end of August. All 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_20 m^{-2}.

The creeping bentgrass putting green was inoculated on 17 July 2002 and 4 August 2003 by dispersing rye grain inoculum (described above) infested with an *R. solani* AG 2-2 IIIB isolate across the experimental area to encourage brown patch development. The 2003 tall fescue sites in Rolesville, NC were inoculated with the same *R. solani* AG 1 isolate, RS 1, on 4 August, 2003. The 2002 tall fescue sites did not receive additional inoculum.

Turf quality was assessed visually by estimating the overall uniformity, density and color within each plot in 2002 every two weeks at all sites except the Hendersonville site, which was evaluated on 18 June, 16 and 30 July, 13 August, and 12 September. Turfgrass quality was quantified using a 1 to 9 scale (9 = best, 5 = minimally acceptable). Percent turf area exhibiting brown patch was assessed by percentage of plot surface area exhibiting brown patch symptoms. AUDPC was calculated as described above. Disease incidence (DI), AUDPC, and turf quality data, were subjected to analysis of variance and means separation using the Waller-Duncan k-ratio t test (k=100).

RESULTS

Fungicide sensitivity

Fosetyl-Al and phosphorus acid did not inhibit mycelial growth of *R. solani* AG 1, *R. solani* 2-2 IIIB, *R. zeae*, or *P. aphanidermatum* at the concentrations tested.

Azoxystrobin (-SHAM) inhibited mycelial growth of all isolates, but it did not inhibit radial growth of *R. solani* 2-2 IIIB or *R. zeae* by 50 %. Mean EC_{50} values were obtained for all four pathogens in the azoxystrobin (+SHAM) treatment (Table 2.2). Effective concentrations for the *R. solani* AG 2-2 IIIB, *R. solani* AG 1, *P. aphanidermatum*, and *R. zeae* isolates were 1.27, 0.04, 0.012, and 0.145 $\mu g\ ml^{-1}$, respectively.

Greenhouse Study

Turf quality (TQ) was significantly lower in the $–PO_4$ treatments prior to the initiation of phosphorous acid treatments. Mean TQ values ranged from 6.0 in the $– PO_4$ treatments to 8.0 in the $+ PO_4$ (data not shown). Turf quality values of PO_4-deficient turf improved following the initiation of phosphorous acid treatments. Average TQ values of the $– PO_4$ treatments increased from 6.3 prior to foliar phosphorous acid application to 7.4 prior to inoculation (data not shown). All phosphorous acid rates increased the elemental phosphorus (P) content of leaf tissue (Table 2.3). Tissue P composition increased in the $– PO_4$ treatments from 0.9 $g\ kg^{-1}$ in the 0.0 $g\ m^{-1}$ (control) rate to 2.1 $g\ kg^{-1}$ in the $+ PO_4$ control treatment. In $+PO_4$ treatments, P composition also increased from 2.1 $g\ kg^{-1}$ in the $+ PO_4$ control to 3.2 $g\ kg^{-1}$ after 3 applications of phosphorous acid applied at the 1.5 $g\ m^{-2}$ rate.

Phosphorus acid did not reduce brown patch in tall fescue at any of the applied rates (data not shown). Area under disease progress curve values were not significantly different among phosphorous acid rates. Phosphate effects ($P < 0.05$) were present at days 5, 7 and 9 following inoculation with *R. solani*. At each of the aforementioned rating dates, the $– PO_4$ treatments exhibited increased disease incidence (day 3=18%; day 5=24%) compared to the $+ PO_4$ treatments (day 3=10%; day 5=17%). The rate of

phosphorous acid applied also affected DI early in the epidemic (days 5 and 7). Disease incidence was significantly greater on day 5 and 7 in the pots treated with 0.5, 1.0, and 1.5 g m^{-2} phosphorous acid compared to the untreated control.

Field Studies

Creeping bentgrass quality in response to both phosphorous acid treatments was not different compared to the control regardless of rate (Table 2.5) during 2002 or 2003. In 2002, there was a decrease in TQ from 8.0 to minimally acceptable levels (4.8 to 5.5) in late August. The azoxystrobin treatment maintained a consistently high TQ throughout the summer. Initial TQ ratings for 2003 were lower compared to 2002, but a similar decrease in TQ over the summer occurred across all treatments. The lower initial TQ ratings in 2003 were due to an infestation of black algae (*Cynobacteria*) before initiation of treatments.

Brown patch incidence was higher in 2002 compared to 2003 due to unusually cool weather conditions in 2003 (Table 2.7). Azoxystrobin provided excellent control of brown patch in 2002 and 2003. Brown patch symptoms were evident in all plots, including azoxystrobin, 8 days after the plot area was inoculated with *R. solani* in 2002. During 2003 few patches were observed, and they did not persist longer than 2 rating periods (Table 2.7).

Turf quality ratings in tall fescue treated with phosphorous acid in 2002 and 2003 followed a similar trend as in creeping bentgrass (Tables 2.4 and 2.6). Mean TQ of the phosphorous acid treatments and the untreated control declined throughout the summer from acceptable to unacceptable levels. There were some differences in site conditions and the health of these areas prior to the initiation of treatments. The Hendersonville site

in 2002 experienced a severe brown patch epidemic followed by an extended drought that resulted in unacceptable turf quality in the azoxystrobin plots. The 2002 Candor site had poor initial TQ values due to extended drought and limited irrigation. However, over time TQ in the azoxystrobin treatments improved from 5.0 in mid July to acceptable TQ levels (7.0) by the end of September. Weather conditions were more favorable for tall fescue growth in 2003 due to moderate temperatures and plentiful rainfall. Mean TQ values remained acceptable (>6.5) throughout July in all treatments. After inoculation on 4 August, overall TQ decreased and DI increased in all treatments except the azoxystrobin plots. Azoxystrobin was very effective in reducing the incidence of brown patch and resulted in maintaining or improving the overall health and quality of tall fescue and creeping bentgrass.

DISCUSSION

The primary objective of this study was to evaluate the effect of phosphorous acid on diseases caused by *Rhizoctonia solani* in tall fescue and creeping bentgrass. Results from the fungicide sensitivity tests indicate that phosphorous acid and fosetyl-Al have little direct toxicity toward mycelial growth of *R. solani* AG 1, *R. solani* AG 2-2 IIIB, *R. zeae*, and *P. aphanidermatum in vitro*. Previous studies also reported that phosphorous acid compounds have very low direct toxicity to fungal pathogens (11). Fenn and Coffey (11) reported that the concentration of phosphorous acid necessary for 50 % inhibition of *R. solani* was >552 µg ml^{-1} and 138 µg ml^{-1} for *P. aphanidermatum*.

The only treatment that was effective in inhibiting colony growth of the pathogens tested was azoxystrobin plus SHAM. These results indicate that *R. solani* and *R. zeae* are

capable of utilizing an alternative respiration pathway *in vitro* and, therefore, growing in the presence of azoxystrobin alone. The expression of the alternative oxidase pathway in the presence of strobulurin fungicides has been well documented in different genera of fungi (1, 22, 23).

There is evidence to indicate that the level of phosphate in amended agar and within plant tissues can affect the uptake and efficacy of phosphonate products and phosphorous acid (11, 3, 21, 2). The greenhouse portion of this study demonstrated that phosphorous acid is actively absorbed by the plant and accumulated in shoot tissue (Table 2.3). However, the form of phosphorous within the foliar tissue is unknown. The lack of disease suppression across plant PO_4 levels and phosphorous acid rates indicates that phosphorous acid was not active or was not present in high enough concentrations to inhibit disease development. Although we did not assay turf plants for the production of phytoalexins or other molecules associated with plant defense mechanisms, we did not observe evidence of plant defense response based on symptom development and disease incidence.

The *in vivo* phosphorous acid treatments often resulted in initial phytotoxicity and much slower plant growth rates (data not shown). Similar side effects from phosphorous acid treatments have been reported in other plants (13, 7). Phosphorous acid compounds added to the solution culture in hydroponic tomatoes and peppers were shown to reduce leaf area, leaf dry weight, stem dry weight, and root dry weight by over 50% compared to controls that received phosphate treatments.

Results from field studies in tall fescue and creeping bentgrass in 2002 and 2003 further indicate that phosphorous acid is not effective in reducing the severity of

Rhizoctonia diseases or enhancing turf quality compared to the untreated turf or fungicide azoxystrobin. Phosphorous acid treatments did have reduced AUDPC as compared to the control; however, overall turf quality of the phosphorous acid treatments did not differ from untreated control plots in any of the tests conducted. The TQ ratings do not take into account initial plant responses to treatments. Similar to observations from the greenhouse studies, phytotoxicity to the phosphorous acid sprays was frequently observed in the high (1.4 g m^{-1}) rates and occasionally in the low (0.7 g m^{-1}) treatments. The phytotoxicity was more severe in the creeping bentgrass plots, but was also observed in tall fescue.

In conclusion, the effectiveness of phosphorous acid as a fungicide to control *Rhizoctonia* diseases in tall fescue and creeping bentgrass turf was limited based on these observations. The efficacy of these compounds in reducing mycelial growth *in vitro* and reducing symptom development *in vivo* was minimal as compared to standard fungicides. There was some suppression of disease development in the field compared to the control, but the level of phytotoxicity and reduced plant growth detract from the overall effectiveness of phosphorous acid treatments. In addition, phosphorous acid applications should not be considered adequate substitutes for phosphonate fungicides or phosphate fertilizers in these turf systems.

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Table 2.1. Treatments, rates, and application intervals for tall fescue and creeping bentgrass plots for 2002 and 2003.

