WAITEA AND *RHIZOCTONIA* PATHOGENS OF SEASHORE PASPALUM – THE ROLE OF SALINITY IN DISEASE EXPRESSION AND CHARACTERIZATION OF A NEW *WAITEA CIRCINATA* VARIETY CAUSING BASAL LEAF BLIGHT

By

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To my wife and best friend Rosemary, my greatest source of support and encouragement and to God for all his blessings and guidance in my life

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LIST OF ABBREVIATIONS

bp	base pairs (of a nucleotide sequence)
d	days
h	hour or hours
FAWN	Florida Automated Weather Network
SP	Seashore paspalum
UCAG	Unidentified *Ceratobasidium* anastomosis group
UWC	Unidentified *Waitea circinata* variety

Abstract of Dissertation Presented to the Graduate School
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WAITEA AND *RHIZOCTONIA* PATHOGENS OF SEASHORE PASPALUM – THE ROLE OF SALINITY IN DISEASE EXPRESSION AND CHARACTERIZATION OF A NEW *WAITEA CIRCINATA* VARIETY CAUSING BASAL LEAF BLIGHT

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Seahore paspalum is growing in popularity in different regions of the world as a high quality warm season turfgrass with the added benefit of tolerance to salinity. A great diversity of *Rhizoctonia*-like isolates were recovered from eight seashore paspalum fairways across middle to Southern Florida including but not limited to *R. solani* AG 2-2LP, the causal pathogen of large patch disease. The most diverse group of isolates recovered were *Waitea circinata* varieties. Soil temperatures, canopy temperatures, soil pH and soil electrical conductivity (as an indicator of salinity and sodium levels) was measured in conjunction with the isolates. Through the use of statistical models, soil and canopy temperatures and increasing salinity were significant indicators of increased recovery of *R. solani* 2-2LP and *W. c*. var *zeae* from seashore paspalum tissue.

Through the seashore paspalum sampling program, a new pathogen was isolated and identified. *Waitea circinata* var. *prodigus* causes a basal leaf blight of seashore paspalum. In controlled environment pathogenicity studies, symptoms progressed most rapidly on creeping bentgrass and roughstalk bluegrass. ‘SeaDwarf’ and ‘SeaIsle

Supreme' seashore paspalum and bermudagrass were also affected. Disease symptoms of basal leaf blight progresses from a foliar necrosis on the basal leaves to progressing rapidly up the turfgrass plant. Profuse pink to yellow mycelia with small salmon to yellow aggregate sclerotia developed in culture most rapidly at temperatures of 25 to 35°C.

Irrigation studies with four concentrations of sodium chloride (0, 10,000, 15,000 and 20,000 ppm) coupled with inoculations of pathogenic strains of *Chyrsorhiza zeae* and *R. solani* AG 2-2LP were performed on potted 'SeaDwarf' seashore pasplum compared to mock-inoculated turfgrass which served as an untreated comparison. Significant decreases in turf quality and increases in turf damage resulted as salinity increased and when inoculated with *C. zeae* and *R. solani* AG 2-2LP. Dry weight was not a good consistent indicator of pathogenicity with either *C. zeae* or *R. solani* but did decrease when *C. zeae* inoculation was coupled with salt levels of 15,000 ppm and above. There were no interactions, positive or negative, between saline water and SP quality or turf damage caused by *R*. *solani* or *C*. *zeae.*

CHAPTER 1
REVIEW OF LITERATURE

Seashore Paspalum

Paspalum vaginatum Swartz is a diploid (2n = 20) species in the Poaceae with a dark green foliage forming a dense canopy (Morton, 1973; Duncan and Carrow, 2000b; Duncan, 2003). The genus *Paspalum* includes halophytic, salt-water-tolerant grasses with several reported places of origin from 1759 to 1983 (Duncan and Carrow, 2000b; Duncan, 2003). Seashore paspalum (SP), *Paspalum vaginatum* Swartz, most probably originates from South Africa and is found growing naturally along seashores, coastal riverbanks subject to periodic flooding, as well as in brackish waters (McCarty, 2005). Seashore paspalum produces a root system with deep, dense rhizomes and stolons more robust than bermudagrass, *Cynodon dactylon* (L.) Pers. A warm season, perennial turfgrass species, SP has a fine to medium leaf texture with folded leaf vernation, no auricles and pointed leaf blade tips. Leaves have large, compact sheaths containing short ligules (0.5 mm long) with a pubescent, broad collar. SP stems are smooth and flattened producing long, dense rhizomes with short internodes. Stolon nodes are described as especially pubescent. Although seed head production can be prolific, reproduction is handicapped due to self-incompatibility between the same genotypes . Flowering culms range from 8 to 60 cm in height, producing two to three smooth spikelets ranging in size from 3.5 to 4.0 mm long (Beard, 2002).

Paspalum vaginatum is part of a diverse group of grasses, subfamily Panicoideae, and in the tribe Panicea, which include such genera as *Axonopus* (common and tropical carpetgrass), *Digitaria* (crabgrass), *Panicum* (torpedograss), *Pennisetum* (kikuyugrass) and *Stenotaphrum* (St. Augustinegrass) in temperate to tropical climates (Watson and

Dallwitz, 1992). Because of their durability and pleasing aesthetic properties, St. Augustinegrass and kikyugrass are maintained as desirable turfgrasses in lawns, industrial parks and sometimes as borders around golf course fairways. The genus *Paspalum*, contains noted species *Paspalum notatum* Flűgge (bahiagrass) and *Paspalum distichum* L. (saltwater couch or siltgrass) (Watson and Dallwitz, 1992; Smiley et al., 1994). Bahiagrass is a utility turf species with poor quality due to its slow recuperative potential, abundant production of seed heads and limited climate range encompassing warm-humid climates representative of tropical and sub-tropical areas (Beard and Beard, 2005).

Paspalum vaginatum is a warm season species which utilizes the C_4 photosynthetic pathway, dicarboxylic acid cycle, for energy capture and assimilation of carbon dioxide (CO_2) (Hull, 1992). Cool season grasses utilize a reductive pentose phosphate cycle or C_3 pathway. The C_4 versus C_3 classification is based on the number of carbon atoms in the first stage of CO_2 assimilation during photosynthesis. During periods of elevated temperatures coupled with high light intensity, warm season C_4 grasses are more efficient than C_3 grasses in the ability to store CO_2 within the bundle sheath cells of the chloroplasts (Nobel, 1991). C_3 grasses incur higher photorespiration rates under these conditions because, in the absence of stored CO_2, oxygen (O_2) competes with CO_2 for the primary enzyme binding site necessary for carbon assimilation. Thus total respiration in C_3 grasses can exceed photosynthesis during these periods of extended high temperatures and high light intensities leading to increased environmental stress and an overall turfgrass decline or death (Hull, 1992).

Following breeding efforts, SP was introduced into the golf course and lawn care markets in 1996 as a high quality turfgrass species with new cultivars requiring fewer environmental inputs (e.g. nitrogen fertilizer and irrigation water) compared to other turfgrass species and also having fewer pest problems (Allar, 2001; Daniel, 2003; Duncan, 1996; Duncan and Carrow, 2000a; Kuo and Fermanian, 2001; Williams, 2002). With its robust root and rhizome system, SP can survive on as little as 30 to 50 percent of the water required by bermudagrass. Seashore paspalum also has a higher tolerance to low light levels and water logged soils than bermudagrass. These factors give SP a favorable environmental profile (Huang et al., 1997; Duncan and Carrow, 2000b; McCarty, 2005). The decreased water requirement of SP, compared to bermudagrass, is more pronounced when low quality water such as saline water or effluent water versus potable water is used to irrigate the grass (Huang, 1997). Lee et al. (2004) reported differences between varieties of SP in their tolerances to drought and saline stress. Seashore paspalum was noted to be highly salt tolerant and cabable of thriving in areas damaging to other turfgrass species (Dudeck and Peacock, 1985; Lee et al., 2004). In salt-affected ecosystems where only saline or re-cycled water is available for irrigation, SP may be the only option for high quality turfgrass. Seashore paspalum is a vigorous growing turfgrass species capable of having a desirable color and good mowing-stripe characteristics but is noted to be prone to thatch accumulation (McCarty, 2005). A number of cultivars, based on ecotypes selected for their adaptation to various environments, now exist for use in turfgrass markets. Some of these cultivars are better adapted to higher heights of cut in fairways while others can tolerate lower heights of cut on putting greens. Seashore paspalum uses can range from low

maintenance, such as a remediation turfgrass species in high salt areas, to higher maintenance uses on golf courses.

As of September 1999, it was reported that there were 16,743 golf courses in the United States, Florida having more than any other state at 1228 golf courses (verbal discussion with Todd Lowe, USGA Agronomist, personal communication 2007). At the time (2011), there were estimated to be forty or more golf courses in Florida with SP and several courses in use or being constructed with SP in the Caribbean. Due to its noted poor cold tolerance, SP's ideal geographical range overlaps with bermudagrass in tropical or subtropical areas of the world where freezing temperatures are transient or short in duration and severity (Duncan and Carrow, 2000a; Duncan & Carrow, 2000b; Duncan, 2003; McCarty, 2005).

Scaptericus vicinus, the tawny mole cricket, can be extremely damaging to bermudagrass, and many golf courses routinely apply insecticides to manage mole crickets. Braman (2004) indicated that SP was more tolerant to tawny mole crickets than was bermudagrass. At the time of the introduction of SP to the turfgrass industry, turfgrass breeders speculated that SP would have few fungal disease problems especially when established in saline environments, because fungi have poor salt tolerance (Duncan and Carrow, 2000b).

Very little research exists pertaining to disease susceptibility of SP. Because SP tolerates salt levels that would severely damage most other grasses and broadleaf species, the use of salt as a herbicide or nematicide has been suggested (Hixson et al., 2004, Wiecko, 2003). Hixson et al. (2004) investigated the effects of saline water treatments on control of sting nematode, *Belonolaimus longicaudatus*, a significant pest

of SP, in addition to effects of saline water on root length. Research with the parasitic nematodes *Belonolaimus longicaudatus* and *Hoplolaimus galeatus* , the lance nematode, indicated SP supported similar population counts to those supported by bermudagrass, suggesting similar susceptibility between the grasses (Hixson et al., 2004). In the amended salt water treatments there was a definitive range of 10 to 15 dS m^{-1} that resulted in increased densities of *B. longicaudatus*, whereas reproduction and feeding decreased at levels above 25 dS m^{-1}. Root stunting due to *B. longicaudatus* occurred at salinity levels of 0 to 10 dS m^{-1}, but decreased at 25 dS m^{-1}. *Gaeumannomyces graminis* var. *graminis*, an ectotrophic, root-infecting fungus that has been associated with warm season turfgrasses, was identified as a pathogen of 'SeaIsle 1' SP (Elmore et al., 2002). Dollar spot, caused by *Sclerotinia hom*oeocarpa F. T. Bennett, has been reported to cause up to 95% turfgrass death on bahiagrass pasture fields (Blount, 2002). Dollar spot also affects SP on US golf courses and was reported in China as a disease of *Paspalum vaginatum* (Smiley et al., 1994, Lv et al., 2010). The degree of susceptibility of *Paspalum vaginatum* to the various pathogenic *Rhizoctonia* spp. is not well known (Morton, 1973). A study by Canegallo and Martin (2006) demonstrated that *Rhizoctonia solani* AG 2-2LP, caused large patch disease on several cultivars of SP in coastal South Carolina. Leaf and sheath spot caused by *Rhizoctonia zeae* has been diagnosed by the Univ. of Florida Rapid Turf Diagnostics lab (P.F. Harmon *personal communication*). Leaf and sheath spot was among the most diagnosed disease on the samples of turfgrass submitted in 2006.

Rhizoctonia

The genus *Rhizoctonia* was first noted by de Candolle in 1815 to consist of imperfect fungi that produced uniform sclerotia and were associated with roots of plants

(Sneh et al., 1998). The name *Rhizoctonia* was derived from the Greek words ‘rhiza’ meaning root, and ‘ktonos’ meaning to murder or destroy. *Rhizoctonia* is classified in the Fungi Imperfecti. *Thanatephorus,* the teleomorph of *Rhizoctonia, is a* Hymenomycete in the Basidiomycotina. *Rhizoctonia* species are noted to be pathogens of economic concern on both monocots and dicots encompassing a wide host range of important agronomic crops including but not limited to cereals, fruits, legumes, vegetables, and many annual, perennial, herbaceous and woody ornamental plants (Farr et al., 1989). Some *Rhizoctonia* species are non-pathogenic and develop mycorhizal, symbiotic relationships with tropical orchids (Farr et al., 1989, Otero et al., 2002).

Classification of *Rhizoctonia* species historically has been based on the number of nuclei per hyphal cell, ability of hyphae to anatamose, and characteristics such as sclerotial morphology and mycelial pigmentation (Sneh et al., 1998). A number of species with binucleate hyphal cells, such as *Rhizoctonia cerealis*, and many diverse multinucleate species that have been subdivided into variety of designations and anastomosis groups, have at one time been included in the genus (Sanders et al., 1978; Martin et al., 1983a; Martin and Lucas 1984a; Hyakumachi et al., 1998; Tomosa-Peterson and Trevathan, 2007).

Rhizoctonia hyphae are uniform in size exhibiting consistent acute and right angle branching near the distal septum of younger hyphae with constriction at the point where branching occurs. Sclerotia are survival structures that some *Rhizoctonia* species form (Hyakumachi et al., 1998). Sclerotia, if produced, can vary considerably in color and size. Monilioid cells, swollen cells similar to sclerotia, produced by many isolates of *R.*

solani and binucleate *Rhizoctonia* spp. also can serve as survival structures in soil, organic matter or thatch (Sneh et al., 1998).

The sexual state of some *Rhizoctonia* species has been observed and differences in the teleomorphs have led to separate and distinct teleomorphic nomenclatures based on morphological differences in basidia, basidiospores, and sterigmata (Sneh et al., 1998, Stalpers and Andersen, 1996). The taxonomy of teleomorphs and anamorphs can be confusing because different names are assigned for perfect and imperfect states of the same fungal species. Several taxonomic revisions have stemmed from studies where the teleomorph stages of *Rhizoctonia* species were induced and characterized (Moore, 1987; Parmeter et al., 1967; Oniki et al., 1985).

Molecular biologists have pioneered a new approach to identification and classification of *Rhizoctonia* species, varieties and subgroups (Parmeter et al., 1967; Hayakawa et al., 2006; Hyakumachi et al., 1998; Yokoyama et al., 1985). Martin (1987) introduced a rapid method for differentiation of multi-nucleate versus bi-nucleate *Rhizoctonia* spp. associated with turfgrass diseases. Fungal hyphae were incubated up to 24 h on agar-coated microscope slides and subsequently stained with a DNA-binding probe, 4,6'-diamidino-2-phenylindole (DAPI). DAPI staining causes nuclei within fungal cells to fluoresce when viewed with ultraviolet light microscopy.

A major scientific breakthrough involving the purification of heat-stable DNA polymerase coupled with oligonucleotide synthesis led to the ability to amplify specific regions of DNA through repetitive replication cycles in a process known as polymerase chain reaction (PCR) (Arnheim and Erlich, 1992; White et al., 1990; Smyth, 1992). The use of PCR has greatly facilitated the identification and differentiation of fungal species,

varieties and strains. This major advancement came with the discovery of methods to read portions of the DNA sequence of an organism through a process known as sequencing. As an example, internal transcribed spacer (ITS) regions exist as non-functional and variable sequences between highly conserved sequences encoding ribosomal subunits (rDNA as a whole). Comparisons between sequences published within shared information banks such as GenBank (http://www.ncbi.nlm.nih.gov/genbank) have led to a greater understanding of the evolutionary relationships of fungal species.

Restriction Fragment Length Polymorphism (RFLP) analysis utilizes differences between homologous DNA sequences, namely the differing locations of restriction enzyme sites, to identify genetic differences between isolates without the need for sequencing. Through the use of specialized digestive restriction enzymes that cut or 'digest' DNA at these specialized sites, different fragment lengths result. These fragments are then separated by size electophoretically and visualized with ultraviolet light after staining with ethidium bromide. Use of specific restriction enzymes, such as Hap II (HpaII), have been used to cut DNA within specific regions to assist in the identification and differentiation of *Rhizoctonia* spp. into varieties with a shared teleomorph, such as *Waitea circinata* var. *circinata*, *W. c.* var. *zeae* and *W. c.* var. *oryzae* (Toda et al., 2005; de la Cerda et al., 2007).