	Tall Fescue		Creeping Bentgrass	
Treatment	Rate	Interval	Rate	Interval
	g m^{-2}	d	g m^{-2}	d
H_2PO_3	0.7	28	0.7	14
H_2PO_3	1.4	28	1.4	14
Azoxystrobin	0.12	28	0.06	14
Control	0.00	28	0.00	14

Table 2.2. Predicted effective concentration (EC_{50}) values of selected fungicides calculated by non-linear regression of $\log_{10}$ concentration and radial growth.

Isolate	Fungicides			
	Fosetyl-Al	Phosphite	Azoxystrobin (-SHAM)	Azoxystrobin (+SHAM)
	EC_{50} (mg L^{-1})			
R. solani AG 1	>10	>10	0.095	0.04
R. solani 2-2 IIIB	>10	>10	>10	1 .27
R. zeae	>10	>10	>10	0.145
P. aphanidermatum	>10	>10	0.31	0.012

Table 2.3. Elemental phosphorus composition of harvested tall fescue leaf tissue prior to inoculation with *R. solani* AG 1.

H_2PO_3 Rate ($g\ m^{-2}$)	Elemental P: Phosphate (-)[a] ($g\ kg^{-1}$)	Elemental P: Phosphate(+) ($g\ kg^{-1}$)
0	0.9a*	2.1d
0.5	1.4b	2.7e
1.0	1.9c	3.1f
1.5	2.1d	3.2f

*Mean separations based on Waller-Duncan k-ratio t test (P=0.05).
[a] Phosphate deficiency induced by fertilization with modified Hoagland solution for 3 weeks prior to inoculation.

Table 2.4. Turf quality of tall fescue plots in response with different rates of phosphorous acid at Hendersonville, North Carolina and Candor, North Carolina during summer 2002 (Scale ranging from 1 to 9 with 9 = ideal turf; 1 = bare ground; and 5 = minimally acceptable).

		Turf Quality Ratings					
Hendersonville:							
Nutrient	Rate	18-Jun	16-Jul	30-Jul	13-Aug	12-Sept	TQ Average
H_2PO_3	0.7	7.5a	5.7b	4.0b	3.4b	1.9b	4.5b
H_2PO_3	1.4	7.6a	5.9b	4.0b	3.6b	1.5b	4.3b
Azoxystrobin	0.12	7.7a	7.8a	6.6a	5.4a	4.7a	6.5a
Control		7.6a	6.0b	4.1b	3.3b	1.9b	4.5b
Candor:							
		18-Jul	1-Aug	20-Aug	3-Sept	20-Sept	TQ Average
H_2PO_3	0.7	5.0a	4.8b	6.0b	5.8c	5.5bc	5.4b
H_2PO_3	1.4	5.0a	4.8b	5.3c	5.8c	5.1c	5.2c
Azoxystrobin	0.12	5.4a	6.0a	7.0a	7.6a	7.0a	6.7a
Control		5.3a	4.8b	5.8b	6.3b	5.4bc	5.5b

Mean separations based on Waller-Duncan k-ratio t test (P=0.05).

Table 2.5. Turf quality ratings of 'A-4' creeping bentgrass plots with different rates of phosphorous acid at North Carolina State Turfgrass Research Station, Raleigh, North Carolina during summer 2002 and 2003 (Scale ranging from 1 to 9 with 9=ideal turf; 1=bare ground; and 5=minimally acceptable).

2002:

		Turf Quality Ratings							
Nutrient	Rate	16-Jun	5-Jul	12-Jul	24-Jul	9-Aug	15-Aug	24-Aug	TQ Average
H_2PO_3	0.7	8.0a	7.5ab	6.5c	5.9bc	5.6b	5.8c	4.8b	6.3b
H_2PO_3	1.4	8.0a	6.8d	6.5c	5.6c	5.7b	5.8c	5.5b	6.3b
Azoxystrobin	0.06	8.0a	7.8a	8.0a	7.5a	8.0a	8.0a	7.3a	7.8a
Control		8.0a	7.8a	7.0bc	6.6ab	5.5b	5.4c	5.0b	6.5b

2003:

		17-Jun	01-Jul	16-Jul	31-Jul	14-Aug	28-Sep	10-Sep	TQ Average
H_2PO_3	0.7	7.5a	7.0a	6.0c	6.25c	6.25c	5.5c	6.0b	6.35b
H_2PO_3	1.4	7.25a	7.25a	5.0d	6.25c	6.5b	5.5c	6.0b	6.25b
Azoxystrobin	0.06	7.0a	7.0a	8.0a	8.0a	8.0a	7.5a	7.5a	7.57a
Control		7.0a	7.0a	7.0b	6.75c	5.25d	6.25b	6.25b	6.5b

Mean separations based on Waller-Duncan k-ratio t test (P=0.05).

Table 2.6. Turf quality ratings of tall fescue plots with different rates of phosphorous acid at Rolesville, NC, during summer 2003 (Scale ranging from 1 to 9 with 9=ideal turf; 1=bare ground; and 5=minimally acceptable).

		Turf Quality Ratings					
Nutrient	Rate	03-Jul	16-Jul	31-Jul	14-Aug	28-Aug	TQ Average
Field 1:							
H_2PO_3	0.7	8.0a	8.0a	7.25b	6.0b	5.25b	6.9b
H_2PO_3	1.4	8.0a	8.0a	7.25b	5.75b	5.75b	6.95b
Azoxystrobin	0.12	8.0a	8.0a	8.0a	8.0a	8.0a	8.0a
Control		8.0a	8.0a	7.25b	6.0b	6.0b	7.05b
Field 2:							
H_2PO_3	0.7	7.0a	7.0b	7.25ab	6.0b	5.50b	6.55b
H_2PO_3	1.4	7.0a	6.75b	6.50b	6.0b	5.50b	6.35b
Azoxystrobin	0.12	7.0a	7.75a	7.50a	7.75a	7.75a	7.55a
Control		7.0a	6.75b	6.50b	5.50b	5.25b	6.0b

Mean separations based on Waller-Duncan k-ratio t test (P=0.05).

Table 2.7. Brown patch incidence and area under the disease progress curve (AUDPC) of *Rhizoctonia solani* in 'A-4' creeping bentgrass plots with different rates of elemental phosphite at North Carolina State Turfgrass Research Station, Raleigh, North Carolina during summer 2002 and 2003.

2002:

		Brown Patch Incidence, %					
Nutrient	Rate	12-Jul	24-Jul	9-Aug	15-Aug	24-Aug	AUDPC
H_2PO_3	0.7	0a	5.0b	12.5bc	21.0	47.5c	167b
H_2PO_3	1.4	0a	12.5c	14.0c	22b	36.3b	154b
Azoxystrobin	0.06	0a	2.5a	0.0a	0.0a	0.0a	3.8a
Control		0a	4.0b	10.0b	28.8b	45.0c	208c

2003:

Nutrient	Rate	01-Jul	16-Jul	28-Jul	18-Aug	02-Sep	10-Sep	AUDPC
H_2PO_3	0.7	0.0a	0.0a	5.0b	19.0b	15.0b	6.0b	204b
H_2PO_3	1.4	0.0a	0.0a	1.75b	20.8b	21.5b	6.0b	195b
Azoxystrobin	0.06	0.0a	0.0a	0.0a	0.0a	7.5a	7.5a	10.0a
Control		0.0a	5.25b	11.75c	32.0c	23.8b	6.25b	338c

Mean separations based on Waller-Duncan k-ratio t test (P=0.05).

CHAPTER 3

STRUCTURE AND DYNAMICS OF *RHIZOCTONIA* POPULATIONS IN CREEPING BENTGRASS IN RESPONSE TO MANAGEMENT PROGRAMS

Structure and dynamics of *Rhizoctonia* populations in creeping bentgrass in response to management programs

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ABSTRACT

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Populations of *Rhizoctonia* species within a creeping bentgrass putting green were monitored over time in response to selected management programs. Fourteen programs, including combinations of fungicides and nutrients, were initiated in 1.52 m^2 plots of 'L-93' creeping bentgrass arranged in a randomized complete block with four replications. Twelve soil cores were taken from each plot on each of 8 sampling dates in 2002 and 7 sampling dates in 2003. The first sample was taken each season prior to initiation of treatments and then on 14 day intervals. Soil cores were placed on alkaline water agar for selective isolation of *Rhizoctonia* species. *Rhizoctonia zeae* and *R. solani* anastomosis group AG 2-2 IIIB were recovered from the soil and thatch samples. Isolates of *R. zeae* were identified morphologically, while isolates of *R. solani* AG 2-2 IIIB were identified

to anastomosis group (AG) by pairing with a known AG 2-2 IIIB tester isolate. All isolates of AG 2-2 IIIB were paired in all combinations within and across sampling dates to identify isolates to the clonal level. Of 103 isolates collected during 2002, 74 were *R. solani* AG 2-2 IIIB and 29 were *R. zeae*. Twenty-one clones were characterized in the AG 2-2 IIIB population, with seven of the clones recovered on multiple sampling dates. During 2003, 21 isolates of *R. zeae* and 67 isolates of *R. solani* AG 2-2 IIIB were obtained. Sixteen of the 21 clonal groups from 2002 also were recovered in 2003, including all seven of the clones that were isolated on multiple sampling dates in 2002. The efficiency of *Rhizoctonia* isolation and the number of clones collected decreased following the initiation of fungicide applications, but clones did not respond differently to fungicide applications. All 21 clones exhibited similar EC_{50} values to mancozeb, chlorothanlonil or azoxystrobin, thus these fungicides did not induce shifts in the clonal structure of the *R. solani* 2-2 IIIB population.