Random amplification of polymorphic DNA (RAPD) analysis uses PCR in a non-template-specific way (Williams et al., 1990). Short, random nucleotide sequences, or primers, are used, and template DNA is subjected to PCR amplification. Large template DNA is required as this increases the likelihood of amplification. This technique is

useful for determining new or divergent species within large populations. Knowledge of the target DNA sequence is not needed with RAPD analysis. If successful, multiple random fragments bands will result following gel electrophoresis. The size and location of these bands across multiple isolates are used to identify genetic diversity within and between species. RAPD PCR analysis was used to group genetically similar *Rhizoctonia* isolates and to identify variability in the *W. circinata* varieties that led to the identification of a new pathogen of bentgrass (Toda et al., 1999; Toda et al., 2005).

Taxonomic reclassifications based largely on the increased understanding of the evolutionary relationships of these similar-looking fungi have resulted in the proposal of several new anamorphic genera, each associated with a distinct teleomorph. For *Rhizoctonia-like* fungi, Moore (1987) suggested the name *Moniliopsis* because the epithet *Rhizoctoni*a was not published according to the International Rules of Botanical Nomenclature, and *Moniliopsis* predated the name *Rhizoctonia*. However, due to extensive reference in mycological and plant pathological literature, the name *Rhizoctonia* has been conserved (Stalpers et al., 1998). In an effort to associate a separate and distinct anamorph with each known teleomorph, the name *Rhizoctonia* is limited to fungi that have a *Thanatephorus* teleomorph, while the name *Ceratorhiza* has been proposed for fungi with a *Ceratobasidium* teleomorph (Moore 1987). Similarly the name *Chrysorhiza* has been proposed as the anamorphic genus for *Waitea* teleomorphs (Stalpers and Andersen, 1996). Although the *Rhizoctonia* names are still commonly used by plant pathologists, the taxonomic epiphets and synonyms used in this dissertation are listed in Table 1-1. Interchageable use of anamorphic and teleomorphic nomenclature will be used in this dissertation.

Rhizoctonia and Turfgrass

Rhizoctonia was first noted to cause brown spots on creeping bentgrass putting greens in Pennsylvania by Piper and Coe (1918). The disease was described as damaged turfgrass associated with white mycelium and as not responding to lime or fertilizer. An "unpleasant" dark smoky ring leading to sclerotia formation also was noted. *Rhizoctonia* spreads chiefly via the growth of mycelium from sclerotia in the soil, thatch, or below ground turfgrass tissues and causes disease in much the same way today. Visual symptoms of infected turfgrass can vary significantly depending on the causal *Rhizoctonia* species as well as the target turfgrass host species (Smiley et al., 1994; Couch, 1995). Symptoms may appear as yellow to brown rings or patches that may expand under an environment conducive to the pathogen but not necessarily ideal for the host.

"*Rhizoctonia*" associated diseases of turfgrass are referred to by a number of names such as brown patch, brown ring patch, foliar blight, large patch, leaf and sheath spot, sheath blight and yellow patch (Aoyagi et al., 1998; Burpee and Martin, 1992; Couch, 1995, Martin and Lucas, 1984a, Smiley et al., 1994, Toda et al., 2005; Vargas, 1994). *Chrysorhiza zeae*, *R. oryzae*, *Ceratorhiza cerealis*, and six anastomosis groups (AG) of *R. solani* are cited as pathogenic on turfgrass (Burpee and Martin, 1992; Haygood and Martin 1990; Martin and Lucas, 1984a.).

Rhizoctonia solani

The most common and widespread pathogenic *Rhizoctonia* is *Rhizoctonia solani* Kuhn, teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk. *Thanatephorus cucumeris* can be differentiated from similar teleomorphs based on the shape and size of the sterigmata and basidiospore (Sneh et al., 1998). Unfortunately the sexual stage

of *R*. *solani* is rarely observed in turfgrass, and it is difficult to induce in culture. Therefore, diagnostic differentiation via sexual structures is not practical.

Rhizoctonia solani has the widest host range and has been the most studied *Rhizoctonia* species that infects turfgrasses. Infection by *R. solani* has been reported to occur on over twelve turfgrass species, and it is unlikely that any species of turfgrass is immune (Couch, 1985).

On cool-season turfgrass species, *R. solani* causes the disease brown patch also known as *Rhizoctonia* blight (Couch, 1995). Disease is most severe in poorly drained soils and with turfgrass stimulated by nitrogen (Hearn, 1943, Couch, 1995). The ideal temperature range at which *R. solani* infects cool season turfgrass was determined to be between 20 to 30^{o}C (Burpee and Martin, 1992). *R. solani* AG 1-IB and AG 2-2 were observed to grow optimally in vitro at a temperature of 27^{o}C, while AG-4 and AG-5 grew optimally at 25^{o}C and 21^{o}C respectively. Wet conditions that persist for at least 10 h or high relative humidity conditions, greater than 95%, coupled with night temperatures above 20°C, were ideal for *Rhizoctonia solani* (AG 1) infection (Smiley et al., 1992).

On warm season turfgrasses *R. solani* is an active pathogen from early fall to late spring in the Southern areas of the United States (Couch, 1995; Martin, 2000). *R. solani* AG-2-2IV was observed infecting warm season turfgrasses at temperatures below 20°C (Burpee and Martin, 1992). Disease symptoms are most noticeable as large patches of dead and damaged turfgrass are slow to green-up in the spring following winter dormancy (Ayogai et al., 1998; Ayogai et al., 1999; Canegello and Martin, 2007). *Rhizoctonia solani* AG 2-2IV and AG 2-2LP are associated with leaf, crown and stolon rot symptoms of large patch on warm season turfgrass species

(Burpee and Martin, 1992; Ayogai et al., 1998; Martin, 2000; Blazier and Conway, 2004; Canegallo and Martin, 2007; Hyakumachi et al., 1998; Martin, 2000). Large patch disease symptoms from *R. solani* can be very severe on zoysiagrass , St. Augustinegrass, bermudagrass, centipedegrass and SP (Aoyagi et al., 1998; Canegallo and Martin, 2007; Couch, 1995; Hurd and Grisham, 1983; Hyakumachi et al., 1998; Smiley et al., 1994).

Isolates of *Rhizoctonia solani* can be separated into groups based on host pathogenicity or distinguished utilizing anastomosis groupings (AG) (Ogoshi, 1987). Determination of anastomosis compatibility of two isolates entails the use of differential media or amended water agar to induce hyphal fusion between the isolates and then observing the subsequent reaction of the fused cells (Carling and Sumner, 1992; Sneh et al., 1998; Yokoyama et al., 1988). To test a *R. solani* sample, a representative "tester" strain from a known AG group is compared to an "unknown" isolate. There are several different methods for pairing these isolates such as on water agar in petri dishes, on microscope slides coated with agar, on cover slips, or placement of cellophane on agar media (Carling et al., 1987; Hyuakumachi et al., 1988; Ogoshi, 1987, Parmeter et al., 1967; Sneh et al., 1988; Zhang and Dernoeden, 1995). A system was proposed by Carling et al. (1988) to categorize the degrees of anastomosis between *Rhizoctonia solani* isolates with '0' being no reaction, '1' representing hyphal contact and attachment with no membrane fusion, '2' representing fusion between hyphae with possible membrane fusion but cells often die, and '3' is complete fusion of hyphae and membranes without death. Perfect fusion, where hyphae from each isolate pair with visible passing of nuclei between the pairs occur, indicates that the unknown

isolate is in the same AG group as the known “tester” strain (Carling et al., 1988; Carling and Sumner, 1992; Sneh et al., 1998).

Rhizoctonia solani has been assigned to different anastomosis groups and subgroups based on *in vitro* morphological differences such as mycelial color and formation of sclerotia (Burpee and Martin, 1992; Hyakumachi et al., 1998; Hayawaka et al., 2006; Tomosa-Peterson and Trevathan, 2007). Zhang and Dernoeden (1995) demonstrated that culture characteristics can be reliable in identification of the different *Rhizoctonia solani* AG groupings. Hyakumachi et al., (1998) utilized RFLP analysis on ribosomal DNA in the nuclear coded genes to segment the previous AG 2-2 groups pathogenic on turfgrass designating the subgroup AG 2-2LP, *Rhizoctonia solani* AG 2-2LP grew optimally in vitro at temperatures of 25^{o}C compared to 28^{o}C for AG 2-2IIIB and AG 2-2IV.

The four *R. solani* AG groups identified as pathogens on turfgrass include two subgroups of AG 1 (IA and IB), three subgroups under AG 2 (2IIIB, 2IV and 2LP), AG 4, and AG5 (Zhang and Dernoeden, 1995, Burpee and Martin, 1992; Martin and Lucas, 1984a, Hyakumachi et al.,1998). *R. solani* AG-4 was isolated by Martin and Lucas (1983a) and identified as a pathogen of perennial ryegrass (*Lolium perenne*), bermudagrass ‘Tifway 419’ [*Cynodon dactyon* (L.) Pers. X *Cynodon transvaalensis* (Burtt-Davy)] and red fescue (*Festuca rubra*). Tomosa-Peterson and Trevathan (2007) explored pathogenicity of AG-4 and AG 1-IB reporting high rates of infection on creeping bentgrass (*Agrostis palustris* Huds.).

Culture characteristics such as pigmentation, zonation, sclerotia, moniloid cells, and growth rates at various temperatures can help distinguish between *R. solani* groups

that are pathogenic to turfgrass (Carling and Sumner, 1992; Ogoshi, 1987; Oniki, 1986). *Rhizoctonia solani* AG 1-IA is brown to light brown in culture with large sclerotia (2 to 5 mm) whereas *R. solani* AG 1-IB has mycelia darker brown in pigmentation with smaller irregular sclerotia (Sneh et al., 1998). *Rhizoctonia solani* AG 2-2IIIB has buff to dark brown pigmentation with distinct sclerotial formation and zonation in culture while AG 2-2IV and AG 2-2LP both exhibited dark brown aerial mycelial growth, no zonation, and no sclerotial formation in culture (Hyakumachi et al., 1998).

Rhizoctonia zeae* and *Rhizoctonia oryzae

When investigating 42 isolates obtained in Japan from rice, corn or paddy soil, three isolates of *R. zeae* and *R. oryzae* were induced to develop the same perfect state named *Waitea circinata* (Oniki et al., 1985). *Waitea circinata* Warcup & Talbot was classified as a hymenomycete similar to *Thanatephorus* but differing in basidiospores that are unable to repetitively germinate on shorter horn-like sterigmata (Warcup and Talbot, 1962). Gunnell (1986) discovered that the teleomorph of *R. zeae* and *R. oryzae* was *Waitea circinata* but also reported variability in basidiospore morphology between the species. Additionally Gunnell noted structural variability in the anamorphic sclerotia of each species. Thus the teleomorphs of *R. zeae* and *R. oryzae* were assigned varietal names under *Waitea circinata* to recognize these differences. The establishment of anastamosis group *oryzae* (WAG-O) and anastamosis group *zeae* (WAG-Z) was proposed (Zhang and Dernoeden, 1995; Sneh et al., 1998). It is common for researchers to use conserved DNA sequences to assign a teleomoprphic name in the absence of direct observation of the sexual structures (de la Cerda et al., 2007; Toda et al., 2005; Toda et al., 2007). Molecular methods utilizing phylogenetics has validated separation *W. circinata* from *T. cucumeris*. *Waitea circinata* and *T. cucumeris*

share the same Phylum (Basidiomycota) but differ in Class, Order and Family (Lawrey et al., 2008). *Waitea circinata* has been placed in the Class Agaricales, Order Corticiales and Family Corticiaceae whereas *T. cucumeris* is placed in the Class Hymenomycetes, Order Ceratobasidiales and family Ceratobasideaceae (Lawrey et al., 2008). Bruns et al., 1998, placed *W. circinata* in the Class Agarimycetes, closely related to an ectomycorhizal fungus of oak seedlings, *Piloderma croceum*.

Variety designations were proposed to recognize differences within the *W. circinata* species as they relate to the anamorphic nomenclature (e.g. *W. c.* var. *circinata*, *W. c.* var. *oryzae* and *W. c*. var. *zeae*; *R. circinata* var. *circinata*, *R. circinata* var. *oryzae* and *R. circinata* var. *zeae*) (Leiner and Carling, 1994). This proposed nomenclatural change from *R. zeae* and *R. oryzae* to varieties *R. c.* var. *zeae* and *R. c.* var. *oryzae* has been utilized by some but ignores the designation and association of *Chrysorhiza* as the anamorphic genus of *Waitea circinata* (Piryatmojo et al., 2002a; Toda et al., 1999; Toda et al., 2005; de la Cerda et al., 2007).

Chrysorhiza zeae (Voorhees) Andersen and Stalpers was a significant pathogen of the *Poaceae* family whereas *W. circinata* was once thought to only be saprophytic (Warcup and Talbot, 1962; Burpee and Martin, 1992; Stalpers and Andersen, 1996; Garcia et al., 2008). *Chrysorhiza zeae* Voorhees, teleomorph = *Waitea circinata* Warcup & Talbot, in addition to being a major pathogen of corn (*Zea*), oats (*Avena*), sunflower (*Helianthus*), soybean (*Glycine*), and wheat (*Triticum*), was noted as a pathogen of warm and cool season turfgrass species (Haygood and Martin, 1998; Martin and Burpee, 1992; Martin et al., 1983b).

Chrysorhiza zeae is white to buff or salmon to pink in culture with reddish, ball shaped sclerotia in artificial media (Sneh et al., 1998; Smiley et al., 1994). Leaf and sheath spot occurs at temperatures higher than typically associated with diseases caused by *R. solani* (Couch 1995). Leaf and sheath spot has been referred to as "hot weather" brown patch by some turfgrass professionals based on the pathogen's optimal growing temperature of 30^{o}C and above (Martin and Lucas, 1984a; Burpee and Martin, 1992; Elliottt, 1999). *Chrysorhiza zeae* exhibits greater tolerance to the benzimidazole and dicarboximide class of fungicides than *R. solani* (Carling et al., 1990; Elliott, 1999; Martin et al., 1984b; Royals, 2002). This differential sensitivity to fungicides such as thiophante-methyl, a benizimidazole, effective against *R. solani*, may result in population increases in insensitive *Rhizoctonia*-like populations such as *C. zeae* where these *Rhizoctonia* selective fungicides are used (Elliott 1999).

Rhizoctonia oryzae fits the concept of *Chrysorhiza,* similar to *C. zeae* having the *Waitea circinata* teleomorph, but the original name was not published according to the tenets of the International Code of Botanical Nomenclature (Andersen and Stalpers, 1994). Supporting taxonomic work regarding Latin diagnoses and descriptions are required before *R. oryzae* can be recognized as *Chrysorhiza oryzae*, however referencing *R. oryzae* as *W. c.* var *oryzae* is valid.

Rhizoctonia oryzae Ryker & Gooch (1938) was first noted as a foliar disease on rice in Louisiana distinctly different from *C. zeae*. *Rhizoctonia oryzae* grows rapidly at temperatures of 30^{o}C and 32^{o}C. *Rhizoctonia oryzae* is white to salmon to pink in culture with small aggregate salmon colored sclerotia that form on the PDA agar surface (Smiley et al., 1994; Zhang and Dernoeden, 1995; Gunnell, 1998; Sneh et al., 1998).

There is less information on the pathogenicity of *R. oryzae* on turfgrass compared to *C. zeae*. *Rhizoctonia oryzae* was demonstrated to cause a sheath rot and foliar lesions on centipedegrass and St. Augustinegrass progressing over five days in pathogenicity studies (Haygood and Martin, 1990). Growth of a collection of isolates (Elliott 1999) became limited at 20°C while Tomosa-Peterson and Trevathan (2007) found optimal temperatures for growth to be 26 and 28°C for two isolates obtained from corn.

Salinity Issues

Water quality is an important issue that can ultimately dictate agronomic decisions in turfgrass management especially during periods of drought or in areas of the world where rainfall is limited. High soluble salts can have detrimental effects on the function of turfgrass roots. Salinity, whether due to proximity to bodies of salt water, indicative of coastal areas, or due to presence or accumulation of salts in irrigation water or soil, is becoming a limiting factor in development of golf courses around the world and in Florida (Neylan, 2007). Golf courses, and the game of golf, were devised to utilize land unsuitable for agriculture, pasture or other uses in Scotland (Beard, 2005). According to a 2003 U.S. EPA report, over 300 Superfund sites, formerly contaminated properties, landfills, or abandoned hazardous dump/waste sites, have been converted to golf courses, playgrounds, parks, sports fields, etc. as of 2002. With approximately 23% of the world's cultivated land being classified as saline soils, these areas have limitations for typical agriculture (Szaboles, 1989). Seashore paspalum has been demonstrated to withstand heavy salt, CsCl, treatments with no deleterious effects when compared to zoysiagrass and St. Augustinegrass (Kuo, 2001). The ability to utilize saline soils for production of turfgrass is especially attractive as agricultural food production is restricted due to the limited range of salt tolerant food crops. Some regions of the world

have limited or no access to quality irrigation water outside of recycled or reclaimed water, which can be high in salts. Ocean waterfront areas are a magnet for tourism, and SP is noted as a turfgrass that can be planted on the banks of seaside estuaries (Allar, 2001). Rapid population growth is occurring in arid areas where soil and water salinity put increased demands on available potable water (Marcum, 2004). Saline soils and water are problems that increase as human populations increase. There is an increasing need for arable land for agriculture as well as for urbanization. Accordingly, SP is attractive as the most salt tolerant warm season turfgrass species (Allar, 2001; Duncan and Carrow, 2000b).

Electrical conductivity (EC) is used to determine the total concentration of soluble salts in water. EC is measured in decisemens/m (dS m^{-1}) or as total dissolved salts (TDS) in ppm (Marcum, 2004). Salt water is approximately 55 dS m^{-1} whereas salinity of irrigation water can be classified as low, medium, high and very high, ranging from less than 0.25 to greater than 2.25 ds m^{-1}. Irrigation with saline water, due to high evaporative demand, can result in quick salt accumulation in the soil profile. Reliance on medium saline irrigation water, alone, without rainfall or flushing of salts, can accumulate salts. Accounting for evaporation, a 2.5 cm irrigation event of water with an EC of 2 dS m^{-1} will deposit 3 kg of salt onto a 1000 ft^2 area (Marcum, 2004). The main hazardous component of high EC irrigation water is sodium, Na+, which can accumulate in the root zone, displacing essential nutrients such as magnesium (Mg^{2+}) and calcium (Ca^{2+}) directly or via precipitation with bicarbonates or carbonates (Richards, 1954).

While tolerant to high salinity, it has been noted that continued irrigation of SP with high saline water or accumulation of salts in soil can be a stress detrimental to turf

quality (Duncan and Carrow, 2005a; Berndt, 2005). Duncan and Carrow (2005b) noted that depending on the level of salinity, application rates of growth regulators such as trinexapac-ethyl, required for maintenance of SP, will vary as water high in salinity has a growth suppressive effect on SP. Reduced shoot growth and elongation of 'SeaDwarf' SP resulted from tests with saline water irrigation causing complete stolon emergence suppression at EC_w levels about 19.1 ds m^{-1} (Berndt, 2005). Indications from Berndt's study are that in order to maintain the highest quality turf, irrigation of SP with the freshest water possible should be performed as often as possible. More importantly, it is noted that while tolerant to saline water, SP can be stressed by too much or too long of a reliance on high salinity irrigation. While exhibiting 50% reduction in top growth when irrigated with an 18.4 ds m^{-1} high salt water mixture, 'FSP-1' SP exhibited enhanced rooting (Peacock and Dudeck, 1985). This effect varies depending on the variety of SP. Salinity is generally regarded as a stress to most turfgrass species. Salt injury might be misdiagnosed as disease, whereas salt and disease management should be addressed together (Yenny, 1994).

The salt tolerance of SP, while high in comparison to bermudagrass, varies among cultivars and can still be damaging at certain levels (Berndt, 2005; Raymer and Braman, 2005). Duncan and Carrow (2000a) stated that SP has few pathogen problems in comparison to other warm season turfgrass species since fungi do not function optimally at high salt levels. High soluble salts, however, have been implicated as enhancing disease symptoms from *Pythium aphanidermatum* and *Labyrinthula terrestris* on cool-season turfgrass species (Camberato et al., 2005; Martin et al., 2002; Rasmussen, 1988). Outside of these two pathogens, little has been published on the

effects of salt on pathogens of turfgrass. There is indication that use of salts may decrease pathogenicity of certain pathogens in other crops. Use of Potassium chloride, KCl, while not effective in vitro against *Fusarium graminearum*, did reduce disease incidence of stalk rot of corn when applied to infested soil at 113 to 225 kg ha^{-1} (Liu et al., 2007). Asparagus, a salt tolerant plant, is susceptible to Fusarium crown and root rot caused by *Fusarium oxysporum* and *Fusarium proliferatum*. Studies by Elmer (1992, 2003 and 2004) demonstrated that applications of NaCl at 560 to 1,120 kg ha^{-1}, versus KCl, KNO_3, NH_4NO_3 or $Ca(NO_3)_2$, suppressed disease development while significantly increasing fresh weight and root health of asparagus with subsequent reductions in colony-forming units of both pathogens isolated from roots. Disease suppression of Fusarium crown rot of asparagus was not due to direct fungicidal activity but due to enhanced host resistance likely caused by increases in Mn-reducing bacteria.

The salt tolerance of SP has been exploited to address weeds and other turfgrass problems. Various weeds such as crabgrass can be controlled fairly effectively through the use of varying concentrations of seawater mixed with potable water (Pool, 2006). Early breeding work with SP has been in soils or areas not deemed high in salinity, therefore the role of salinity as it relates to virulence of fungal pathogens of SP has not been explored.

Table 1-1. “Rhizoctonia” taxonomy and nomenclature for fungal isolates in this dissertation

Causal fungus (pathogen) nomenclature		
Anamorph name	basionym	Teleomorph
Rhizoctonia solani[1]	*Moniliopsis solani*	*Thanatephorus cucumeris*[2]
Ceratorhiza cerealis	*Rhizoctonia cerealis*	*Ceratobasidium cereale*
Rhizoctonia oryzae	*Monioliopsis oryzae*	*Waitea circinata* var. *oryzae*[3,4]
Rhizoctonia circinata	----	*Waitea circinata* var. *circinata*[7]
----	----	*Waitea circinata* var. *prodigus*[8]

[1] Moore, 1987
[2] Ryker and Gooch, 1938
[3] Oniki et al., 1985
[4] Warcup and Talbot, 1962
[5] Stalpers and Andersen, 1996
[6] Stalpers, et al., 1998
[7] de la Cerda and Wong, 2007
[8] Kammerer et al., 2011

CHAPTER 2
SURVEY FOR *RHIZOCTONIA* SPECIES ISOLATED FROM SEASHORE PASPALUM GOLF COURSES IN FLORIDA

Introduction

In 2010, there were at least twenty five golf courses in Florida grassed with 'SeaDwarf' seashore paspalum and possibly a few more with other cultivars such as 'Aloha', 'Excalibur', 'SeaIsle I', 'SeaIsle 2000', 'Platinum', 'SeaIsle Supreme', and 'SeaSpray' (Zinn, 2010). Seashore paspalum (SP) requires different cultural practices compared to bermudagrass including an aggressive aerification program to manage the biomass produced over time (McCarty, 2005; Zinn, 2010).

Diseases on SP and the causal pathogens have not been fully explored. In comparison to salinity and light studies, basic pathology work with SP is minimal with few peer-reviewed publications. Little is known about the distribution, ecology and impact of fungal populations on established SP turf. Seashore paspalum was stated to be susceptible to a number of pathogens causing major diseases such as dollar spot and large patch (Canegallo and Martin, 2007). Curvularia blight and take all root rot were cited as contributing to failure of SP greens (Duncan and Carrow, 2005). *Gaumannomyces graminis* var. *graminis* is a noted pathogen of SP (Elmore et al., 2002). Zinn (2010) acknowledges that in humid environments diseases such as *Pythium*, large patch, brown patch, fairy ring, *Rhizoctonia* and especially dollar spot can be more problematic to SP versus when grown in an arid environment.

In 2010, dollar spot caused by *Sclerotinia homoeocarpa* F. T. Bennett was reported as a serious disease of SP (Lv et al., 2010). While noted for its low nitrogen requirement, increasing the nitrogen and potassium fertility for SP can be an advantageous cultural practice to minimize dollar spot infestations (Smiley et al., 1994;

Couch, 1995; Duncan and Carrow, 2000b). Outside of increased dollar spot tolerance, nitrogen applications to SP resulted in increased turf quality, another feature highly regarded in the golf course market (Kopec et al., 2005). Unfortunately, increased nitrogen fertility for dollar spot can result in a more conducive environment for *Rhizoctonia* diseases of turfgrass (Bloom and Couch, 1960; Burpee, 1995).

To date, utilization of SP for golf courses around the world has been most prevalent in tropical to sub-tropical climates representative of the environment in Florida. In humid environments like Florida, *R. solani* is known to be a major pathogen of significance on a wide range of plants (Farr et al., 1981). In addition to nitrogen rich turfgrass, temperatures ranging from 21 to 32°C coupled with persistent wet conditions for at least 10 h are ideal for *R. solani* infection of cool season turfgrasses (Smiley et al., 1992). On warm season turfgrasses, especially in the transition zone areas of the United States, *R. solani* can be very problematic (Couch 1995, Martin 2000). *R. solani* AG 2-2LP has optimal growth temperatures at 25°C while *R. solani* AG 2-2IIIB and *R. solani* AG 2-2IV grow optimally at 28°C (Hyakumachi et al., 1998). The disease susceptibility of several cultivars of SP was limited to research efforts with *Rhizoctonia solani* AG 2-2LP in South Carolina (Canegallo 2007).

Couch (1995) observed infection of warm and cool-season turfgrasses by *Chrysorhiza zeae* was more likely with higher temperatures, daytime temperatures of 28 to 33 °C, compared to infection by *R. solani*. These high temperatures are more conducive for growth of *C. zeae*, than for *R. solani* (Martin and Lucas, 1984a; Burpee and Martin, 1992; Elliottt, 1999). Leaf and sheath spot disease, caused by *C. zeae*, can

result in symptoms sometimes referred to as “hot weather” brown patch at temperatures of 30°C.

*Rhizoctonia oryz*ae grows rapidly at temperatures of 30°C and 32°C, similar to *C. zeae* (Ryker and Gooch, 1938). Information on the pathogenicity of *R. oryzae* in turfgrasses is limited compared to *C. zeae*.

Salinity and scarcity of quality irrigation water is noted as a major limiting factor in golf course development in Florida and around the world (Neylan, 2007). Evidence suggests that changes in salinity affects photosynthetic parameters, leaf temperature, osmotic and leaf water potential, rate of transpiration and relative leaf water content (Sultana et al., 1999). High soluble salts can negatively affect the osmotic potential and corresponding ability of turfgrass roots to take up water. The tolerance of SP to high salinity is well documented; however, continued reliance on irrigation with high saline water is a stress that can be detrimental to turf quality (Duncan and Carrow, 2005a; Berndt, 2005). On cool-season turfgrass species, diseases caused by *Pythium aphanidermatum* and *Labyrinthula terrestris* were more severe on turf irrigated with water containing high soluble salts compared to low levels of salts (Camberato et al., 2005; Martin et al., 2002; Rasmussen, 1988). Precipitation and displacement of essential nutrients with bicarbonate and carbonates is associated with high EC irrigation water containing sodium (Richards, 1954). The correlation of the incidence of disease caused by *Rhizoctonia*-like fungi on SP and fluctuating soil EC values is unknown.

The role of soil pH in infection of turfgrass by fungal pathogens is not fully understood. Take-all disease of wheat, caused by *Gaumannomyces graminis* var. *tritici*, is alleviated by acidification of alkaline soils (Ownley et al., 1991). Nutrient

imbalances and deficiencies can cause stress on turfgrass. Severity of brown patch, caused by *R. solani*, was observed to be greater on creeping bentgrass ranging from a pH 5.6 to 9.0 when nitrogen levels were high (Bloom and Couch, 1960). Sharp increases in soil pH resulted in increased spring dead spot and bermudagrass decline disease severity on bermudagrass in Alabama (Hagan, 1997). High soil pH resulted in declines in populations of beneficial bacteria found to be suppressive to *G. graminis* var. *tritici* colonization of wheat roots (Bull et al., 1991, Ownley et al., 2003). Some of these suppressive bacteria reduce manganese to unavailable forms as soil pH levels exceed 7. Applications of calcium to SP, which can increase soil pH, has been a common practice in the maintenance of turfgrasses as a means of alleviating sodium accumulation (Duncan, 1996).

The objectives of this study were to: 1. Determine the incidence and identities of *Rhizoctonia*-like fungal species infecting SP fairways in Florida; 2. Calculate correlation coefficients for *Rhizoctonia*-like fungus isolation and environmental and edaphic factors.

Materials and Methods

Turfgrass Sampling

Eight SP golf course fairways and/or roughs across the state of Florida were sampled nine times, approximately every two months, from August 2007 to January 2009 to obtain a two-year ecological survey focusing on *Rhizoctonia* and *Rhizoctonia*-like fungi. The nine sampling periods were; September 4 – 5, 2007 (Sept 07), October 29 – 30, 2007 (Oct 07), January 3 – 4, 2008 (Jan 08), March 3 – 4, 2008 (Mar 08), May 5 – 6, 2008 (May 08), July 8 – 9, 2008 (July 08), August 25 – 26, 2008 (Aug 08), November 3 – 4, 2008 (Nov 08), and January 5 – 6, 2009 (Jan 09). One cup-cutter plug sample, approximately 15 cm in depth and 10.75 cm in diameter, was removed from

each golf course fairway or rough where patches, rings, foliar blight, thinning of the foliar canopy or some degree of localized chlorosis and/or necrosis occured. The samples were immediately placed in a polyethylene bag on ice. Each of the eight golf courses were located in Florida; two in Boca Raton, Fort Myers, two in Naples, Sarasota, Vero Beach, and West Palm Beach. Four SP cultivars were sampled; 'SeaIsle 2000' at Boca West (Boca Raton), 'SeaDwarf' at Hammock Bay (Naples), Old Palm Golf Club (West Palm Beach), Parkland Golf Course (Boca Raton), The Plantation (Fort Myers) and Tuscany Reserve (Naples), 'SeaIsle Supreme' at The Oaks Club (Sarasota), and 'SeaIsle I' at Vero Beach Country Club (Vero Beach) (Figure 2-1).

Temperature, soil pH and EC determination

Corresponding agronomic data were collected in conjunction with each sample removed from each golf course. Soil temperature to a 6-cm-depth was recorded with an analog soil thermometer, and canopy temperature was determined using a Fluke 61 Infrared thermometer (Fluke Corp., China) from the same location from which a cup-cutter sample was removed. In the laboratory, soil was shaken from the cup-cutter sample and collected. Turf samples were washed of any remaining soil and dead organic matter. Soil samples (10 cc) were air-dried then added to 20 mL of deionized water (~24ºC) and stirred in a 100 cc cup. The slurry mixture of soil and water was allowed to settle for 15 minutes then stirred again directly prior to taking pH readings using a calibrated Corning® pH meter 120 (Corning Inc., Corning, NY). Soil electrical conductivity, EC, values representative of sodium levels were taken using an Oakton® Conductivity/TDS/OC Meter CON 11 Series (Eutech Instruments – Singapore) on a µS cm^{-1} scale set for 23ºC.

Fungal isolations

Turf samples were transported on ice in a styrofoam cooler for no more than three days from collection to processing. In the laboratory, using a knife, all cup cutter turf samples were sliced approximately 0.5 cm below the soil surface separating the underground stolons, rhizomes, and roots from the above-ground leaves, sheaths and stolons. Sixteen 1.5 to 2 cm above-ground and sixteen below-ground tissue pieces displaying some form of chlorosis or necrosis, collected from each plug, were surface sterilized in 70% ethanol for 30 seconds and rinsed twice for 30 seconds with sterilized de-ionized water. Using sterile tissue paper, all tissue samples were patted dry and transferred to Petri dishes containing either water agar [15 g Bacto agar (Difco Laboratories, Detroit, MI)/Liter de-ionized water], one-fifth-strength potato dextrose agar [7.8 g PDA (Difco),10 g Bacto agar, 150 µg rifampicin (Fisher Scientific Co., Fair Lawn, NJ), 0.5 g ampicillin (Sigma Chemical Co., Steinheim, Germany)/Liter de-ionized water)], or PDA + thiophanate-methyl (Topsin-M 70WP, Cerexagri Inc., King of Prussia, PA) [39 g PDA, 0.1 g thiophanate-methyl, 150 µg rifampicin, 0.5 g ampicillin/Liter de-ionized water]. Four pieces of above ground tissue and four pieces of below ground tissue from each sample were placed on each media type. The tissue samples were incubated at 25ºC and observed for up to 5 d for non-sporulating, *Rhizoctonia*-like colonies and hyphae (Sneh et al., 1991). Hyphal tip transfers of characteristic colonies were made to PDA + rifiampicin + ampicillin ((39 g PDA + 150 µg rifamipicin + 0.5 g ampicillin)/Liter de-ionized water) to obtain pure cultures of the fungi.