INTRODUCTION

Rhizoctonia species are associated with multiple foliar diseases of warm- and cool-season turfgrasses (Couch; 1995; Haygood and Martin, 1990; Burpee and Martin, 1992, Smiley et al., 1992). Techniques have been developed to rapidly identify these pathogens to the species level (Martin, 1987), which is important in disease management because *Rhizoctonia* species vary in sensitivity to certain fungicides (Martin et al., 1984a; Royals, 2002). Six anastomosis groups (AG's) or subgroups of *R. solani* have been associated with turfgrass diseases (Burpee and Martin, 1992). The most common subgroup found on creeping bentgrass (*Agrostis palustris*) putting greens is AG 2-2 IIIB (Burpee and Martin, 1992; Zhang and Dernoeden, 1995). However, little is known about

the structure and diversity of AG 2-2 IIIB populations on a putting green and how this diversity may influence the efficacy of disease management programs.

Identification to the AG level can been done by observation of morphological characteristics, hyphal fusion with ‘tester’ isolates, or genetic markers that target specific regions of the genome (Sneh et al, 1998; Cubeta and Vilgalys, 1997). Pairing techniques have been developed for identification of isolates to specific AG groups commonly associated with turfgrasses (Zhang and Dernoeden, 1995). Identification of the organism to this level is important for management recommendations, but it does not provide information on the structure of populations.

Studies of *R. solani* population structure have been conducted in agricultural cropping systems using both anastomosis pairings and molecular markers (MacNish et al., 1997; Ceresini et al., 2002a; Ceresini et al., 2002b; Ceresini et al., 2003). Ceresini et al. (2002a) focused on the genetic structure of *R. solani* AG 3 populations associated with potato and tobacco in eastern North Carolina. Populations from potato and tobacco differed in their genetic structure. All thirty-two isolates from potato had a distinct AFLP phenotype, and 28 somatic compatibility groups (clones) were present in the sample population. In comparison, of 36 AG 3 isolates from tobacco, there were 28 distinct AFLP phenotypes and only eight clones. Based on these results, the authors concluded that *R. solani* AG 3 populations exhibited a clonal structure. Increased clonal diversity in the potato populations were attributed to the introduction of inoculum on potato seed pieces (Ceresini et al., 2002a).

A study of *R. solani* AG 8 populations in cereals and legumes in West Australia using hyphal anastomosis and pectic zymograms demonstrated that different clones could

be identified within isolates from the same pectic zymograms (MacNish et al., 1993). For example, using pectic zymograms, 5 groups were detected within the AG 8 population. With hyphal anastomosis, different clones were identified within each zymogram group and some of the clones were distributed over a broad geographical region; individual clones were found in Washington, Oregon and West Australia. Results from these studies support a population model that includes both clonal individuals and some individuals that evolve through recombination (Ceresini et al., 2002a; Ceresini et al., 2002b; MacNish et al., 1993).

The perennial nature of turfgrass swards and the organic matter associated with grasslands provide a favorable environment for fungal growth and survival. There have been a limited number of studies on the population structure of *Rhizoctonia* species in managed perennial grass systems. A study conducted on creeping bentgrass in South Carolina characterized and identified shifts in artificially inoculated populations of *R. solani* and *R. zeae* in response to fungicide application and environmental conditions (Royals, 2002). Results indicated that *R. zeae* may become the dominant pathogen when temperatures consistently exceed 32 °C (Royals, 2002).

The combination of conventional somatic pairing techniques with the use of molecular markers has proven to be a successful approach for characterizing *R. solani* populations. However, there is limited information that specifically addresses the structure of *R. solani* AG 2-2 IIIB populations in any cropping system. Creeping bentgrass putting greens are among the highest valued turfgrass areas and *R. solani* AG 2-2 IIIB is one of the major pathogens that affects the quality of these areas. Therefore, the objectives of this study were to: (1) characterize the clonal structure of *R. solani* 2-2

IIIB isolates in a creeping bentgrass putting green using anastomosis pairings and molecular markers over a 2-yr period; and (2) to determine if shifts in the populations of *R. solani* AG 2-2 IIIB are induced by imposing selected disease management programs.

MATERIALS AND METHODS

Isolate collection. Isolates of *Rhizoctonia* species were collected from a 'L-93' creeping-bentgrass putting green located at the North Carolina State University Turfgrass Research Field Laboratory in Raleigh, North Carolina during the summers of 2002 and 2003. Management programs were initiated on 19 June 2002 and 18 June 2003. Treatments included the use of the fungicides chlorothalonil (Daconil Ultrex 82.5WDG, Syngenta Crop Protection, Greensboro, NC), fosetyl-Al (Aliette 80WDG, Bayer Environmental Sciences, Montvale, NJ) + mancozeb (Fore 80WP, DowAgroSciences, Indianapolis, IN), and azoxystrobin (Heritage 50WG, Syngenta Crop Protection, Greensboro, NC) alone or rotated on 14 day schedules in all possible combinations (Table 3.1). In addition, a nutrient treatment (H_2PO_3 + $MnSO_4$) was applied alone or in combination with each fungicide regime (Table 3.1). Treatments were applied to 1.52 m^2 plots on 14 day intervals with the final treatment occurring on 30 August, 2002 and 25 August, 2003. The experimental design was a randomized complete block with 4 replications. All treatments were applied with a CO_2-powered boom sprayer at 276 kPa using flat fan nozzles (TeeJet 8004, R & D Sprayers, Opelousas, LA) calibrated to deliver 81.5 ml H_20 m^{-2}.

Three soil cores (2 cm diameter, 1.27 cm deep) were taken from each plot prior to initiation of fungicide treatments and again on 14 day intervals after initiation of treatments. In 2002, samples were collected on 7 and 26 June, 10 and 25 July, 8 and 23

August, 6 September, and 10 October. In 2003, samples were collected on 9 and 25 June, 8 and 22 July, 5 and 18 August, and 30 September (Table 3.1). Four subsamples were taken from each soil core and the 12 subsamples were placed on alkaline water agar (AWA) and incubated at room temperature (22 to 25 °C) for 48 hrs. The AWA medium contained 15 g of Bacto Agar (Difco), 100 mg streptomycin sulfate, 100 mg penicillin G, and 0.8 ml of 1N NaOH per liter of deionized water. Actively growing hyphae were transferred to Petri dishes containing potato dextrose agar (PDA, Difco, Detroit, MI).

Identification and pairing of isolates. Isolates of *R. zeae* were identified based on the morphological characteristics outlined by Sneh et al. (1998) and Burpee and Martin (1992). The *R. solani* AG 2-2 IIIB isolates were initially identified using the morphological characteristics described by Zhang and Dernoeden (1995) and were then paired with a known AG tester isolate to confirm the placement in this AG. The agar medium (Gutierrez et al. 1998) used for detection of somatic incompatibility reactions was based on a medium developed for study of *Sclerotium cepivorum* (Earnshaw and Boland, 1997) and *Sclerotinia sclerotiorum* (Kohn et al., 1991; Kohn et al., 1991). The pairing medium consisted of dilute potato dextrose agar (Difco PDA, 30 g/liter,Detroit, MI), to which red food-color dye (McCormick & Co., Hunt Valley, MD 21030) was added at a concentration of 1.5 ml liter^{-1}. A very thin layer of medium was poured in 9-cm Petri dishes. Colonized agar disks were placed 3 to 4 cm apart on fresh plates of indicator media and incubated at room temperature for 7 days. Plates were observed daily to ascertain the type of hyphal interaction. *Rhizoctonia solani* AG 2-2 IIIB isolates were identified by the presence of a killing or C2 reaction between hyphal cells of the field isolates and the tester isolate. When the *R. zeae* isolates were paired with the AG 2-2

IIIB tester isolate, massive cell death occurred and a macroscopic red indicator line developed between the organisms. Isolates collected from 2002 were paired in all combinations within and across sampling dates and the 2003 isolates were paired against all clonal groups identified from 2002.

Genetic markers. Genetic relationships among clones were assessed using ribosomal DNA (rDNA) sequences. Each isolate was grown for ~7 days at room temperature ~25 °C in 2 ml of potato dextrose broth. The mycelial suspension was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 5 min at 11,000 rpm. The mycelial plug was harvested and genomic DNA was extracted using the Easy-DNA Kit (Invitrogen Corp., Carlsbad, CA). The DNA solutions were treated with RNase (40 $\mu g\ \mu L^{-1}$) and incubated at 37 °C for 30 min. Samples were analyzed for purity by spectophotometry and standardized to a concentration of 50 ng DNA ml^{-1}.

Polymerase chain reaction (PCR) amplification of rDNA internal transcribed spacer (ITS) regions ITS1, 5.8S rRNA, and ITS2 was performed using primers ITS4 and ITS5 (White et al., 1990). PCR reactions were 50 µl in volume and consisted of 20mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTPs, 200nM of each primer, 1.25 U of Taq polymerase (Invitrogen Corp., Carlsbad CA), and 50 ng of genomic DNA. Thermal cycling conditions involved 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 10 min. Amplification products were purified with a Qiaquick PCR Purification Kit (Quigen Inc., Valencia, CA). Cycle sequencing reactions were performed using an ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems Inc., Forester City, CA). Following the removal of unincorporated dye using Centrisep

columns (Princeton Separations Inc., Adelphia, NJ) the samples were dried and sent to the University of Iowa DNA Facility (Iowa City, IA) for analysis. Homologous sequences were identified through a BLAST search of the GenBank database (National Center for Biotechnology Information). Sequences were aligned using the Clustal W method in MegAlign 5.05 (DNASTAR Inc., Madison, WI) and adjusted by visual examination. A phylogenic tree was constructed in MEGA v2.1 using the Neighbor-Joining algorithm from genetic distances calculated using the Kimura Two-Parameter Model.