Morphological Characterization and Groupings

Colony morphology was observed and noted up to 3 weeks following a 5 mm mycelia plug transfer to full-strength PDA. Isolates were maintained in the dark at 25°C.

Upon colony and microscopic inspection, all isolates that closely resembled each other were grouped by growth rates, colony patterns, presence or absence of; pigment/colors, moniloid cells, sclerotia, sclerotia shape and color, and hyphal growth characteristics (on the surface, below or above the agar), and branching patterns. One or more isolates from each group were selected for DNA sequencing. Isolates were transferred to sterilized oat seed which was colonized over 2 to 3 weeks, dried in autoclaved paper bags, and stored at 15°C.

Molecular DNA methods and isolate identification

Twenty-five isolates from the paspalum sampling events, five isolates from bermudagrass, one from annual bluegrass, one from perennial ryegrass and one from zoysiagrass were characterized (Table 2-1). Ribosomal DNA (rDNA) including ITS1, the 5.8S ribosomal subunit, and ITS2 of one or more fungal isolates from each distinct morphological group was sequenced after polymerase chain reaction (PCR) amplification (White, 1990). Additional sequences of related fungi were retrieved from GenBank.

To extract DNA, approximately 50 mg of aerial mycelia was removed from each fungal colony and macerated for one minute with a 0-3200 SPM Mini-Bead beater at medium speed (Biospec Products, Bartlesville, OK) in 20 µL of sterile distilled de-ionized water. The macerated samples were processed using a QIAGEN® Quick Clean-Up DNA extraction kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions.

Template DNA from each isolate was PCR-amplified. The 50 µL PCR reaction mixture had 20 pM of each oligonucleotide primer (ITS1 and ITS4), approximately 100 ng of template DNA, and 25 µL of RED Extract-N-Amp PCR reaction mix (Sigma-

Aldrich, St. Louis, MO) (White et al.,1990). An Eppendorf® AG 22331 thermocycler (Hamburg, Germany) was programmed for an initial 94°C - 3 minute cycle followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute with one last cycle of 72°C for 10 minutes and a 4°C indefinite holding cycle. Using 5 µL of PCR product, electrophoresis was performed in a 1.5% agarose gel with 0.005% ethidium bromide. Amplification was confirmed by the presence of a 650 to 700 bp band under UV light.

The PCR product was cleaned with the QuickLyse Miniprep 250 kit (Qiagen Inc., Valencia, CA) according to manufacturer's directions. Following cleaning, the amplified PCR products for each isolate were inserted into *Escherichia. coli* and cloned utilizing a Topo TA Kit (Invitrogen, Carlsbad, CA). Five transformed white bacterial clones per isolate were cultured in Luria Bertani (LB) broth at 37°C in a shaker incubator for 14 to 16 h. Plasmids were extracted from bacterial clones with a miniprep kit according to manufactures directions (QIAGEN QuickLyse Miniprep kit, Qiagen Inc., Valencia, CA).

The purified plasmids were submitted to the Interdisciplinary Center for Biotechnology Research at the University of Florida in Gainesville, FL for sequencing. Sequences were aligned using the Clustal W method in Mega Build 4.02 (Center for Evolutionary Functional Genomics, Tempe, AZ). Consensus sequences were derived using 3 or more clone sequences for each isolate after removing the primer sequences.

Using Mega 4.02, a phylogenetic tree was constructed using a Neighbor-Joining algorithm for the Kimura Two-Parameter Model (Burpee et al., 2003). Bootstrap values were determined based on 1,000 random samples of the data set. Two sequences from GenBank were included, NUK-3BG from creeping bentgrass, and a sequence for

Sclerotinia homeocarpa, accession number GU002301. Isolates were identified based on the closest-known sequences in GenBank based on Blast searches.

Correlation of Edaphic Factors and Isolation Frequency of Fungi

The golf course (site), date sampled, soil temperature, canopy temperature, soil pH, soil EC and whether an isolate was obtained from above or below ground turfgrass tissue were recorded for each isolate recovered. Mean isolation frequency (isolates per sample) was calculated as was the mean salinity, pH, soil, and canopy temperatures associated with each sample site. Data were subjected to a general linear model and means were separated using Fischer's LSD, $\alpha = 0.05$.

Isolates were identified and grouped as *Waitea circinata*, *Thanatephorus cucumeris*, and *Ceratobasidium* spp. Random effects with significance at Pr>F at 0.01, 0.05 and 0.10 for the variables of soil temperature, canopy temperature, soil EC and soil pH were identified. Five year averages from September 1, 2005 to November 1, 2009 were obtained for soil temperatures and rainfall from archived data collected by the Florida Automated Weather Network (FAWN) for the Fort Lauderdale, FL. Correlation coefficients were assessed between these averages and isolate recovery, observed EC readings and soil temperatures.

For environmental and edaphic correlations, data were analyzed utilizing the GLIMMIX (General Linear Mixed model) procedure, and Pearson correlation coefficients were calculated for all *Rhizoctonia*-like isolates. Statistical analyses were performed using statistics analysis software (SAS) for Windows version 8.02 (Cary, NC).

Results

Isolates Recovered – Phylogenetics, Morphology and Descriptions

Seventy-four isolates resembling *Rhizoctonia* with hyphae that had right- and acute- angle branching were recovered from the eight SP golf courses over nine sampling events August 2007 to January 2009. Twenty-five of these isolates were identified through DNA sequencing (Table 2-1). All sequenced isolates were within the *Ceratobasidium*, *Thanatephorus* and *Waitea* genera. Of the 25 sequenced, approximately 43% of isolates were *Thanatephorus cucumeris*, 28% were *Waitea circinata*, and 24% were *Ceratobasidium* (Table 2-2).

A phylogenetic tree was constructed using the DNA sequences of the twenty-five SP isolates, sequences obtained from additional isolates, and sequences retrieved from GenBank from outside this study are listed in Table 2-1 (Figure 2-2). Isolates recovered from SP were identified in 9 of 11 clades; three associated with *W. circinata* clades, two with *T. cucumeris*, and four with *Ceratobasidium*. *Waitea circinata* var. *prodigus* was recovered five times over four sampling dates from three different golf courses. There were four distinct clades of *Ceratobasidium* isolates belonging to different AG groups. One of the *Ceratobasidium* clades was a new clade 95% related to *Ceratobasidium* sp. AG-E. Within *T. cucumeris* two AG groups were identified, AG 2-2LP, the causal pathogen of large patch and AG 2-2IIIB, the causal pathogen of brown patch on cool-season turfgrasses. The *T. cucumeris* AG 2-2LP isolates obtained from SP were within the same clade as isolates obtained from perennial ryegrass and zoysiagrass. There was less distance separation between the clades of the *Ceratobasidium* and *Thanatephorus* isolates between these genera and the *Waitea circinata* varieties.

Initial growth for the *T. cucumeris* isolates, both *T. cucumeris* AG 2-2LP and AG 2-2IIIB, was observed to be much slower at 25°C as compared to the *W. circinata* isolates on full strength PDA. The young individual hyphal strands produced abundant, tight 90° angle branches. After 28 d, the mycelia darkened to a milk-chocolate, brown color. None of the *T. cucumeris* isolates produced sclerotia. After the mycelia began to turn brown, moniloid hyphae were observed. (Figure 2-3).

The *Ceratobasidium* isolates were variable in appearance producing light brown to gray colored mycelia in 28 d cultures on full strength PDA (Figure 2-4). Some produced concentric rings of mycelia. The unknown *Ceratobasidium* sp. anastomosis group (UCAG) was the only C*eratobasidium* group to produce black sclerotia 3 to 6 mm in diameter and irregular shaped on the agar surface. It is not known if this isolate is a turfgrass pathogen.

Initial growth of the *Waitea circinata* varieties resulted in less consistent 90° acute-angle branching, more likely to be 60 to 75°, and less branching with greater distance between each branch compared to *Thanatephorus* or *Ceratobasidium* isolates (Figure 2-5). *W. c.* var. *prodigus* mycelia developed a slight yellow to pink color after filling the petri dish within 1 to 2 weeks. None of the *Waitea circinata* varieties developed brown pigmentation. Both the *W. c.* var. *zeae* and *W. c*. var *oryzae* isolates produced mycelia limited to the agar surface. in contrast to the *W. c.* var. *prodigus* which had floccuse, prolific aerial mycelia that clumped together and attached to the underside of the petri dish lid. Isolates of all the *W. circinata* varieties produced sclerotia embedded in the agar, including the *W. c*. var *circinata* isolate (picture not shown). The *W. c.* var *zeae* isolates had uniform shaped sclerotia whereas the *W. c.*

var *oryzae* and *W. c.* var *prodigus* isolates produced irregular sclerotia. Sclerotia of the *W. c*. var *prodigus* isolates ranged from a cream yellow, orange, or salmon color, irregular or spherical in shape, and were embedded in the agar in clumped or catenulate arrangements.

Means and Isolate Recovery Frequencies

A mean of one *Rhizoctonia*-like isolates was recovered from samples taken with a range of isolates from 0 to 4 isolates per sample (Table 2-3). Mean EC values ranged from a minimum of 34 up to a maximum of 1871 µS/cm.

The Oaks Club location had the highest mean recovery of total *Rhizoctonia*-like isolates. The Oaks Club and The Plantation had the highest recovery rate of *T*. *cucumeris* isolates. The Oaks Club also had the highest mean recovery of *W*. *circinata* isolates (Table 2-4). The Oaks Club had the highest mean EC values over the nine sampling events. Vero Beach Country Club had the highest mean recovery of *Ceratobasidium* isolates. Boca West and Tuscany Reserve had among the highest pH levels. Hammock Bay and Tuscany Reserve had the lowest mean recovery of total *Rhizoctonia-like* isolates, Hammock Bay having the lowest recovery of *T*. *cucumeris* isolates. Parkland had the lowest recovery of *W*. *circinata* isolates.

The sampling period that delivered the highest mean recovery of *Rhizoctonia*-like isolates and *T*. *cucumeris* isolates was Jan 08 which also subsequently had the lowest mean soil and canopy temperatures (12 and 14°C respectively) of the nine sampling periods (Table 2-5). The sampling period that resulted in the highest recovery of *W*. *circinata* varieties was Sep 07 which also had among the highest soil and canopy temperatures (29 and 36°C respectively). There were no differences among sampling periods for mean EC values. The soil and canopy temperatures were closely aligned

(Figure 2-6). Canopy temperatures were higher than soil temperatures with the Jan 08 and Jan 09 periods representing a steep drop from the previous sampling period. Looking at the 5 year average of historical soil temperatures from the FAWN for Fort Lauderdale, FL, the highest frequency of *T. cucumeris* isolate recovery coincides with the lowest soil temperatures of approximately 21°C (Figure 2-7). Recovery of *W. circinata* isolates was not as concentrated around a specific temperature range as was *T. cucumeris* isolates.

Isolate Recovery Correlations

There was a correlation between recovery of *Rhizoctonia-like* isolates and the variables, soil temperature, canopy temperature and soil EC (Table 2-6) utilizing a General Linear Mixed Model (Glimmix). When the data were graphed in excel (data not shown) the trend was one of increasing total isolate recovery with decreasing soil and canopy temperatures and increasing soil salinity (increasing EC values). *T. cucumeris* AG 2-2LP recovery was correlated to decreasing soil and canopy temperatures. With the GLIMMIX model, *W. c.* var. *zeae* and *W. c.* var. *oryzae* isolation was correlated to increasing canopy temperatures and increasing EC values respectively at $P< 0.10$. *Waitea circinata* var. *oryzae* isolation frequency correlated with increasing salinity at $P< 0.05$, with the Pearson model (Table 2-7). All correlation models indicated the strongest variable correlations being between soil and canopy temperatures (data not shown). Recovery of one isolate was not correlated with recovery of another isolate.

Discussion

The recovery of three different genera of *Rhizoctonia-like* fungi representing nine distinct phylogenetic clades from SP golf course fairways in Florida is indicative of a diverse group. While temperatures of 25°C may be ideal for in vitro growth of *T.*

cucumeris AG 2-2LP (Hyakumachi et al., 1998), recovery of isolates, specifically *T. cucumeris* AG 2-2LP was most correlated to soil temperatures of 12 – 21°C and canopy temperatures of 14 – 24°C for the sampled SP. Other genera, such as the *W. circinata* varieties represent other problematic pathogens of significance in SP. There was a wider diversity of isolates in the *W. circinata* varieties and *Ceratobasidium* groups than with *T. cucumeris*.

Plants are defined as salt tolerant based on the EC_e (electrical conductivity mean of saturated paste soil extracts from the rootzone) threshold required to cause reductions in yield below what is normally achieved under nonsaline conditions (Carrow and Duncan, 1998; Lee et al., 2005). 'FSP-3' SP was noted to demonstrate a 10% shoot growth increase up to 14 dS/m, before a decrease occurred (Peacock and Dudeck, 1985). This was more than ten times greater than the highest EC level detected in the sampling program. Soil EC levels were low to moderate for the golf courses in this study (34 – 1871 µS/cm), and there was good recovery of isolates through the sampling period.

Though SP has an inherently high tolerance for salt (Duncan and Carrow, 2000b; Duncan, 2003; Lee et al., 2004), results in this sampling program indicate that increasing salinity may increase the likelihood for infection and subsequent disease from *T. cucumeris* AG 2-2LP and *W. c.* var. *oryzae* based on the correlation models on recovery of these pathogens. *Thanatephorus cucumeris* AG 2-2IIIB, the causal pathogen of brown patch on cool-season turfgrass, was identified three times, all on below ground SP tissue. This is the first known report of occurrence of *T. cucumeris* AG 2-2IIIB on SP. One of the *T. cucumeris* AG 2-2IIIB isolates recovered from the SP

produced brown patch symptoms when inoculum was introduced to potted creeping bentgrass (data not presented). It is not known if *T. cucumeris* AG 2-2IIIB is pathogenic to SP. It is of interest to note that *T. cucumeris* AG 2-2LP, a pathogen of warm season turfgrass, was recovered from symptomatic cool season turfgrass, perennial ryegrass tissue (isolate SK-SgPR). The perennial ryegrass, where this isolate was recovered, was seeded over a bermudagrass tee box.

Waitea circinata varieties represent potential problematic pathogens on SP as the most widely detected variety, *W. c.* var. *zeae*, has been diagnosed from seashore paspalum disease samples (Stiles et al., 2008) in addition to being a noted disease of significance on bermudagrass (Haygood and Martin, 1998; Martin et al., 1983b; Martin USGA report). *W. c*. var. *oryzae* and a new pathogen, *W. c.* var. *prodigus*, were isolated multiple times. This new variety was most closely related to what was published as isolate NUK-3BG, an unknown *Rhizoctonia* species related to *Waitea circinata* recovered from creeping bentgrass and Kentucky bluegrass in Japan, and entered in GenBank as *Waitea circinata* var. *agrostis* (Toda et al., 2007). *Waitea circinata* var. *prodigus* was demonstrated to be a new pathogen causing a basal leaf blight of SP (Kammerer et al., 2010). With the exception of *W. c.* var. *oryzae*, retrieval of all the *W. circinata* varieities were obtained from above-ground tissue.

For the *Ceratobasidium* isolates, the separation of total isolates from SP resulted in a range of diversity similar to the *W. circinata* isolates. Some *Ceratobasium* isolates are noted as beneficial in mycorhizal relationships (Otero et al., 2002). As contrasted to the *W. circinata* isolates, most of the *Ceratobasidium* isolations were recovered from

below ground tissue, roots and rhizomes. It is not known if any of these isolates were pathogenic or beneficial.

In this study, it was common for one cup-cutter sample to yield two, and sometimes more, different isolates. It is unknown whether these isolates collectively translate into increased or decreased disease symptoms, but their co-existence can pose challenges in regards to fungicide selection as some fungicides control one fungus versus the other more efficiently (Royals, 2002; Elliott, 1999; Carling et al.,1990; Martin, 1984b; Blazier 2004).

The canopy temperature results indicate that monitoring temperature levels may be a worthwhile indicator that turfgrass managers can utilize in monitoring for large patch. Increasing canopy temperatures corresponding to the optimal growing temperature of 30°C for *W. c.* var. *zeae* may indicate an increase in risk for leaf and sheath spot (Martin and Lucas, 1984a; Burpee and Martin, 1992; Elliottt, 1999). The EC levels, indicative of soil sodium levels versus overall salts is another environmental variable that may warrant monitoring as an additional potential stress that correlated with isolation of *T. cucumeris* AG 2-2LP and *W. c*. var. *oryzae* from SP in this study.

Golf courses in Florida and across the country are increasingly utilizing effluent, reclaimed or run-off water which may contain high soluble salts. Utilizing historical data for rainfall from the FAWN, and looking at correlation models with the isolates recovered, recovery of *T. cucumeris* AG 2-2LP was highest during the drier, cooler periods of the year (Figure 2-8). Low rainfall necessitates irrigation which may result in increased salinity over time.

Table 2-1. Species utilized in this study and identification from sampling effort (or otherwise noted) utilizing rDNA internal transcribed spacer (ITS) region sequences for genetic characterization

Isolate no.	Host[a]	Origin	Date collected	Identity	GenBank accession no.
01 SK-SMd	Annual bluegrass	Reston, VA	25 Apr 2008	*Waitea circinata* var. *circinata*	FJ154894
05 SK-BA1	'SeaIsle 2000' SP	Boca Raton, FL	4 Sep 2007	*Waitea circinata* var. *zeae*	HM597140
09 SK-4OA3-1/5	'SeaIsle Supreme' SP	Sarasota, FL	4 Sep 2007	*Waitea circinata* var. *zeae*	HM597139
10 SK-5OB-TM1	'SeaIsle Supreme' SP	Sarasota, FL	6 May 2008	UCAG[b]	
14 SK-3OA-TM	'SeaIsle Supreme' SP	Sarasota, FL	30 Oct 2007	*Waitea circinata* var. *zeae*	HM597141
15 SK-BG-UFC	'Tifeagle' bermudagrass	Gainesville, FL	7 Jan 2008	UCAG	
18 SK-HBB1W	'SeaDwarf' SP	Naples, FL	30 Oct 2007	*Ceratobasidium* sp. AG-L	
36 SK-OA-W3-I	'SeaIsle Supreme' SP	Sarasota, FL	9 July 2008	*Waitea circinata* var. *prodigus*	HM597147
39 SK-HBA1	'SeaDwarf' SP	Naples, FL	9 July 2008	*Waitea circinata* var. *oryzae*	HM597138
41 SK-4OPB-1/5/1	'SeaDwarf' SP	Palm Beach Gardens, FL	9 July 2008	*Ceratobasidium* sp. AG-G	
42 SK-0821 BG	'Tifeagle' bermudagrass	Columbia, SC	24 Aug. 2008	*Waitea circinata* var. *oryzae*	HM597137
43 SK-0821 BG2	'Tifeagle' bermudagrass	Columbia, SC	24 Aug. 2008	*Thanatephorus cucumeris* AG 2-2IIIB	
44 SK-PSA-TM4	'SeaDwarf' SP	Ft. Myers, FL	4 Jan 2008	*Waitea circinata* var. *prodigus*	HM597146
45 SK-OA-TM1	'SeaIsle Supreme' SP	Sarasota, FL	4 Jan 2008	*Waitea circinata* var. *prodigus*	HM597144
46 SK-HBA-W1	'SeaDwarf' SP	Naples, FL	4 Jan 2008	*Waitea circinata* var. *prodigus*	HM597143
47 SK-0821-BG3	'Tifeagle' bermudagrass	Columbia, SC	24 Aug 2008	*Waitea circinata* var. *oryzae*	
59 SK-OA-W1-II	'SeaDwarf' SP	Sarasota, FL	4 Nov 2008	*Waitea circinata* var. *oryzae*	HM597135
60 SK-4BWB-W1	'SeaIsle 2000' SP	Boca Raton, FL	6 Jan 2009	*Waitea circinata* var. *oryzae*	HM597134
61 SK-PB-TM1	'SeaDwarf' SP	Boca Raton, FL	6 Jan 2009	*Ceratobasidium* sp.	HM597133
64 SK-VBA2-1/5	'SeaIsle I' SP	Vero Beach, FL	21 Dec 2007	*Ceratobasidium* sp. AG-G	
66 SK-PMA-WA1	'SeaDwarf' SP	Ft. Myers, FL	26 Aug 2008	*Waitea circinata* var. *prodigus*	HM597145
67 SK-BWA-W3	'SeaIsle 2000' SP	Boca Raton, FL	26 Aug 2008	*Ceratobasidium* sp AG-Q	
68 SK-4VBB4-W1	'SeaIsle I' SP	Vero Beach, FL	4 Sept 2007	*Ceratobasidium* sp. AG-L	
69 SK-3OB1W	'SeaIsle Supreme' SP	Sarasota, FL	30 Oct 2007	*Thanatephorus cucumeris* AG 2-2IIIB	HM597131
70 SK-OA-W1-I	'SeaIsle Supreme' SP	Sarasota, FL	4 Nov 2008	*Waitea circinata* var. *oryzae*	HM597136
74 SK-820 BG	'Tifeagle' bermudagrass	West Palm Bch, FL	10 July 2008	*Waitea circinata* var. *zeae*	HM597142
75 SK-VBB4-1/5-I-2	'SeaIsle I' SP	Vero Beach, FL	2 Mar 2008	*Thanatephorus cucumeris* AG 2-2LP	
76 3BWA-TM2	'SeaIsle 2000' SP	Boca Raton, FL	6 Jan 2009	*Thanatephorus cucumeris* AG 2-2LP	HM597132
78 SK-3OA-1/5-2	'SeaIsle Supreme' SP	Sarasota, FL	4 Nov 2008	*Thanatephorus cucumeris* AG 2-2LP	
79 SK-4VBB1-1/5	'SeaIsle I' SP	Vero Beach, FL	9 July 2008	*Ceratobasidium* sp. AG-G	
81 SK-3PMA-W4	'SeaDwarf' SP	Ft. Myers, FL	4 Nov 2008	*Thanatephorus cucumeris* AG 2-2LP	
82 SK-EL-2-II	'Emerald' zoysiagrass	Atlanta, GA	18 Sept 2008	*Thanatephorus cucumeris* AG 2-2LP	
83 SK-SgPR	Perennial ryegrass	Ponte Vedra Bch, FL	7 Jan 2008	*Thanatephorus cucumeris* AG 2-2LP	
84 GU002301[c]	'Salam' SP	Foshawn, China	24 Aug 2010	*Sclerotinia homoeocarpa*	GU002301
91 NUK-3BG[c]	Creeping bentgrass	Aichi, Japan	June 1999	*Waitea circinata* var. *agrostis*	AB213567

[a] Annual bluegrass (*Poa annua* L.), SP: SP (*Paspalum vaginatum* Swartz), bermudagrass (*Cynodon dactylon* (L.) Pers.), zoysiagrass (Zoysia japonica Steud.), perennial ryegrass (Lolium perenne L.), creeping bentgrass (*Agrostis stolonifera* L.)

[b] UCAG = Unidentified *Ceratobasidium* Anastomosis Group

[c] DNA sequence and other information were obtained from GenBank.

Table 2-2. Isolate recovery frequencies and SP tissue location

Isolates	Total recovery	Above ground tissue	Below ground tissue
Thanatephorus cucumeris	32	23	9
T. cucumeris AG 2-2LP	28	23	5
T. cucumeris AG 2-2IIIB	4	0	4
Ceratobasidium spp.	18	4	14
Waitea circinata	24	21	3
W. c. var. *zeae*	10	10	0
W. c. var. *oryzae*	9	6	3
W. c. var. *prodigus*	5	10	0
Total	74	48	26

Table 2-3. Mean of *Rhizoctonia*-like fungus isolation recovered from seashore paspalum tissue, soil EC (salinity), soil pH, soil temperature, and canopy temperatures

Parameter*	Mean	Std Dev	Median	Minimum	Maximum
Rhizoctonia-like isolates	1	1	1	0	4
EC (µS/cm)	285.4	381	135	34	1871
pH	7.5	0.4	7.5	6.6	8.3
Soil temperature (°C)	23.4	6.2	24	7.5	34.4
Canopy temperature (°C)	28.6	10.1	28.4	3.9	55.6

*n = 72 (eight golf courses and nine sampling events)

Table 2-4. Mean *Rhizoctonia*-like isolate recovery from golf course sites, EC and pH over nine sampling events

Golf course and location[a]	All *Rhizoctonia*-like isolates	*Thanatephorus cucumeris* isolates	*Waitea circinata* isolates	*Ceratobasidium* isolates	EC (µS/cm)	pH
The Oaks Club, Sarasota	1.8 a	0.7 a	0.9 a	0.2 c	868.6 a	7.4 b
Vero Beach CC, Vero Beach	1.4 b	0.3 d	0.3 c	0.8 a	277.8 c	7.5 ab
Boca West, Boca Raton	1.1 c	0.6 b	0.2 d	0.3 b	99.8 c	7.7 a
The Plantation, Ft. Myers	0.9 d	0.7 a	0.2 d	0 e	143 c	7.5 ab
Old Palm, West Palm	0.9 d	0.1 f	0.6 b	0.2 c	126.8 c	7.5 ab
Parkland, West Palm	0.8 e	0.4 c	0 e	0.3 b	82.1 c	7.4 ab
Hammock Bay, Naples	0.7 f	0.2 e	0.2 d	0.2 c	574.3 b	7.5 ab
Tuscany Reserve, Naples	0.7 f	0.3 d	0.2 d	0.1 d	110.9 c	7.7 a

[a] Means followed by the same letter are statistically equivalent according to GLM (t-tests using Fisher's LSD, α = 0.05).

Table 2-5. Mean recovery frequencies of total and individual *Rhizoctonia*-like isolates recovered, soil and canopy temperatures, EC, and pH values from SP golf courses over nine sampling events

Sampling period[a]	All *Rhizoctonia*-like isolates	*Thanatephorus cucumeris* isolates	*Waitea circinata* isolates	*Ceratobasidium* isolates	Soil temp (°C)	Canopy temp (°C)	EC (µS/cm)	pH
Sept 07	1.0 c	0 g	0.9 a	0.1 e	29 a	36 ab	247	7.6 abc
Nov 07	0.9 d	0.1 f	0.2 d	0.5 b	25 b	30 bc	388	7.7 ab
Jan 08	1.9 a	1.2 a	0.4 c	0.2 d	12 e	14 d	276	7.3 cd
Mar 08	0.6 e	0.5 c	0 f	0.1 e	18 d	25 c	214	7.5 bcd
May 08	0.5 f	0.2 e	0.1 e	0.1 e	25 b	33 ab	228	7.2 d
July 08	0.9 d	0.1 f	0.4 c	0.4 c	30 a	35 ab	226	7.6 abc
Sept 08	0.9 d	0 g	0.1 e	0.7 a	30 a	38 a	197	7.2 d
Nov 08	1.0 c	0.4 d	0.5 b	0.1 e	21 c	23 c	378	7.7 ab
Jan 09	1.6 b	1.1 b	0.4 c	0.1 e	21 c	24 c	415	7.9 a

[a] Means followed by the same letter are statistically equivalent according to GLM (t-tests using Fisher's LSD, α = 0.05).

Table 2-6. Glimmix (General Linear Mixed model) for mean incidence of selected fungus on SP tissue vs. soil temperature, canopy temperature, EC and pH

	soil temperature (°C)		canopy temperature (°C)		EC (µS/cm)		pH	
Fungal isolates	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F
All *Rhizoctonia*-like isolates	8.12	0.006**	7.24	0.009**	8.81	0.004**	0.03	0.8572
Thanatephorus cucumeris AG 2-2LP	41.04	<0.0001**	27.44	<0.0001**	2.38	0.1273	0.83	0.3658
Thanatephorus cucumeris AG 2-2IIIB	0.20	0.6556	0.53	0.4681	0.04	0.8519	0.99	0.3221
All *Ceratobasidium* isolates	1.20	0.2772	0.13	0.7234	0.01	0.9267	0.53	0.4703
W. c. var. *zeae*	1.74	0.1918	2.90	0.0931*	2.14	0.1483	0.04	0.8332
W. c. var. *oryzae*	0.29	0.5901	1.09	0.2993	3.80	0.0554*	1.37	0.2446
W. c. var. *prodigus*	2.29	0.1349	1.24	0.2695	0.01	0.9109	3.28	0.0746*

*, ** Significant at P < 0.10, 0.01 respectively.

Table 2-7. Pearson correlation coefficients for mean incidence of selected fungi on SP tissue vs. soil temperature, canopy temperature, EC and pH.

Fungus	Soil temperature (°C) corr.	Pr>R	Canopy temperature (°C) corr.	Pr>R	EC (µS/cm) corr.	Pr>R	pH corr.	Pr>R
All *Rhizoctonia-like* isolates	-0.32	***	-0.31	***	0.33	***	0.02	-
Thanatephorus cucumeris AG 2-2LP	-0.61	***	-0.53	***	0.18	-	0.11	-
Thanatephorus cucumeris AG 2-2IIIB	0.05	-	0.09	-	0.02	-	-0.12	-
All *Ceratobasidium* isolates	0.13	-	0.04	-	0.01	-	-0.09	-
W. c. var. *zeae*	0.16	-	0.20	*	0.17	-	0.02	-
W. c. var. *oryzae*	-0.06	-	-0.12	-	0.23	**	0.14	-
W. c. var. *prodigus*	-0.18	-	-0.13	-	0.01	-	-0.21	*

*, **, *** Significant at P<0.10, 0.05, 0.01 respectively.

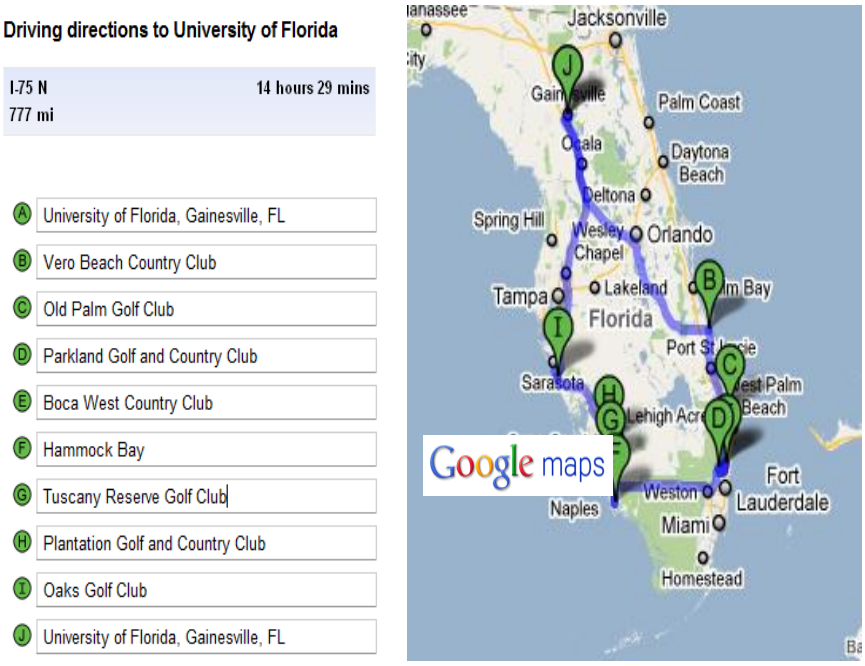

Figure 2-1. Locations and distances between golf courses utilized in this survey.