Fungicide Sensitivity Studies

A single representative isolate of each clonal group was screened for sensitivity to azoxystrobin (Heritage 50 WG, Syngenta) amended with salicylhydroxamic acid (SHAM), mancozeb (Fore 80 WP, DowAgroSciences), and chlorothalonil (Daconil Ultrexx 82.5WDG, Syngenta). PDA was amended fungicides after autoclaving to establish concentrations of 0.0, 0.1, 1.0, and 10.0 μg a. i. ml^{-1}. SHAM was dissolved in acetone and added to all concentrations of azoxystrobin (+ SHAM treatments) at a concentration of 0.1 % (v/v), including a PDA + SHAM control.

Each isolate was grown for 7 days on 2% water agar then transferred to the center of amended PDA in 9 cm diameter Petri dishes. Cultures were incubated at 24 °C for 48 h, and radial growth of the colony was measured. The diameter of the colony was measured in two perpendicular directions and the mean diameter was determined. Effective concentration values, representing the fungicide concentration that reduced radial growth by 50 % (EC_{50}), were estimated by nonlinear regression of the percentage of growth inhibition on log_{10} transformed fungicide concentration (SAS Institute Inc.,

Cary, NC). Each fungicide x isolate combination was replicated three times and the experiment was repeated twice.

RESULTS

Identification and pairing of isolates. Of the 103 isolates collected during 2002, 74 were *R. solani* AG 2-2 IIIB and 29 were *R. zeae*. In the summer of 2003, 67 isolates of *R. solani* AG 2-2 IIIB and 21 isolates of *R. zeae* were collected (Table 3.2). Recovery of both *R. solani* and *R. zeae* was highest prior to initiation of fungicide treatments and at the final sample date that occurred 5 to 6 wks after the final fungicide application. Lowest recovery of both species occurred during the summer months, between early July and early September (Table 3.2). Populations of *R. solani* AG 2-2 IIIB appeared to recover faster than *R. zeae*, as observed with the high number of isolates recovered once fungicide applications were stopped (Table 3.2).

Pairings among the 74 isolates of the AG 2-2 IIIB collected in 2002 revealed the presence of 21 clonal groups based on observation of the C2 and C3 anastomosis reactions (Carling, 1996, Cubeta and Vilgalys, 1997). Seven of the 21 clones were collected on multiple sampling dates (Table 3.3). Sixteen of the 21 clonal groups from 2002 also were recovered in 2003 (Table 3.3); no new clones were identified. All seven of the clonal groups that appeared in multiple samplings in 2002 were recovered in 2003 (Table 3.3).

Clonal group RS-21 exhibited a C3 anastomosis reaction when paired with our AG 2-2 IIIB tester isolate RH-5. RH-5 was introduced to putting green sampled during fungicide evaluation trials conducted in the summers of 1999 and 2000 (H.C. Wetzel,

personal communication). Isolates compatible with RS-21 were obtained on 8 August, 2002 and 9, 25 June and 5 August, 2003 (Table 3.2).

Isolates were recovered from the non-fungicide treated plots on 5 of 8 sampling dates in 2002 and on all dates in 2003 (Table 3.4). There were no 2-2 IIIB isolates collected from the azoxystrobin plots (treatment 3) after fungicide treatments were initiated either year; however, isolates were collected on the final sampling dates 10 October, 2002 and 30 September, 2003 that occurred 5 to 6 wks after the last fungicide treatment.

Phylogenic Relationships

A BLAST search of the GenBank database identified three *R. solani* AG 2-2 IIIB isolates (GenBank accession no. AY154311, AB054658, and AB054857), and two *R. solani* AG 2 subgroups AG 2-2 LP and AG 2-2 IV (GenBank accession no. RS0238167 and AB054868) for comparison. *Rhizoctonia solani* AG 4 and AG 3 (GenBank accession no AY154307 and AY154319, respectively) were used as outgroups. In addition to the clones tested, two other AG 2-2 IIIB isolates, RH-46 and RH-67 (courtesy of H.C. Wetzel, Syngenta Crop Protection) recovered from 'Cato/Crenshaw' creeping bentgrass were sequenced and included in the analysis.

Sequences from the clones RH 1, RH2, RH3; RH 5, RH6, RH7, RH9, RH10, RH11, RH12, RH13, RH14, RH16, RH18, RH19, RH20, RH21 and the other AG 2-2 IIIB isolates RH46, RH67, AY154311, AB054658, AB054857, were highly similar (Table 3.6). Genetic distances within the AG 2-2 IIIB subgroups ranged from .008 to .002 according to the Kimura Two-Parameter Model (Table 3.5). Phylogenetic analysis placed all AG 2-2 isolates in a single clade with 100 % bootstrap support (Fig. 3.1). The

AG 2-2 LP and AG 2-2 IV isolates are separated from the AG 2-2 IIIB isolates by 92 % bootstrap. Additional structure was also observed within the AG 2-2 IIIB clade, but these subclades did not have significant bootstrap support. The *R. solani* AG 3 and AG 4 isolates formed a distinct clade that were not similar (> 0.04) to each other or the AG 2 groups and subgroups.

Fungicide Sensitivity

All fungicides tested inhibited mycelial growth of all *R. solani* AG 2-2 IIIB isolates. Analysis of variance conducted on mean EC_{50} values indicated that the isolates of the different clones responded similarly to fungicides in agar medium (Table 3.6). However, significant differences among fungicides were detected, with mean EC_{50} values for mancozeb, chlorothalonil and azoxystrobin (+SHAM) of 2.44, 0.85, and 0.38 $\mu g\ ml^{-1}$, respectively (Table 3.7).

DISCUSSION

The population of *Rhizoctonia* species recovered from a creeping bentgrass putting green was composed of *R. solani* AG 2-2 IIIB and *R. zeae*. In both years, the most frequently recovered organism (> 70% of all isolates) was *R. solani* AG 2-2 IIIB. Since this organism is the primary pathogen of creeping bentgrass (Burpee and Martin, 1992; Zhang and Dernoeden, 1995) it is not surprising that it was the most frequently recovered organism in this study. Brown patch was observed on the test green in both seasons, but soil cores were not taken from active disease patches. This may indicate that the pathogen may be active, or at least surviving, in grass prior to occurrence of symptoms. Similar observations were reported for *R. solani* isolates that induce large patch in zoysiagrass (Aoyagi et al., 1998).

Within the 79 isolates of *R. solani* AG 2-2 IIIB recovered in 2002, 21 different clones were identified based on the occurrence of the C2 or C3 anastomosis reaction in pairings (Carling, 1996, MacNish et al., 1997). A similar level of clonal diversity was observed among the isolates recovered in 2003, with 16 clones identified. All 16 of the clones recovered in 2003 were observed at least once in 2002. Although 21 clones were identified in 2002, only seven of these clones were recovered on more than one sampling date, indicating that certain clones are more prevalent in the AG 2-2 IIIB population. This conclusion was supported by the 2003 observations, when all seven of these clones from 2002 were again recovered. As observed in 2002, there were seven clones recovered on multiple sampling dates in 2003. However, only three of these seven clones were repeats of clones recovered on multiple sampling dates in 2002. It is not known whether these three clones were predominant across both years because of their aggressiveness on 'L-93' creeping bentgrass or because they produce more effective survival structures.

Isolation of some clones was dependent on sampling date. For example, clones RS 17 and 18 were only recovered late in the year in 2002 and 2003. In contrast, clones RS 2, 4, 9, and 10 were only recovered early in the season. Isolation of other clones appeared to be independent of sampling date. For example, clones RS 6 and 11 were recovered throughout the sampling period both years of the study. Additional experimentation is needed to determine if the pattern of recovery is due to temperature optima for the isolates or if other environmental or host factors are responsible. Of particular interest was the recovery of RS 21, which was used as our tester isolate in pairings. This isolate, originally collected in Iowa and introduced to the green as artificial inoculum in 1999 and 2000, was recovered on one date in 2002 and on three dates in

2003. Thus, it is clear that an introduced organism can survive multiple years on a creeping bentgrass green. Similar observations were made for *R. solani* introduced into an agricultural soil on infested seed pieces (Ceresini et al. 2003). It is unclear how the other clones were introduced and became established on the green.

Recovery of *Rhizoctonia* species was greatest before and after the fungicide treatments were applied to the green. Frequency of recovery dropped rapidly for *R. solani* and *R. zeae* in all fungicide plots, but not in control plots or in plots that received only nutrient amendments. This drop in recovery was probably due to a suppression of the pathogen, as populations recovered to pretreatment levels within 5 to 6 weeks after the last fungicide application for *R. solani* AG 2-2 IIIB. The *R. zeae* populations did not recover to levels equivalent to those found prior to the initiation of fungicide treatments during September as did the *R. solani* population. This difference in recovery may have been due to a higher temperature optima for *R. zeae* than for *R. solani* (Voorhees, 1934).

Resistance to certain classes of fungicides in isolates of *Pyricularia grisea* and *Sclerotinia homoeocarpa* has been reported in managed turfgrass (Vincelli, 2002; Miller et al., 2002). From our results, it is apparent that fungicides impacted the recovery of *R. solani* and *R. zeae* in creeping bentgrass. However, applications of the fungicides mancozeb + fosetyl-Al, azoxystrobin, and chlorothalonil did not alter the clonal structure of the population of *R. solani*. The effectiveness of all fungicides and the similar EC_{50} values of all isolates indicate that fungicide sprays did not cause a significant shift in population structure of *R. solani*.

This study is the first to examine clonal structure of *R. solani* AG 2-2 IIIB within a stand of creeping bentgrass. Previous studies have evaluated *R. solani* AG 3 and AG 8

population structure on a local and regional scale. Results of these studies support models that include both clonal survivability and genetic diversity (Ceresini et al., 2002a; Ceresini et al., 2002b; Ceresini et al., 2003; MacNish et al., 1997). The results from this work suggest that the clones are persistent across multiple years in creeping bentgrass and that management practices that include nutrients and fungicides did not affect the clonal structure of the AG 2-2 IIIB population. Thus, other factors such as environmental conditions may have a greater influence on the clonal structure in these systems.