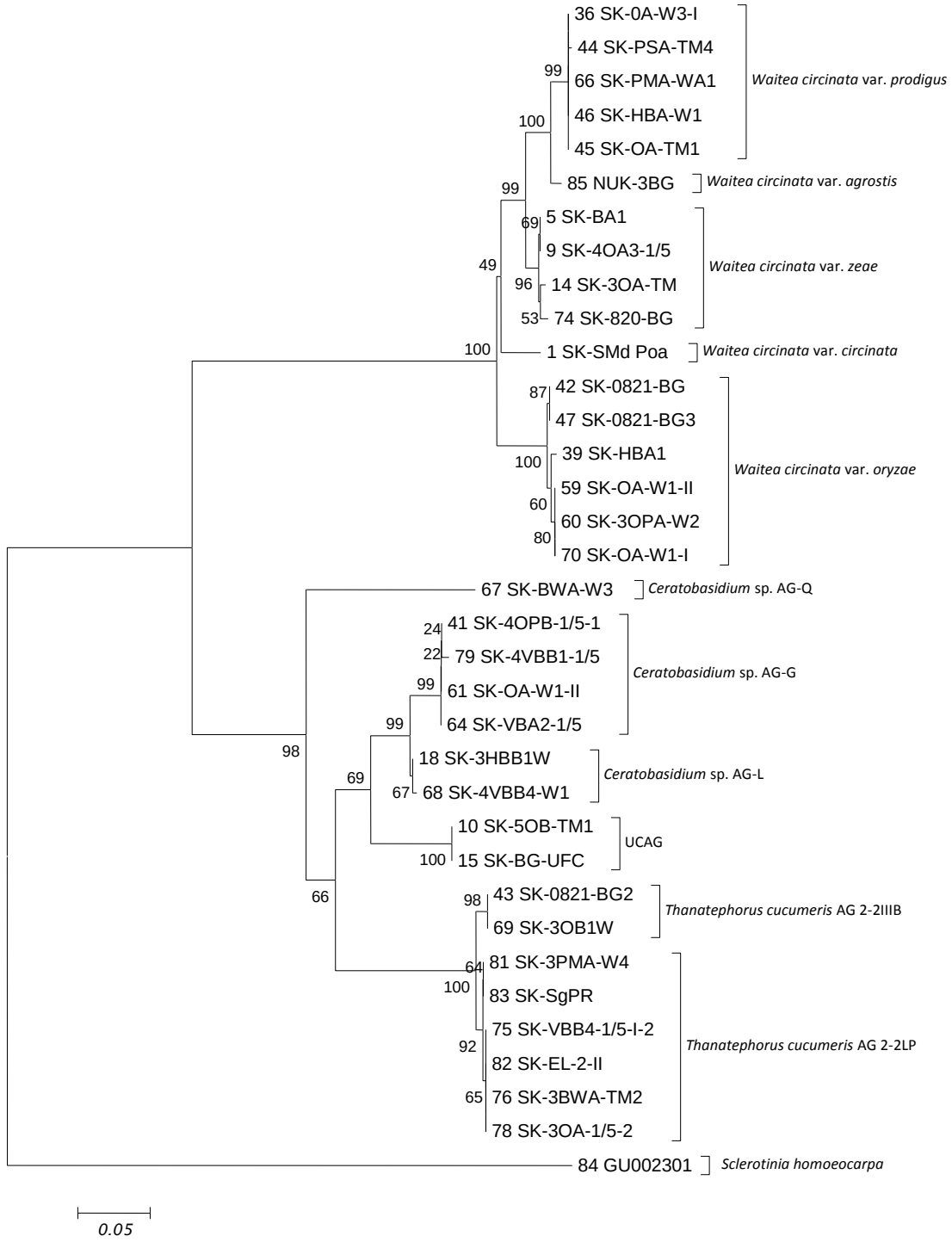

Figure 2-2. Phylogenetic distance tree comparing nucleotide sequences from the rDNA of isolates of *Thanatephorus*, *Waitea*, and *Ceratobasidium* (UCAG – unknown *Ceratobasidium* anastomsis group).

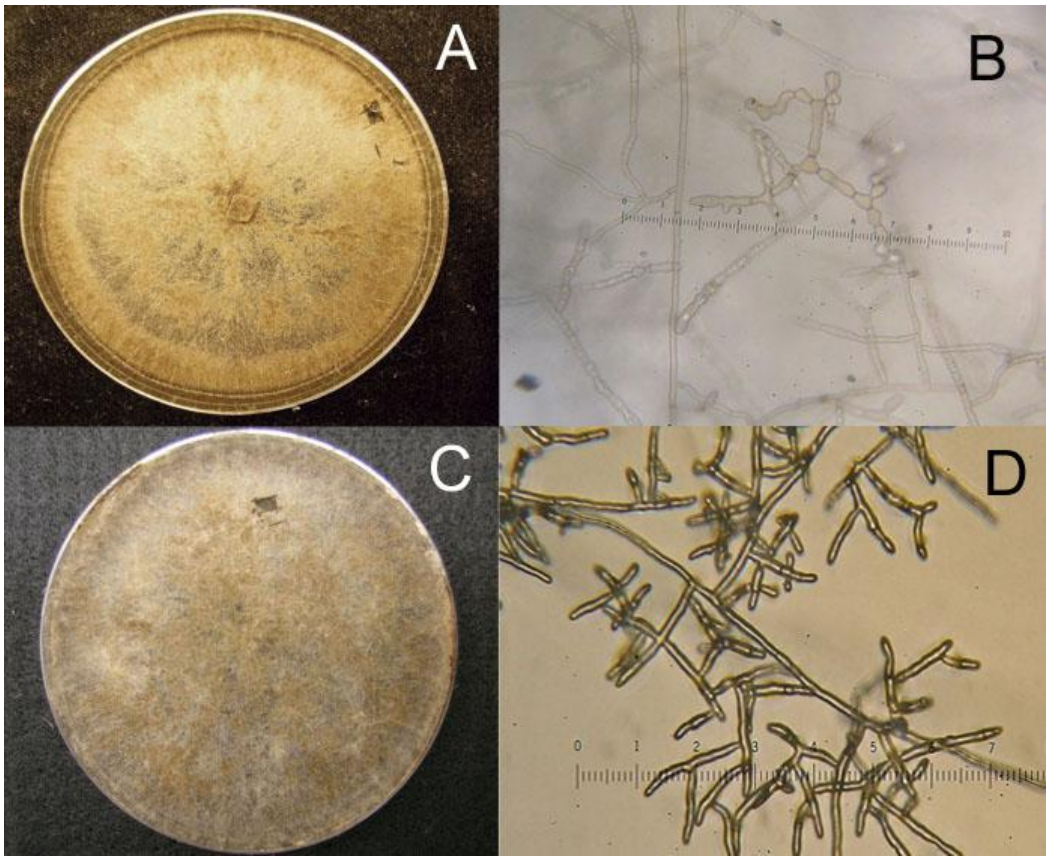

Figure 2-3. *Thanatephorus cucumeris* (*Rhizoctonia solani*) 28 d culture isolates. A) *Rhizoctonia solani* AG 2-2IIIB. B) under 100X magnification. C) *Rhizoctonia solani* AG 2-2LP. D) under 100X magnification.

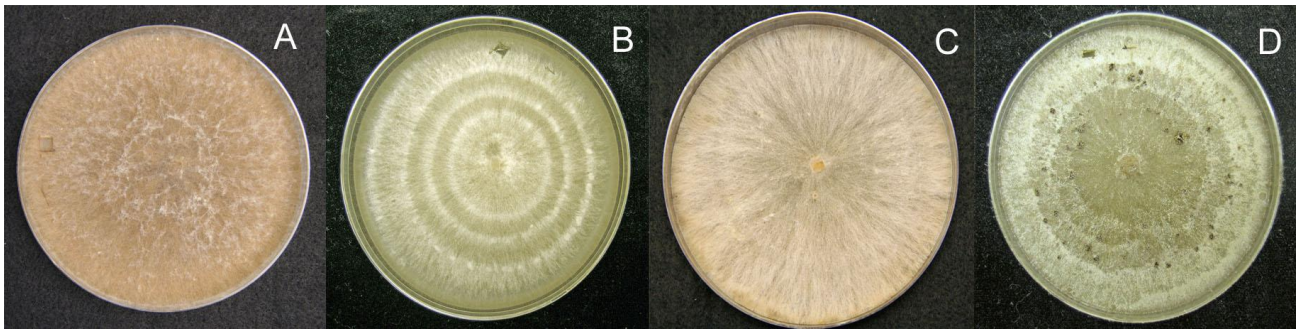

Figure 2-4. *Ceratobasidium* spp. 28 d colony characteristics. A) *Ceratobasidium* sp. AG-G. B) *Ceratobasidium* sp. AG-L. C) *Ceratobasidium* AG-Q. .D) unidentified *Ceratobasidium* anastomosis group.

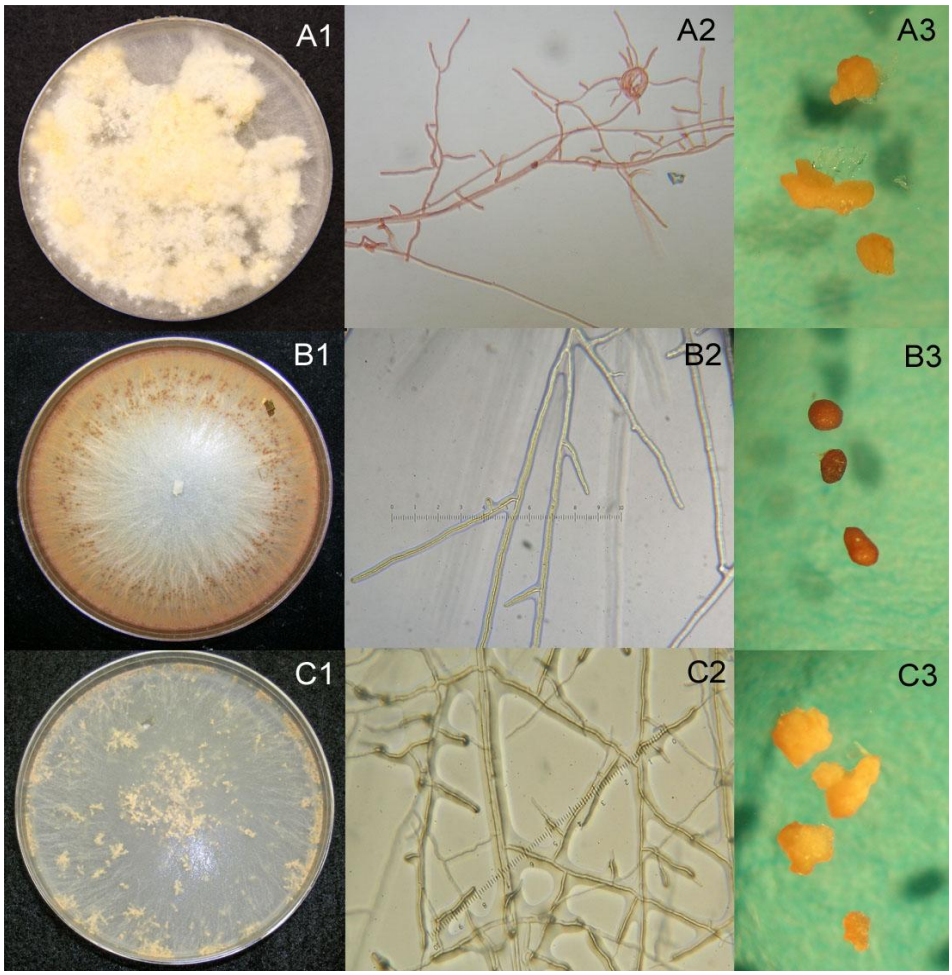

Figure 2-5. *Waitea circinata* varieties. A1) *W*. *c*. var. *prodigus*; colony, A2) 40X – stained safranin-O, A3) sclerotia. B1) *W*. *c*. var. *zeae*; colony, B2) 100X, B3) sclerotia. C1) *W*. *c*. var. *oryzae* colony, C2) 100X, C3) sclerotia.

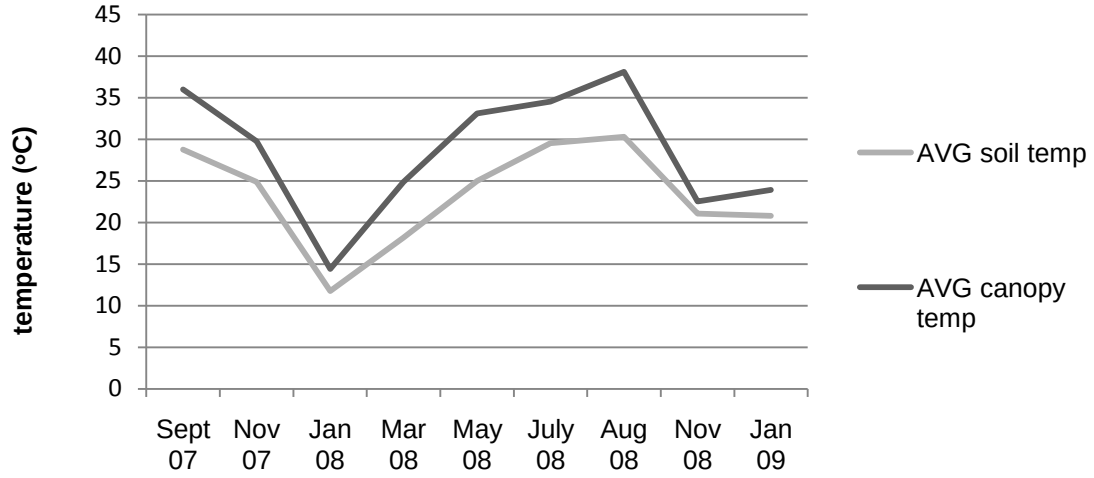

Figure 2-6. Average soil temperatures (6 cm depth) compared to average turf canopy temperatures on eight South Florida SP fairways over a two year period.

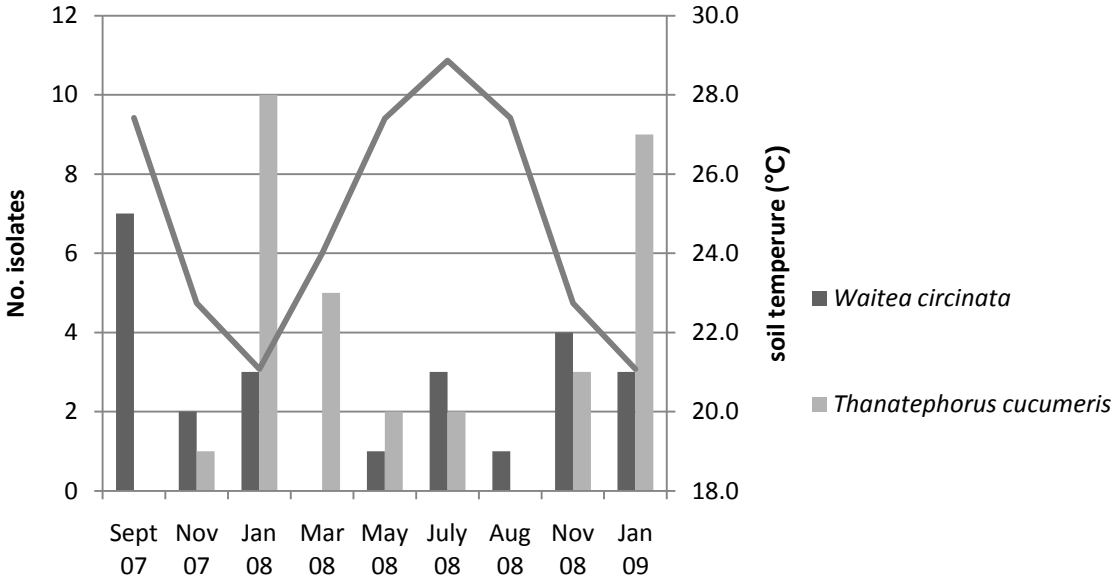

Figure 2-7. Number of *Thanatephorus cucumeris* and *Waitea circinata* isolates recovered compared to historical average 5-year data from the FAWN for Fort Lauderdale, FL for soil temperatures at -10 cm.

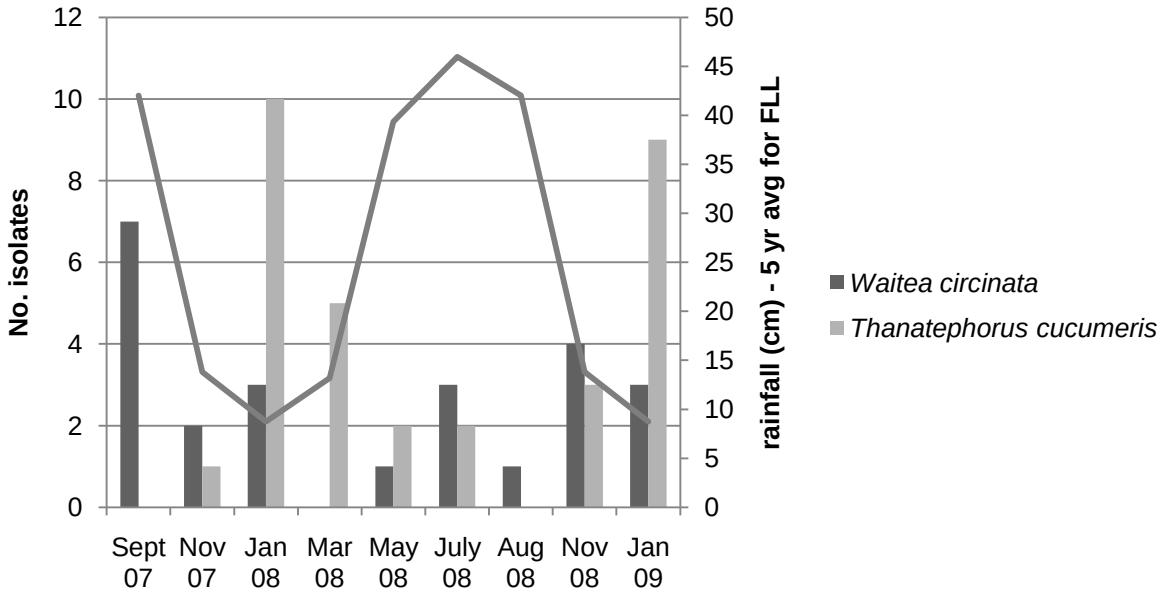

Figure 2-8. Number of *Thanatephorus cucumeris* and *Waitea circinata* isolates recovered compared to historical average 5-year data from the FAWN for Fort Lauderdale, FL for rainfall.

CHAPTER 3[1]
IDENTIFICATION OF A NEW *WAITEA CIRCINATA* VARIETY CAUSING BASAL LEAF BLIGHT OF SEASHORE PASPALUM

Introduction

Seashore paspalum (SP), *Paspalum vaginatum* Swartz, is a warm season grass best adapted to tropical and subtropical climates (Morton, 1973). The species is halophytic (saltwater tolerant) and has been found in South Africa growing along seashores and in brackish waters, but the origin of the grass is uncertain (Duncan 2003; Morton 1973). A number of synonyms has been recognized since 1759 such as *Paspalum distichum*, *P*. *foliosum*, *P. reptans*, and *P*. *reimarioides,* to name but a few (Duncan and Carrow, 2000). Seashore paspalum produces a very robust root system with deep, dense rhizomes and stolons (McCarty, 2005). Suitable uses range from low maintenance or remediation turfgrass for saline soils to higher maintenance plantings like lawns and golf course turf (Duncan and Carrow, 2000). It is a vigorously-growing plant capable of producing a dense, dark green canopy suitable for amenity turfgrass uses. Seashore paspalum was introduced into the turfgrass market in 1996 as a "next-generation" turf for golf courses that requires less water and fertilizer inputs to maintain a high quality foliar canopy compared to other warm season turfgrass species.

Seashore paspalum is susceptible to a range of fungal pathogens and nematodes that affect other warm season turfgrass species. In greenhouse studies, SP was found to be as susceptible as bermudagrass, *Cynodon dactylon* (L.) Pers., to the parasitic nematodes *Belonolaimus longicaudatus* and *Hoplolaimus galeatus* (Hixson et al., 2004).

[1] This chapter was previously published in Plant Disease 95: 515-522 (Kammerer, S. J., Burpee, L. L., and Harmon, P. F. 2010. Identification of a new *Waitea circinata* variety causing basal leaf blight of seashore paspalum.)

Samples of SP exhibiting dollar spot symptoms caused by *Sclerotinia homoeocarpa* F. T. Bennett are routinely received in the University of Florida Extension Plant Disease Clinic (Philip Harmon, *personal communication*). Dollar spot has been reported as a disease of *P. vaginatum* in China (Lv et al., 2010). Take all root rot, caused by *Gaeumannomyces graminis* (Sacc.) Arx & Oliver var. *graminis*, an ectotrophic, root-infecting fungus of warm season turfgrass, also has been diagnosed on SP (Elmore et al., 2002). Large patch, caused by *Rhizoctonia solani* AG 2-2 'LP', occurs frequently on SP and may require preventative fungicide applications to prevent turfgrass loss (Ayogai et al., 1999; Haygood and Martin, 1990; Martin 2000).

In 2007, an unusual disease of SP was observed on three golf courses in Florida. The disease was observed three times during the coldest period of the year for South Florida, January, but also twice during the hotter periods of the year, July and September. Symptoms were observed on SP during cold and hot weather, following low and high fertility, and during wet and dry periods. Symptoms included irregular-shaped, blighted patches of turfgrass foliage ranging from 15 to 150 cm long and 5 to 90 cm wide. A *Rhizoctonia*-like fungus, tentatively identified as *Waitea circinata,* was isolated from necrotic leaves and stolons from samples taken from these locations. *Waitea circinata* is recognized as the teleomorph of a number of *Rhizoctonia*-like fungi (Andersen and Stalpers 1994; de la Cerda et al., 2007; Leiner and Carling, 1994; Stalpers and Andersen, 1996; Toda et al., 2005; Toda et al., 2007; Warcup and Talbot, 1962). Previous work has suggested the use of variety designations for *W. circinata* to further differentiate the species based on biological and molecular genetic differences. Varieties *agrostis*, *circinata*, *oryzae*, and *zeae* (de la Cerda et al., 2007; Leiner and

Carling, 1994; Lv et al., 2010; Sharon et al., 2008; Toda et al., 2007) cause distinct diseases on graminaceous hosts but have not been reported on SP. The objective of this study was to assess the biology, pathogenicity, and genetic similarities of isolates of *W. circinata* sampled from SP in Florida.

Materials and Methods

Turfgrass Sampling and Isolate Maintenance.

Eight golf courses in South Florida with either 'SeaIsle 2000', 'SeaDwarf', or 'SeaIsle Supreme' SP fairways were visited nine times approximately every two months from August 2007 to January 2009. One sample,10.75 cm in diameter and approximately 15 cm in depth, was removed from an area exhibiting foliar blight or thinning on each of the eight golf course fairways or roughs at each of the nine visits (Figure 3-1). Each sample was placed in a polyethylene bag, and the bagged samples were placed on ice. The soil and dead organic matter were washed from each sample. Samples were sliced in half approximately 0.5 cm below the soil surface, separating the underground stolons, rhizomes, and roots from the above-ground leaves and stolons.

Sixteen above-ground and sixteen below-ground necrotic tissue pieces (each approximately 1.5 to 2 cm in length) were removed from each plug, surface-sterilized in 70% ethanol for 30 seconds and rinsed twice for 30 seconds with sterilized, de-ionized water. Samples were blotted dry on sterile tissue and transferred to Petri dishes containing either water agar [15 g Bacto agar (Difco Laboratories, Detroit, MI)/Liter de-ionized water], one-fifth-strength potato dextrose agar [⅕-PDA: 7.8 g PDA (Difco),10 g Bacto agar, 150 µg rifampicin (Fisher Scientific Co., Fair Lawn, NJ), 0.5 g ampicillin (Sigma Chemical Co., Steinheim, Germany)/Liter de-ionized water)], or PDA +

thiophanate-methyl (Topsin-M 70WP, Cerexagri Inc., King of Prussia, PA) [39 g PDA, 0.1 g thiophanate-methyl, 150 µg rifampicin, 0.5 g ampicillin/Liter de-ionized water]. Samples were incubated at 25°C and were observed for up to 5 d. Non-sporulating, *Rhizoctonia*-like colonies with hyphae that had characteristic right- and acute- angled branching were transferred to PDA + rifampicin + ampicillin (Sneh et al., 1991). Additional hyphal tip transfers were made to obtain pure cultures. Isolates were maintained in the dark at 25°C. For extended storage, isolates were transferred to sterilized oat seed that had been autoclaved for three consecutive days for 30 minutes each time. The inoculated oat seed was incubated for 2 to 3 weeks, dried in autoclaved paper bags, and stored at 15°C.

Colony and Fungal Characteristics.

Size, color, and morphology of sclerotia were noted for five uncharacterized *Waitea circinata* (UWC) isolates from diseased SP on four golf courses after the fungi were grown at 25°C for 28 d on PDA. To measure hyphal width and the number of nuclei per cell, isolates were transferred to sterilized microscope slides coated with 2% water agar, and incubated at 25°C in the dark for 2 d. Mycelia on the agar-coated slides were stained with 6 mL 0.5% (w/v) safranin-O (Fisher Scientific, Fair Lawn, NJ) in distilled water, 3.0% (w/v) KOH, and 5 mL glycerol. Diameters of nine hyphal strands per isolate were measured, and nuclei per cell were counted (Bandoni, 1979).

Internal Transcribed Spacer Region DNA Sequencing.

Ribosomal DNA, including the internal transcribed spacer (ITS)1, the 5.8S ribosomal subunit, and ITS2, were sequenced from the five UWC isolates and compared to the sequences of other plant pathogenic fungi including *Thanatephorus*, *Waitea*, *Ceratobasidium,* and *Sclerotinia* spp (Table 3-1). Approximately 50 mg aerial

mycelium from each isolate was removed from agar plates and macerated in 20 µL sterile, distilled, de-ionized water for 1 min with a 0-3200 SPM Mini-Bead beater operated at medium speed (Biospec Products, Bartlesville, OK). A QIAGEN Quick Clean-Up DNA extraction kit (Qiagen Inc., Valencia, CA) was used to extract DNA from the macerated samples according to the manufacturer's instructions. Approximately 100 ng template DNA was used for each 50 µL PCR reaction with 20 pM of each oligonucleotide primer (ITS1 and ITS4) and the RED Extract-N-Amp PCR reaction mix (Sigma-Aldrich, St. Louis, MO) (White et al., 1990). An Eppendorf AG 22331 thermocycler (Hamburg, Germany) was set for an initial 3 min cycle at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min with one final cycle of 72°C for 10 min and a 4°C indefinite holding cycle. Five µL of PCR product were electrophoresed in a 1.5% agarose gel with 0.005% ethidium bromide. The presence of the expected 650 bp amplicon was visualized with UV light after ethidium bromide staining.

DNA Cloning.

The PCR product for each isolate of UWC was cleaned with the QuickLyse Miniprep 250 kit (Qiagen Inc., Valencia, CA) according to the manufacturer's directions. The PCR products for each isolate were inserted into *E. coli* and cloned with a Topo TA Kit (Invitrogen, Carlsbad, CA). Five white bacterial clones per isolate were cultured in Luria Bertani (LB) broth in a shaker incubator at 37°C for 14 to 16 h. The transformed bacterial clones were lysed and cleaned with a miniprep kit according to the manufacturer's directions (QIAGEN QuickLyse Miniprep kit, Qiagen Inc., Valencia, CA).

The purified plasmids were submitted for sequencing to the Interdisciplinary Center for Biotechnology Research at the University of Florida in Gainesville, FL. All

sequences were aligned using the Clustal W method in Mega Build 4.02 (Center for Evolutionary Functional Genomics, Tempe, AZ). After removing the primer sequences, consensus sequences were derived using three or more clone sequences for each isolate. A phylogenetic tree was constructed in Mega Build 4.02 using a Neighbor-Joining algorithm for the Kimura Two-Parameter Model, and a genetic distance matrix was determined (Burpee et al., 2003). Bootstrap values in Mega Build 4.02 were determined based on 1,000 random samples of the data set.

Temperature, Growth Studies.

The five UWC isolates and an isolate of *W. circinata* var. *zeae* and an isolate of *W. circinata* var. *circinata* were evaluated for colony growth rate at each of six temperatures. A mycelial plug (5 mm diameter) was removed from the edge of a colony of each isolate on PDA, transferred to a 9 cm diameter Petri plate of PDA, and incubated at 15, 20, 25, 30, 35, or 40°C in the dark. Colony diameter was measured every 24 h for 4 d or until the colony filled the plate. There were three replicate plates for each isolate, and the test was performed twice. Growth rates (mm per day) per day) were calculated for the first 24 h, and rates were averaged across the six temperatures. Average growth rate data were analyzed with analysis of variance (ANOVA) in SAS version 9.0 (SAS Institute, Cary, NC). Fisher's least significant difference (LSD) test was used to separate means by isolate (P = 0.05).

Pathogenicity Studies.

Inoculations with the five UWC isolates were made onto two cultivars of SP ('SeaDwarf' and 'SeaIsle Supreme'). A qualitative disease incidence assessment was made 3 d after inoculation, and the test was repeated two times. In subsequent inoculation experiments, isolate SK-PSA-TM4 was inoculated onto 'SeaDwarf' and

‘SeaIsle Supreme’ SP, ‘Sonesta’ seeded bermudagrass (*Cynodon dactylon*), and ‘Dark Horse’ roughstalk bluegrass (*Poa trivialis* L.). Disease severity was rated 3 d after inoculation, and the experiment was repeated. In a final inoculation experiment, the five UWC isolates were inoculated onto ‘Penncross’ creeping bentgrass (*Agrostis stolonifera* L.). Disease severity was assessed 3 d after inoculation. The experiment was repeated. In all inoculation experiments, three replications per isolate and host combination were used. Data from multiple repetitions of experiments were combined where statistically appropriate and unless otherwise specified.

Cool season grasses and bermudagrass were seeded into 7.5 cm diameter pots filled with calcine clay (Turface MVP; Profile Products LLC, Buffalo Grove, IL). The grass was grown for at least three weeks with up to one foliar trimming prior to, but not the day of, inoculation. Granular fertilizer was applied once at 0.3 grams per pot (6-12-8 starter fertilizer, Harrell’s LLC, Lakeland, FL).

SP was sprigged into either 7.5 or 10.0 cm diameter pots by placing 5 to 8 stolon pieces of SP on the surface of each pot filled with calcine clay, and the pots were irrigated. The SP was fertilized twice with 6-12-8 fertilizer at 0.3 g per 7.5 cm diameter pot and 0.5 g per 10 cm diameter pot one month after sprigging. The SP was trimmed weekly for approximately 3 months, until the turfgrass canopy covered each pot. Inoculations were made approximately 4 months after sprigging.

Inoculum was produced on oat seed. Fifty grams of seed were soaked in 50 mL distilled water for 24 h in a 250 mL glass flask with a foam plug. The imbibed seed was autoclaved for 30 min once a day for three sequential days. Sterilized seed was inoculated with 5 agar plugs transferred from the leading edge of a 1 day-old PDA

culture of each isolate. Flasks were maintained in a dark incubator at 25ºC for 2 to 3 weeks. Approximately every 2 d, the flasks were vigorously shaken to mix the mycelia with the seed. The colonized oat seed was transferred to sterilized paper bags and was dried for 2 weeks in a fume hood. Inoculum was stored at 5ºC in sterilized 100 mL glass vials sealed with Parafilm.

Turfgrass was inoculated by placing five colonized oat seeds equidistant in the foliar canopy of a pot that had been irrigated with 100 mL water. Each pot was placed in a 30 by 50 cm polyethylene bag containing a moist paper towel to maintain high humidity. The bags were sealed and placed in an incubator for 3 d at 30°C with 12 h light per day. Pots inoculated with sterile oat seed served as a control treatment for all inoculation experiments.

Disease incidence was assessed 3 d after inoculation of the SP cultivars. Disease severity was assessed on pots of turfgrass utilizing the Horsfall-Barratt rating scale for inoculation experiments with SK-PSA-TM4 and the five UWC isolates on bentgrass (Horsfall and Barratt 1945). Data were subjected to logit transformation and converted to a 0 to 100% severity scale after ANOVA, and means separation was performed according to Fisher's LSD utilizing SAS version 9.0. At the conclusion of these studies, eight pieces of host tissue from at least one replication of each inoculated turfgrass species were excised, surface-sterilized with 70% ethanol, rinsed, and transferred to ⅕-PDA and water agar. Isolated fungi were transferred into pure culture and identified by morphological characteristics as described above.

Results

Colony Morphology and Characterization.

A summary of the morphological characteristics of UWC isolates SK-PSA-TM4, SK-PMA-WA1, SK-OA-W3-I, SK-OA-TM1, and SK-HBA-W1 is presented in Table 3-2. All UWC isolates had right- and acute-angle hyphal branching, and produced extremely profuse, light yellow to pink, mycelial growth on PDA (Figure 3-2). The aerial mycelium of each isolate clumped, clustered, and attached to the underside of the Petri dish lid. No basidiospores or other sexual (teleomorphic) structures were either found associated with the symptomatic turfgrass samples or in vitro at any time up to 120 d after isolation. Irregularly-shaped, salmon-colored to brown sclerotia, 3 to >7 mm in diameter were observed embedded in the PDA after 2 to 4 weeks of incubation. Isolate SK-3-OA-W3-I produced sclerotia that were light yellow, while SK-HBA-W1 produced light brown sclerotia. Hyphal diameter averaged 5 µm with six to seven nuclei per cell for each UWC isolate.