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Figure Legend

Figure 1. Phylogenetic tree drawn from the nucleotide sequences of internal transcribed (ITS) region of ribosomal DNA from isolates of *R. solani* AG 2-2 IIIB (RH 1, RH2, RH3; RH 5, RH6, RH7, RH9, RH10, RH11, RH12, RH13, RH14, RH16, RH18, RH19, RH20, RH21, RH46, RH67, AY154311, AB054658, AB054857); *R. solani* AG 2-2 (RS0238167, AB054868, AY270015); *R. solani* AG(3) (AY154319); and *R. solani* AG 4 (AY154307). Tree constructed using Neighbor-Joining Algorithm on sequence data.

Table 3.1. Fungicide treatments, rates, and intervals for 'L-93' creeping bentgrass putting green during the summer of 2002.

Treatment and rate [1]	Interval (days)
1. Daconil Ultrex 82.5WDG 3.2oz………	14
2. Nutrients (H_2PO_3 + $MnSO_4$)…….........	14
3. Heritage 50WG 0.4 oz…………………	14
4. Aliette 80WDG 8.0 oz + Fore 80WP 4.0 oz…………………...	14
5. Daconil Ultrex 82.5WDG 3.2oz + Nutrients (H_2PO_3 + $MnSO_4$)……….	14
6. Daconil Ultrex 82.5WDG 3.2oz + Heritage 50WG 0.4 oz ……………..	14
7. Daconil Ultrex 82.5WDG 3.2oz – Aliette 80WDG 8.0 oz + Fore 80WP 4.0 oz…………..	14
8. Nutrients (H_2PO_3 + $MnSO_4$)- Heritage 50WG 0.4 oz …………….	14
9. Nutrients (H_2PO_3 + $MnSO_4$)- Aliette 80WDG 8.0 oz + Fore 80WP 4.0 oz…………..	14
10. Heritage 50WG 0.4 oz – Aliette 80WDG 8.0 oz + Fore 80WP 4.0 oz…………..	14
11. Daconil Ultrex 82.5WDG 3.2oz – Nutrients (H_2PO_3 + $MnSO_4$)- Heritage 50WG 0.4 oz……………..	14
12. Nutrients (H_2PO_3 + $MnSO_4$)- Heritage 50WG 0.4 oz – Aliette 80WDG 8.0 oz + Fore 80WP 4.0 oz…………..	14
13. Daconil Ultrex 82.5WDG 3.2oz Nutrients (H_2PO_3 + $MnSO_4$)- Heritage 50WG 0.4 oz – Aliette 80WDG 8.0 oz + Fore 80WP 4.0 oz………………….	14
14. Un-treated control……………………	--

[1]Treatments 1 to 4 were applied every spray interval and treatments 5 to 13 consisted of all combinations of a rotational plan that applied different chemicals every 14 days.

Table 3.2. Fungicide applications, sampling dates, isolates collected, anastomosis groupings, and percentage of the *R. solani* 2-2 IIIB isolates collected from the non-treated control plots from each sampling date collected from a single creeping bentgrass putting green during 2002 and 2003.

			Isolates Collected		
Year	Fungicide Applications	Sample Date	*R. zeae*	*R. solani* AG 2-2 IIIB	% of Isolates from Non-treated controls
2002					(%)
		7 Jun	11	18	0
	19 Jun	26 Jun	3	14	6
	2 Jul	10 July	4	11	27
	16 Jul	25 July	2	4	0
	1 Aug	8 Aug	2	5	20
	15 Aug	23 Aug	5	5	60
	30 Aug	6 Sep	0	3	0
		10 Oct.	2	14	14
2003					
		9 Jun	9	14	14
	18 Jun	25 Jun	2	11	18
	2 Jul	8 Jul	2	6	33
	15 Jul	22 Jul	2	7	28
	29 Jul	5 Aug	5	7	43
	11 Aug	18 Aug	3	5	0
	25 Aug	30 Sep	3	17	6

Table 3.3. Sampling dates from 2002 and 2003 and *R. solani* 2-2 IIIB clones (indicated by X) collected from 'L-93' creeping bentgrass at the North Carolina State University Field Laboratory

CLONES	2002								2003						
	Sampling Dates														
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	8
RS 1	X	X	X	--	X	--	--	--	--	X	--	--	--	--	--
RS 2	X	--	--	--	--	--	--	--	--	--	--	--	--	--	--
RS 3	X	--	--	X	--	X	--	--	X	--	--	--	--	--	--
RS 4	X	--	--	--	--	--	--	--	--	--	--	--	--	--	--
RS 5	X	--	X	--	--	--	--	X	X	--	X	--	--	--	--
RS 6	X	X	--	--	--	X	X	X	--	X	--	--	--	--	X
RS 7	X	X	--	X	--	--	--	--	X	--	--	--	--	--	X
RS 8	X	--	--	--	--	--	--	X	X	--	--	--	--	--	--
RS 9	--	X	--	--	--	--	--	--	--	--	--	--	--	--	--
RS 10	--	X	--	--	--	--	--	--	--	X	--	--	--	--	--
RS 11	X	--	X	--	--	X	--	--	X	X	--	X	--	--	X
RS 12	--	--	--	X	--	--	--	--	--	--	--	--	--	--	--
RS 13	X	--	--	--	--	--	--	--	X	--	--	X	--	X	--
RS 14	--	--	X	--	--	--	--	--	X	--	X	--	--	--	--
RS 15	--	--	X	--	--	--	--	--	--	--	X	--	--	--	--
RS 16	--	--	X	--	--	--	--	--	X	--	--	--	--	--	--
RS 17	--	--	--	--	--	--	--	X	--	--	--	--	--	--	X
RS 18	--	--	--	--	--	--	--	X	--	--	--	--	--	--	--
RS 19	--	--	--	--	X	--	--	--	--	X	--	--	--	--	--
RS 20	--	--	--	--	X	--	--	--	--	X	--	--	--	--	--
RS 21	--	--	--	--	X	--	--	--	X	X	--	--	X	--	--

Table 3.4. Treatments and sampling dates from 2002 and 2003 and the number of *R. solani* 2-2 IIIB isolates collected from ‘L-93’ creeping bentgrass at the North Carolina State University Field Laboratory.

Treatment	2002								2003						
	Sampling Dates														
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	8
1	0	0	1	1	0	0	0	4	2	0	0	0	0	0	1
2	2	4	1	0	0	0	2	0	1	0	1	1	2	0	0
3	2	0	0	0	0	0	0	1	0	0	0	0	0	0	1
4	0	0	2	1	0	0	0	1	1	4	1	1	2	0	2
5	0	0	1	0	1	0	0	0	1	2	0	0	1	1	3
6	4	1	0	0	0	0	0	2	2	0	0	1	0	0	0
7	1	2	0	0	0	0	0	0	1	1	1	1	0	0	1
8	0	1	1	0	0	0	0	0	0	0	0	0	0	0	2
9	2	2	0	0	2	2	0	2	1	0	0	0	0	1	3
10	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0
11	2	2	0	0	0	0	1	0	1	2	0	0	0	1	0
12	2	1	0	1	0	0	0	0	0	0	0	0	0	0	2
13	2	0	1	0	0	0	0	2	1	0	0	0	0	1	1
14	0	1	3	0	1	3	0	2	2	2	2	3	2	1	1

Table 3.5. Genetic distance matrix based on sequence data from the internal transcribed spacer (ITS)1, 5.8S rRNA, and ITS2 regions of the rDNA locus from *Rhizoctonia solani* AG (2-2IIIB); (RH1, RH2, RH3, RH5, RH6, RH7, RH8, RH9, RH10, RH11, RH12, RH13, RH14, RH16, RH18, RH19, RH20,RH21, RH46, RH67, AY154311, AB054658, AB054857); *R. solani* AG (2-2) (RS0238167, AB054868, AY270015); *R.solani* AG (3) (AY154319); and *R.solani* AG (4) (AY154307)[a].

	AB054857	AB054658	AB054868	AY154307	AY154311	AY154319	RH46	RH67	RH1	RH10	RH11	RH12	RH13	RH14	RH16	RH18	RH19	RH2	RH20	RH21	RH3	RH5	RH6	RH7	RH9	RS0238167
AB054857																										
AB054658	0.004																									
AB054868	0.017	0.015																								
AY154307	0.114	0.114	0.12																							
AY154311	0.007	0.006	0.017	0.112																						
AY154319	0.105	0.105	0.112	0.081	0.104																					
RH46	0.009	0.005	0.017	0.115	0.008	0.11																				
RH67	0.008	0.006	0.017	0.119	0.003	0.11	0.007																			
RH1	0.006	0.005	0.015	0.117	0.002	0.108	0.006	0.001																		
RH10	0.008	0.006	0.017	0.117	0.003	0.108	0.007	0.003	0.001																	
RH11	0.009	0.008	0.019	0.121	0.005	0.112	0.006	0.004	0.003	0.004																
RH12	0.006	0.002	0.014	0.115	0.005	0.106	0.003	0.004	0.003	0.004	0.006															
RH13	0.009	0.008	0.017	0.118	0.005	0.11	0.009	0.004	0.003	0.001	0.006	0.006														
RH14	0.008	0.003	0.015	0.115	0.006	0.106	0.001	0.006	0.004	0.004	0.004	0.001	0.006													
RH16	0.008	0.006	0.017	0.121	0.003	0.112	0.004	0.003	0.001	0.003	0.001	0.004	0.004	0.003												
RH18	0.006	0.005	0.015	0.118	0.002	0.11	0.007	0.003	0.001	0.003	0.004	0.004	0.004	0.006	0.003											
RH19	0.006	0.005	0.015	0.118	0.002	0.11	0.006	0.001	0	0.001	0.003	0.003	0.003	0.004	0.001	0.001										
RH2	0.006	0.005	0.015	0.117	0.002	0.108	0.006	0.001	0	0.001	0.003	0.003	0.003	0.004	0.001	0.001	0									
RH20	0.008	0.006	0.017	0.117	0.003	0.111	0.007	0.003	0.001	0.003	0.004	0.004	0.004	0.006	0.003	0.003	0.001	0.001								
RH21	0.006	0.005	0.015	0.117	0.002	0.108	0.006	0.001	0	0.001	0.003	0.003	0.003	0.004	0.001	0.001	0	0	0.001							
RH3	0.009	0.008	0.019	0.122	0.005	0.112	0.009	0.004	0.003	0.004	0.004	0.006	0.006	0.007	0.003	0.004	0.003	0.003	0.004	0.003						
RH5	0.012	0.011	0.022	0.128	0.008	0.117	0.012	0.007	0.006	0.007	0.009	0.009	0.009	0.01	0.007	0.007	0.006	0.006	0.007	0.006	0.004					
RH6	0.006	0.005	0.015	0.118	0.002	0.11	0.006	0.001	0	0.001	0.003	0.003	0.003	0.004	0.001	0.001	0	0	0.001	0	0.003	0.006				
RH7	0.009	0.005	0.017	0.115	0.008	0.11	0	0.007	0.006	0.007	0.006	0.003	0.009	0.001	0.004	0.007	0.006	0.006	0.007	0.006	0.009	0.012	0.006			
RH9	0.006	0.005	0.015	0.118	0.002	0.11	0.007	0.003	0.001	0.001	0.004	0.004	0.004	0.006	0.003	0.003	0.001	0.001	0.003	0.001	0.004	0.007	0.001	0.007		
RS0238167	0.019	0.021	0.018	0.132	0.021	0.114	0.022	0.021	0.019	0.021	0.022	0.019	0.021	0.021	0.021	0.019	0.019	0.019	0.021	0.019	0.022	0.026	0.019	0.022	0.019	

[a] Genetic distances were calculated using the Kimura Two-Parameter Model.

Table 3.6. Mean squares from the analysis of variance conducted on clonal *R. solani* AG 2-2 IIIB effective concentration values determined using non-linear regresssion.

Source	df	MSE†
Clone	20	1.5
Fungicide	2	58.5*
Clone X Fungicide	40	1.2

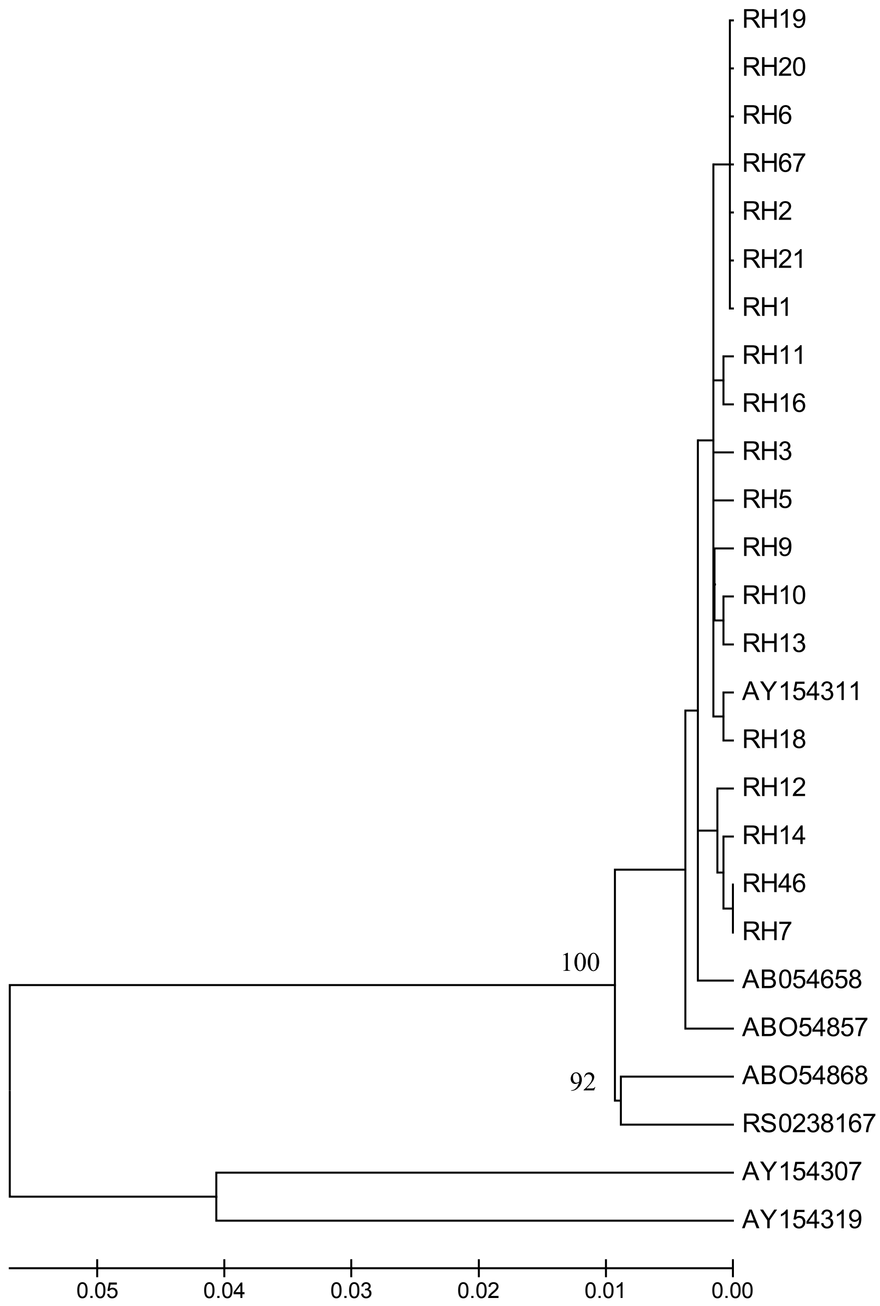
*Indicates significance at the $p \leq 0.05$.
† MSE = mean standard error.

Table 3.7. Mean effective concentration values for fungicides applied.

Fungicide	EC_{50} Value ($\mu g\ ml^{-1}$)	Waller Grouping*
Mancozeb	2.44	A
Chlorothalonil	0.85	B
Azoxystrobin†	0.38	B

* Mean significant difference = 0.46.
† Azoxystrobin treatment was amended with SHAM at 0.1% v/v.

FIGURE 1.



CHAPTER 4

IDENTIFICATION OF *RHIZOCTONIA* SPECIES ASSOCIATED WITH BROWN PATCH OF TALL FESCUE AND THEIR SENSITIVITY TO SELECTED FUNGICIDES

Identification of *Rhizoctonia* Species Associated with Brown Patch of Tall Fescue and Their Sensitivity to Selected Fungicides

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Abstract

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Rhizoctonia species were isolated from leaves of tall fescue exhibiting brown patch symptoms in June 2003. Leaves were collected from > 70 home lawns and commercially managed turf areas, primarily from Wake Co., NC. Leaf sections containing a single brown patch lesion were excised, rinsed and placed on alkaline water agar for selective isolation of *Rhizoctonia* spp. Isolates were identified to species and anastomosis group (AG) based on morphology, somatic compatibility with tester isolates, and rDNA sequencing. Of 224 isolates collected, 88 were *R. solani* AG 1, 105 were binucleate *Rhizoctonia* CAG 1, 14 were *R. zeae*, two were *R. cerealis*, and 15 were not identified to species or AG. One hundred and seventy four of the isolates were assayed for sensitivity

to the fungicides flutalonil, iprodione, tebuconazole, and trifloxystrobin. Mean EC_{50} values varied across fungicides and species, but generally were below 3.3 μg ml^{-1}. The greatest range in sensitivity was to flutalonil, with an EC_{50} value of 0.24 μg ml^{-1} for *R. solani* and > 10 μg ml^{-1} for *R. cerealis*. EC_{50} values were not reached (all > 10 μg ml^{-1}) with trifloxystrobin with or without the addition of SHAM for any of the organisms. The diverse pathogen population structure and the range in fungicide sensitivities observed indicates that response to management strategies in tall fescue may vary with location and environment.

INTRODUCTION

Multiple species of *Rhizoctonia* and binucleate *Rhizoctonia*-like fungi have been found in association with tall fescue (*Festuca arundinacea*) (Burpee 1980, Burpee and Martin 1992, Burpee et al. 1980, Martin and Lucas 1984, Martin et al. 1984). Identification of these fungi to species and anastomosis group (AG or CAG) is important for understanding the effects of these organisms on plant health and for optimizing disease management recommendations (Burpee and Martin 1992). For example, at least six AGs of *R. solani* have been identified from turfgrasses, but the most commonly isolated AG from tall fescue, AG-1 IA, is generally considered to be the cause of *Rhizoctonia* blight (Zhang and Dernoeden, 1995). *Rhizoctonia* blight, or brown patch, is the most damaging disease of tall fescue turf in the Southeastern US (Couch, 1995). Binucleate *Rhizoctonia, R. zeae,* and *R. cerealis* isolates are also pathogenic to tall fescue (Burpee et al., 1980, Martin and Lucas, 1984, Burpee and Martin 1992), but are not typically associated with *Rhizoctonia* blight.

Investigation of the organisms that affect the health of tall fescue is important in characterizing the population structure of *Rhizoctonia* species in natural turf systems. Characterization of *Rhizoctonia* is largely based on morphological characteristics, hyphal anastomosis with known tester isolates, nuclear condition, and DNA characterization (Martin 1987, Sherwood 1969, Zhang and Dernoeden 1995, Salazar et al., 2000, Toda and Hyakumachi, 2004). Pairing of isolates on a thin layer of water agar was determined to be the most efficient method for determining AG groups of *R. solani* (Zhang and Dernoeden, 1995).

Previous studies of *R. solani* population structure have been completed in agricultural cropping systems using both anastomosis pairing and molecular markers (MacNish et al., 1997; Ceresini et al., 2002; Ceresini et al., 2002; Ceresini et al., 2003). Populations of AG 3 from potato and tobacco in eastern North Carolina differed in their genetic structure. All thirty-two isolates from potato had a distinct AFLP phenotype, and 28 somatic compatibility groups were identified based on somatic compatibility on agar media. In comparison, of 36 AG 3 isolates from tobacco, there were only eight distinct groups based on somatically compatibility, and 28 distinct AFLP phenotypes. Based on these observations, it was determined that the *R. solani* AG-3 population structure had a clonal component; isolates from tobacco were primarily clonal, while the potato isolates were clonal based on introduction of inoculum into fields on seed potatoes (Ceresini et al., 2002; MacNish et al., 1997).

A study of *R. solani* AG 8 in West Australia using hyphal anastomosis and pectic zymograms showed that the isolates that demonstrated a C3 (clonal) hyphal reaction had the same pectic zymogram group (MacNish et al., 1993). These clones were found over a

broad geographical region, with individual clones found in Washington, Oregon and Western Australia. Results from these studies support a population model that includes both clonal individuals and some individuals that evolve through recombination (Ceresini et al., 2003; Ceresini et al., 2003; MacNish et al., 1993).

Aside from studies by Martin and Lucas (1984) that characterized *Rhizoctonia* spp. and related organisms collected from leaf litter and organic debris in a stand of tall fescue, little has been done to further characterize the population structure and dynamics of *Rhizoctonia* in tall fescue during brown patch epidemics. The objectives of this study were to identify the species and groups of *Rhizoctonia* present in leaves of tall fescue during brown patch epidemics and to determine if the species or groups vary in their sensitivity to fungicides commonly used for brown patch management.

MATERIALS AND METHODS

Isolate collection and identification. Two hundred and twenty four isolates of *Rhizoctonia* spp. were collected from tall fescue turf stands in June of 2003, from Wake County, North Carolina. The majority of the locations consisted of home lawns that were maintained by commercial lawn care companies. Other locations, such as municipal parks, represented turf under moderate to high levels of management and were comparable in quality of turf in the home lawns.

At each location, leaf blades exhibiting brown patch symptoms were collected from at least three different patches of diseased turf. Leaf sections containing individual lesions were excised, rinsed in sterile H_20 for 30 s, surface disinfested in 10 % Clorox® for 30 s, rinsed again in sterile H_20, and then placed on alkaline water agar (AWA) for selective isolation of *Rhizoctonia* spp. AWA contained streptomycin sulfate (100 μg ml$^-$

^{1}), penicillin G (100 μg ml^{-1}), 1N NaOH (0.8 μl ml^{-1}), and 1.5 % Bacto agar (Difco, Detroit, MI). Petri dishes were incubated at room temperature (22 to 25 C) for 48 hrs then actively growing hyphae were transferred to fresh potato dextrose agar (PDA, Difco) in Petri dishes.

Initially, isolates were separated into different groups based on morphological characteristics (Zhang and Dernoeden, 1995). An agar medium (W. A. Gutierrez and H. D. Shew, unpublished) was used to determine somatic compatibility between isolates and a known *R. solani* AG 1 isolate. The pairing medium is based on the media developed for study of *Sclerotium cepivorum* (Earnshaw and Boland, 1997) and *Sclerotinia sclerotiorum* (Kohn et al., 1990; Kohn et al., 1991). The basal medium consists of 3 % PDA to which red McCormick® food-color dye (McCormick & Co., Hunt Valley, MD 21030) was added to enhance visualization of somatic reactions. *Rhizoctonia solani* AG 1 isolates were identified by observing a microscopic (C2) killing reaction between hyphal cells of field isolates with the AG 1 tester isolate.

Representative isolates were selected from each group of isolates based on anastomosis reactions and morphological characteristics and classified based on ribosomal DNA (rDNA) sequences. Genetic relationships among clones were assessed using ribosomal DNA (rDNA) sequences. Each isolate was grown for ~7 days at room temperature ~25 ^{o}C in 2 ml of potato dextrose broth. The mycelial suspension was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 5 min at 11,000 rpm. The mycelial plug was harvested and genomic DNA was extracted using the Easy-DNA Kit (Invitrogen Corp., Carlsbad, CA). The DNA solutions were treated with RNase (40

μg $μL^{-1}$) and uncubated at 37 ^{o}C for 30 min. Samples were analyzed for purity by spectophotmetry and standardized to a concentration of 50 ng ml^{-1}.

Polymerase chain reaction (PCR) amplification of rDNA internal transcribed spacer (ITS) regions ITS1, 5.8S rRNA, and ITS2 was performed using primers ITS4 and ITS5 (White et al., 1990). PCR reactions were 50 μl in volume and consisted of 20mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTPs, 200nM of each primer, 1.5 U of Taq polymerase (Invitrogen Corp., Carlsbad CA), and 50 ng of genomic DNA. Thermal cycling conditions involved an initial denaturation step at 95°C for 3 min, followed by 33 cycles of 95°C for 30 s, 58 ^{o}C for 1 min, and 72 ^{o}C for 10 min. Amplification products were purified with a Quiquick PCR Purification Kit (Quigen Inc., Valencia, CA). Cycle sequencing reactions were performed using an ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems Inc., Forester City, CA). Following the removal of unincorporated dye using Centrisep columns (Princeton Separations Inc., Adelphia, NJ) the samples were dried and sent to the University of Iowa DNA Facility (Iowa City, IA) for analysis. Sequences homologous to the clonal isolates were identified through a BLAST search of the GenBank database (National Center for Biotechnology Information). All sequences were aligned using the Clustal W method in MegAlign 5.05 (DNASTAR Inc., Madison, WI) and adjusted by visual examination. A phylogenic tree was constructed in MEGA v2.1 using the Neighbor-Joining algorithm from genetic distances calculated using the Kimura Two-Parameter Model.

Fungicide sensitivity. From the original isolates, 171 isolates were selected to determine concentrations of selected fungicides that resulted in 50 % inhibition of mycelial growth

(EC_{50}). The fungicides were trifloxystrobin (Compass 50 WG, Bayer Environmental Sciences, Montvale, NJ) iprodione (26GT 25L, Bayer Environmental Sciences, Montvale, NJ), flutalonil (Prostar 70 WP, Bayer Environmental Sciences, Montvale, NJ), and tebuconazole (Lynx, Bayer Environmental Sciences, Montvale, NJ). PDA was amended with each fungicide to establish fungicide concentrations of 0.0, 0.01, 0.1, 1.0, or 10.0 μg ml^{-1}. Trifloxystrobin was added with and without salicylhydroxamic acid (SHAM) (Avile-Adame and Köller, 2002, Steinfeld et al., 2001). The SHAM was dissolved in acetone and added to all concentrations of trifloxystrobin (+ SHAM) at a concentration of 0.1 % (v/v), including a no-fungicide PDA control.

Each isolate was grown for 7 days on 2 % WA, and then a 6.75 mm diameter plug of colonized WA was transferred to the center of freshly prepared fungicide-amended PDA. Cultures were incubated at 24 C for 48 h, and radial growth of the colony was measured in 2 perpendicular directions. Effective concentration (EC_{50}) values, representing the fungicide concentration that reduced radial growth by 50%, were estimated using nonlinear regression of the percentage of growth inhibition on log_{10} transformed fungicide concentration (SAS 8.2., Cary, NC).

RESULTS

Isolate collection and identification. Identification of isolates from symptomatic tall fescue leaves based on morphological characteristics, somatic compatibility, nuclear condition, and molecular markers indicated there were two major groups of organisms present (Table 4.1). Of 224 isolates collected, 193 resembled *R. solani* based on morphological characteristics. Eighty eight isolates were subsequently identified as *R. solani* AG 1 using anastomosis reactions and DNA sequencing. A large number of

isolates (105) that appeared similar to *R. solani* were identified as binucleate fungi and were found to be closely related to *Ceratobasidium graminearum* (CAG 1) based on sequencing data (Table 4.2). The remaining isolates obtained from active lesions were identified as *R. zeae* (14 isolates) and *R. cerealis* (2 isolates); 15 isolates could not be identified to species or AG, but were similar to *Rhizoctonia* in appearance (Table 4.1).

Phylogenic Relationships

A BLAST search of the GenBank database identified 2 *R. solani* AG 1 isolates (GenBank accession no. AG122138 and AF354979) and a *C. graminearum* (GenBank accession no. AF354086) for comparison of genetic similarity to selected representatives selected from each major group of isolates. An *R. zeae* and *R. cerealis* (GenBank accession no AF222799 and AAF067640, respectively) were used as an outgroup for phylogenetic analysis of the *Rhizoctonia* isolates from tall fescue (Figs. 4.1a and 4.1b).

A phylogenic tree was constructed using representatives from the collection of isolates made from tall fescue and the most closely related entry available on GenBank (Fig 4.1a.) The sequences from the isolates (BP 28, BP 60; AF354086) were similar and formed a distinct clade (Fig. 4.1b). The *R. solani* AG 1, *R. zeae*, and *R. cerealis* isolates all formed distinct clades (Fig. 4.1a). Additional structure was also observed within the AG 1 clade. The *C. graminearum* isolates (BP 28 and BP 60) formed a distinct clade with 100 % bootstrap support (Fig. 4.1b).

Fungicide Sensitivity

Tebuconazole, iprodione, and flutalonil were effective in inhibiting mycelial growth of all isolates tested. Trifloxystrobin did not inhibit mycelial growth with or without the addition of SHAM. Flutalonil, iprodione, and tebuconazole were effective in

inhibiting mycelial growth of *R. solani* AG 1 and *C. graminearum.* For the *R. solani* AG 1 isolates, EC_{50} values were the lowest for flutalonil, followed by tebuconazole and iprodione (Table 4.3). Mean EC_{50} values for *R. solani* were low (below 3.5 mg L^{-1}) for all fungicides except trifloxystrobin. *Rhizoctonia zeae* was more sensitive to tebuconazole (0.24 mg L^{-1}) than flutalonil (1.91 mg L^{-1}) and iprodione (3.44 mg L^{-1}). The *R. cerealis* isolate was sensitive to iprodione and very sensitive to tebuconazole (0.09 mg L^{-1}). The two isolates of *R. cerealis* were not sensitive to flutalonil.

DISCUSSION

The results of this study indicate that the composition of *Rhizoctonia* spp. in tall fescue is diverse and populations are comprised of multiple species including both multinucleate and binucleate *Rhizoctonia* pathogens. Similar results were obtained in a previous study of the organisms present in organic debris and soil in a stand of tall fescue (Martin et al., 1984). However, isolates in this study included only isolates from foliar lesions. We did not detect *R. solani* AG 4 in our study as previously found by Martin et al. (1983).

Our results show that there are two major groups of organisms that are associated with brown patch lesions in tall fescue turf in central North Carolina. The *R. solani* AG 1 and the CAG 1 isolates comprised 39 % and 47 % of the total isolates recovered, respectively. Even though it has been reported that CAG 1 is pathogenic to tall fescue (Martin et al., 1984a; Burpee et al., 1980, Burpee and Martin, 1992) there are no studies that have examined the population dynamics of this group in tall fescue systems during brown patch epidemics. Our results indicate that isolates of CAG 1 can be a common pathogen of tall fescue and the ecology of this group warrants further investigation.

Rhizoctonia zeae and *R. cerealis* were isolated infrequently compared to *R. solani* and CAG 1 and accounted for only 14 % of the total isolates collected. Since all of the isolates were collected in June of 2003, it would be expected that *R. zeae* isolates, which have a temperature optimum of 32 °C, would be active during this period. Recovery of *R. cerealis*, only 2 isolates, during this period is interesting because this species is typically associated with yellow patch, which develops during cooler temperatures (<20 °C) (Burpee, 1980).

Mean EC_{50} values were consistent with previously reported values for *Rhizoctonia* (Tani and Beard, 1997). The most notable exceptions observed were the high level of sensitivity of *R. zeae* and *R. cerealis* isolates to tebuconazole and the lack of sensitivity (EC_{50} >10 mg L^{-1}) of the *R. cerealis* isolates to flutalonil. Differences in fungicide sensitivity within *Rhizoctonia* spp. and groups have been previously reported (Martin et al., 1984a; Royals 2002). The sensitivity of *R. zeae* and *R. solani* to tebuconazole (EC_{50} = 0.24 mg L^{-1} and 2.56, respectively) is also consistent with EC_{50} values reported for *R. zeae* (EC_{50} < 1) and AG 1 (EC_{50} = 2.97) to the DMI fungicide triadimefon (Martin et al., 1984). It is apparent that fungicidal sensitivity varies among species and subgroups of *Rhizoctonia* associated with tall fescue. Therefore, correct identification of the pathogens is important to optimize chemical control.

The population structure of *Rhizoctonia* present in leaves of tall fescue was diverse. Although the majority of the isolates collected were identified as either *R. solani* AG 1 or CAG 1, at one sampling location two CAG 1, two *R. cerealis*, one *R. zeae*, and 3 *R. solani* AG 1 isolates were collected on the same date. This level of diversity at a single site, coupled with the differences in sensitivity to the classes of fungicides

currently used to manage brown patch, indicates that it is important to develop efficient and effective control programs based on *Rhizoctonia* ecology in tall fescue. Further research is needed to clarify the role of CAG 1 as a pathogen causing brown patch of tall fescue.

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Table 4.1. Frequency of isolation of *Rhizoctonia* species from tall fescue active brown patch leaf lesions in June of 2003.

Source	Anastamosis Group	2003[a]
Rhizoctonia solani	AG 1	88
Ceratobasidium graminearum	CAG 1	105
R. zeae	n/a	14
R. cerealis	n/a	2
Unknown[b]	n/a	15

[a]224 isolates recovered in June 2003.

[b]Unknown isolates were similar to *Rhizoctonia* in morphological characteristics but were not closely related using genetic distance calculatations.

Table 4.2. a.) Genetic distance matrix from the internal transcribed spacer (ITS) 1, 5.8S rRNA, and ITS 2 regions of the rDNA locus from *Ceratobasidium graminearum* (CAG 1): (BP28, BP60, AF354086); and **b**.) *R. solani* AG (1) (BPAG1, AG122138, and AF35497)

a.)

	BP28	**BP60**	**AF354086**
BP28	--	--	--
BP60	0.006	--	--
AF354086	0.009	0.003	--

b.)

	AG122138	**AF35497**	**BPAG1**
AG122138	--	--	--
AF35497	0.074	--	--
BPAG1	0.000	0.074	--

Table 4.3. Predicted mean effective concentration (EC_{50}) values of the selected fungicides calculated by non-linear regression models of concentration by radial growth.

		Fungicides			
Isolate	n	Flutalonil	Iprodione	Tebuconazole	Trifloxystrobin (+SHAM)
		EC_{50} ($\mu g\ ml^{-1}$)			
R. solani (AG1)	65	0.24	3.12	2.56	>10
C. graminearum	89	1.00	1.64	2.96	>10
R. zeae	12	1.91	3.28	0.24	>10
R. cerealis	2	>10	1.50	0.09	>10

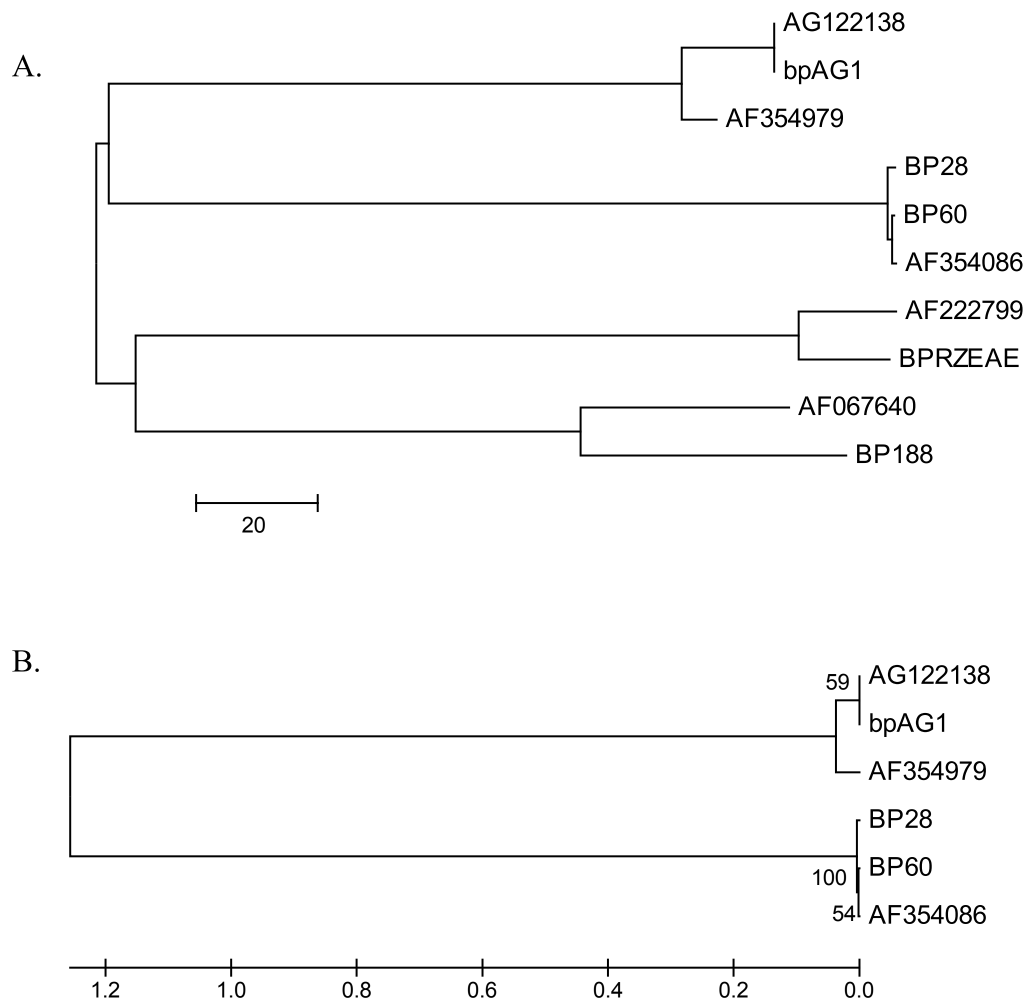

Figure 4.1. (a.) Phylogenic tree drawn from the nucleotide sequences of internal transcribed (ITS) region of ribosomal DNA from isolates of all *Rhizoctonia*-like isolates collected from tall fescue turf. Tree constructed using Neighbor-Joining algorithm on sequence data. (b.) Phylogenic tree drawn from ITS region of the 2 major groupings of *Rhizoctonia* isolates collected. Tree constructed using Kimura Two-Parameter Model.