Maximum growth rates for the UWC isolates occurred at 30°C (Figure 3-3). When comparing rates of growth on PDA encompassing temperatures from 15 to 40º C, SK-PSA-TM4, SK-OA-TM1, and SK-OA-W3-I had statistically similar growth rates that were significantly greater than the growth rates of isolates SK-PMA-W1, SK-HBA-W1, and SK-SMd (Table 3-3). The growth rate of isolate SK-BA-W1 was significantly greater than that of all the other isolates tested. The growth rate of isolate SK-SMd was significantly less than that of all other isolates tested.

rDNA-ITS Sequence Analysis.

Genetic pairwise distances for all ITS sequences according to the Kimura Two-Parameter Model had a range of 0.000 to 1.697, isolate numbers and identities

described in Table 3-1 (Figure 3-4). When pairing sequences, the smaller the number, the greater the similarity between the sequences, and the larger the number the greater the dissimilarity between the sequences. The minimum and maximum genetic distance among the UWC isolates was 0.000 and 0.0021 (100 and 99.79% similarity, respectively) with no more than 1 bp difference in the 589 bp sequenced. A 9 to 14 bp difference was detected between UWC isolates and *W. circinata* var. *agrostis* isolates (Sharon et al., 2006).

The UWC isolates formed a distinct phylogenic clade with 99% support (Figure 3-5). The clade formed by the UWC isolates was closest to the clade formed by *W. circinata* var. *agrostis*, followed by the *W. circinata* var. *zeae* clade, and the *W. circinata* var. *circinata* single-isolate clade. The *W. circinata* var. *oryzae* clade was most distant from the UWC clade.

Turfgrass Pathogenicity Studies.

Both cultivars of SP developed symptoms following inoculation with each of the UWC isolates. Symptoms included water-soaking, chlorosis, and then necrosis of the lower leaf blades and sheaths 3 d after inoculation. Abundant aerial mycelium was observed in the lower leaf canopy for each isolate (Figure 3-6). Symptoms developed in each of the three repetitions of the experiment, and isolates were recovered from symptomatic tissue for each isolate on each SP cultivar. No symptoms were observed in the non-inoculated control treatments.

UWC isolate SK-PSA-TM4 caused an average of 57 to 97% disease severity on ‘SeaDwarf’ SP, ‘Sonesta’ bermudagrass, ‘SeaIsle Supreme’ SP, and ‘Darkhorse’ roughstalk bluegrass after 3 d of incubation at 30°C (Table 3-4). Disease severity was greatest on roughstalk bluegrass. For all the turfgrass cultivars inoculated, the lower

leaves were blighted, and the sheaths turned yellow to brown with a water-soaked appearance. Mycelium was copiously present in the lower and upper turfgrass canopy. The infected turfgrass was unifory blighted, water-soaked in appearance, with little to no healthy tissue remaining 3 d after inoculation. There were no individual or distinct leaf or sheath lesions present. Isolates used in the pathogenicity studies were recovered from necrotic foliage of the various turfgrass species inoculated. The UWC isolates SK-PSA-TM4, SK-OA-TM1, SK-HBA-W1, SK-PMA-WA1, and SK-OA-W3-I caused (mean±standard deviation) 98±1.4, 92±7.0, 88±7.3, 85±13, and 78±15% disease severity (respectively) on creeping bentgrass 3 d after inoculation. Prolific aerial mycelium of each isolate grew throughout the lower turfgrass canopy. Water-soaking and necrosis of the leaf blades and sheaths were visible by day 2. Disease symptoms with all UWC isolates on all turfgrass species began as a lower (basal) leaf blight quickly progressing to an overall crown and foliar blight. No leaf spots, lesions, or sclerotia were observed.

Discussion

The discovery of a 'new' SP disease and associated *Rhizoctonia*-like fungus merits a discussion of the taxonomy of the group. Over the last 150 years, diverse groups of fungal species have been assigned to the form-genus *Rhizoctonia* s. l. (Andersen and Stalpers 1994, de la Cerda et al., 2007, Garcia et al., 2006, Mazzola et al., 1996, Moore 1987, Priyatmojo et al., 2002a). However, taxonomic reclassifications and clarifications have resulted in the development of several new anamorphic genera, each associated with a distinct teleomorphic genus. For example, the anamorphs of *Thanatephorus*, *Ceratobasidium*, and *Waitea* are currently recognized as *Rhizoctonia* (Stalpers et al., 1998), *Ceratorhiza* (Moore, 1987), *and Chrysorhiza* (Stalpers and

Andersen, 1996), respectively. Taxonomy and nomenclature of the teleomorphs of *Rhizoctonia*-like fungi have been based on characteristics of basidiocarps, sterigmata, basidiospores and other sexual structures (Sneh et al., 1991; Stalpers and Andersen, 1996). Teleomorphs of the *Rhizoctonia*-like pathogens of turfgrass rarely are observed, so isolate identification tends to be based on the morphology of anamorphs, which lack asexual spores and fruiting bodies (Burpee and Martin, 1992). Traditionally, characteristics such as mycelial color, the number of nuclei per hyphal cell, and the shape, size and color of sclerotia, have been used to identify these fungi (Toda et al., 2005; Toda et al., 2007). In addition, isolates have been grouped based on the ability of hyphae to anastamose (Carling et al., 2002; Garcia et al., 2006; Kuninaga et al.; 1997, Priyatmojo et al., 2002a; Stalpers et al., 1998; Yokoyama et al., 1985).

The hymenomycete *Waitea circinata* Warcup and Talbot (1962) was described to include fungi similar to *Thanatephorus* but having shorter than *Thanatephorus*, horn-like sterigmata and basidiospores that fail to undergo repetitive germination. Mycelia of *W. circinata* were considered to be saprobic (Warcup and Talbot, 1962) prior to the diagnosis of what is believed to be the anamorph, *Chrysorhiza zeae* (Voorhees) Andersen & Stalpers [= *R. zeae*] (Garcia et al., 2008; Oniki et al., 1985; Stalpers and Andersen, 1996), as a pathogen of several members of the *Poaceae* (Burpee and Martin 1992, Garcia et al., 2008). Isolates of *C. zeae* also have been referred to as *W. circinata* var. *zeae* (de la Cerda et al., 2007; Toda et al., 2005; Toda et al., 2007). The epithet *Chrysorhiza* was created to accommodate fungi with a *Waitea* teleomorph (Stalpers and Andersen, 1996), while *Rhizoctonia* anamorphs are associated with *Thanatephorus* (Stalpers et al., 1998).

Sequences from phylogenetically conserved regions of DNA such as the rDNA ITS region have been used to distinguish varieties of *Waitea circinata* (de la Cerda et al., 2007, Garcia et al., 2008, Leiner and Carling 1994, Sharon et al., 2008). Isolates may be identified based on the similarity of conserved sequences such as ITS1, the 5.8S ribosomal subunit, and ITS2 when teleomorphic structures are not present (de la Cerda et al., 2007, Sharon et al., 2008). The UWC isolates we found associated with SP in Florida appear to be morphologically and genetically distinct from previously described species affecting turfgrass. Morphological characteristics that distinguished the SP isolates from established varieties of *W*. *circinata* included abundant, yellow to pink, aerial mycelium on PDA and salmon to yellow-brown, aggregated sclerotia measuring 3.4 to 7.3 mm in diameter embedded in the agar medium after 2 to 4 weeks of incubation at 30ºC on full-strength PDA. In addition, analyses of rDNA sequences revealed that the isolates from diseased SP formed a clade that was significantly distinct from that of isolates of *W. circinata* vars. *agrostis*, *circinata, oryzae,* or *zeae.* Variability in growth rates, host ranges, temperature optima, disease symptoms, and hyphal and sclerotia color reinforce the differentiation of the UWC clade.

Based on morphological and sequence data, we propose that these isolates from SP comprise a new variety designated *W. circinata* var. *prodigus.* DNA sequence data indicated that isolates of *W. circinata* var. *prodigus* were only 97 to 98% similar to *W. circinata* var. *agrostis* and formed a distinct clade with 99% support in an ITS phylogenetic tree. Isolates of *W. circinata* var. *agrostis* were recovered from diseased creeping bentgrass in Japan (Sharon et al., 2006) They were not observed to produce abundant, yellow to pink mycelium, and the sclerotia were only 1 to 3 mm in diameter

and dark brown in contrast to the sclerotial described above as characteristic of *W. circinata* var. *prodigus.*

Diagnostic differentiation among *Thanatephorus spp.* and *Waitea* spp. anamorphs affecting SP could be assisted greatly by routinely plating samples on both standard and thiophanate methyl-amended (100 μg/mL) agar media. Growth of the LP strain of *R. solani* that causes large patch is inhibited on the amended medium. Isolates of *C. zeae* that cause leaf and sheath spot, and *W. circinata* var. *prodigus* will grow on both media, but the *prodigus* isolates appear more floccose within 48 h of plating. Misidentification of these pathogens could result in unnecessary applications of ineffective fungicides. For example, symptoms caused by *C*. *zeae* or *W*. *circinata* var. *prodigus* could be confused with brown patch or large patch caused by *R. solani*, resulting in ineffective management of the disease with applications of thiophanate-methyl (Elliott 1999). Accurate identification of the causal agent for fungicide selection is an important factor in turfgrass disease management.

The proposed common disease name of 'basal leaf blight', caused by *W. circinata* var. *prodigus,* is reflective of the aggressive blighting caused by the pathogen on the oldest, basal leaves, progressing over time to form diffuse necrotic patches in turfgrass swards. The rapid colonization and blight of four turfgrass species at 30ºC in controlled environmental conditions in this study, and the rapid growth rate of *W. circinata* var. *prodigus* in vitro at 25 to 35ºC suggest this pathogen could be a potential threat to multiple species of turfgrass over a wide range of climatic conditions.

Table 3-1. Isolates of *Waitea circinata* and related fungi that were examined utilizing rDNA internal transcribed spacer (ITS) region sequences

Isolate no.	Host[a]	Origin	Date collected	Identity	GenBank accession no.
01 SK-SMd	Annual bluegrass	Reston, VA	25 Apr 2008	*Waitea circinata* var. *circinata*	FJ154894
05 SK-BA1	'SeaIsle 2000' SP	Boca Raton, FL	4 Sep 2007	*Waitea circinata* var. *zeae*	HM597140
09 SK-4OA3-1/5	'SeaIsle Supreme' SP	Sarasota, FL	4 Sep 2007	*Waitea circinata* var. *zeae*	HM597139
14 SK-3OA-TM	'SeaIsle Supreme' SP	Sarasota, FL	30 Oct 2007	*Waitea circinata* var. *zeae*	HM597141
36 SK-OA-W3-I	'SeaIsle Supreme' SP	Sarasota, FL	9 July 2008	UWC[b]	HM597147
39 SK-HBA1	'SeaDwarf' SP	Naples, FL	9 July 2008	*Waitea circinata* var. *oryzae*	HM597138
42 SK-0821 BG	'Tifeagle' bermudagrass	Columbia, SC	24 Aug. 2008	*Waitea circinata* var. *oryzae*	HM597137
44 SK-PSA-TM4	'SeaDwarf' SP	Ft. Myers, FL	4 Jan 2008	UWC	HM597146
45 SK-OA-TM1	'SeaIsle Supreme' SP	Sarasota, FL	4 Jan 2008	UWC	HM597144
46 SK-HBA-W1	'SeaDwarf' SP	Naples, FL	4 Jan 2008	UWC	HM597143
59 SK-OA-W1-II	'SeaDwarf' SP	Sarasota, FL	4 Nov 2008	*Waitea circinata* var. *oryzae*	HM597135
60 SK-4BWB-W1	'SeaIsle 2000' SP	Boca Raton, FL	6 Jan 2009	*Waitea circinata* var. *oryzae*	HM597134
61 SK-PB-TM1	'SeaDwarf' SP	Boca Raton, FL	6 Jan 2009	*Ceratobasidium* sp.	HM597133
66 SK-PMA-WA1	'SeaDwarf' SP	Ft. Myers, FL	26 Aug 2008	UWC	HM597145
69 SK-3OB1W	'SeaIsle Supreme' SP	Sarasota, FL	30 Oct 2007	*Thanatephorus cucumeris* AG2-2IIIB	HM597131
70 SK-OA-W1-I	'SeaIsle Supreme' SP	Sarasota, FL	4 Nov 2008	*Waitea circinata* var. *oryzae*	HM597136
74 SK-820 BG	'Tifeagle' bermudagrass	West Palm Bch, FL	10 July 2008	*Waitea circinata* var. *zeae*	HM597142
76 3BWA-TM2	'SeaIsle 2000' SP	Boca Raton, FL	6 Jan 2009	*Thanatephorus cucumeris* AG2-2LP	HM597132
90 ATCC MYA-4521 [c]	Culture collection	Manassas, VA	Apr 2009	*Sclerotinia sclerotiorum*	FJ810516
91 NUK-3BG [c]	Creeping bentgrass	Aichi, Japan	June 1999	*Waitea circinata* var. *agrostis*	AB213567
92 DAI-BG [c]	Creeping bentgrass	Hyoga, Japan	July 1998	*Waitea circinata* var. *agrostis*	AB213569
93 OHT-BG [c]	Creeping bentgrass	Chiba, Japan	June 1997	*Waitea circinata* var. *agrostis*	AB213578
94 SHO-BG [c]	Creeping bentgrass	Kanagawai, Japan	July 1999	*Waitea circinata* var. *agrostis*	AB213575

[a] Annual bluegrass (*Poa annua* L.), SP: SP (*Paspalum vaginatum* Swartz), bermudagrass (*Cynodon dactylon* (L.) Pers.), creeping bentgrass (*Agrostis stolonifera* L.)

[b] UWC = previously uncharacterized variety of *Waitea circinata*

[c] DNA sequence and other information were obtained from GenBank.

Table 3-2. Comparison of hyphal and sclerotial characteristics of isolates from symptomatic SP in South Florida

		Hyphae[b]		Sclerotia	
Isolate	Colony morphology[a]	Diameter (µm)	Nuclei	Characteristics	Size (mm)[c]
SK-PSA-TM4	Yellow-pink, aerial clumping, monilioid cells	5 ± 0.5	7 ± 1.1	Many, clumped, irregular, salmon, in agar[d]	5 ± 1.7
SK-PMA-WA1	Yellow-pink, aerial tufted growth	5 ± 0.4	7 ± 1.4	Many, irregular, yellow-salmon, in agar	3 ± 1.0
SK-OA-W3-I	Yellow-pink, aerial clumping, monilioid cells	5 ± 0.6	7 ± 1.7	Sparse to none, yellow, spherical, in agar	5 ± 1.0
SK-OA-TM1	Yellow, aerial clustered clumping, monilioid cells	5 ± 0.3	6 ± 2.5	Sparse, clumped, irregular, yellow-orange, in agar	7.4 ± 2.6
SK-HBA-W1	Yellow-pink aerial clumping	5 ± 0.4	7 ± 1.7	Erratic, abundant, salmon-cream, catenulate, scattered in agar	3.4 ± 1.6

Table 3-3. Mycelial growth rates of *Waitea circinata* isolates after 24 h on potato dextrose agar.

Isolate	Growth rate (mm/day)[a]
SK-BA-W1	22 a
SK-PSA-TM4	20 b
SK-OA-TM1	20 b
SK-OA-W3-I	20 b
SK- PMA-W1	19 c
SK-HBA-W1	17 d
SK-SMd	11 e

[a] Mean (n = 36) growth rate. Isolate SK-BA-W1 is *W. circinata* var. *zeae* and SK-SMd is *W. circinata.* var. *circinata*. Means followed by the same letter are statistically similar according to Fisher's least significant difference (α = 0.05).

Table 3-4. Pathogenicity of *Waitea circinata* isolate SK-PSA-TM4 on SP, bermudagrass, and roughstalk bluegrass

	% Disease severity[a]			
Inoculum	'SeaIsle Supreme' SP[b]	'SeaDwarf' SP	'Sonesta' bermudagrass[c]	'Dark Horse' roughstalk bluegrass[d]
Non-inoculated	10 b	5 b	5 b	3 b
SK-PSA-TM4	69 a	57 a	57 a	97a

[a] Ratings were taken 3 d after inoculation and incubation at 30°C. Means (n = 6) within columns followed by the same letter are statistically similar according to Fisher's least significant difference (α = 0.05).
[b] SP (*Paspalum vaginatum* Swartz)
[c] Bermudagrass (*Cynodon dactylon* (L.) Pers.)
[d] Roughstalk bluegrass (*Poa trivialis* L.)

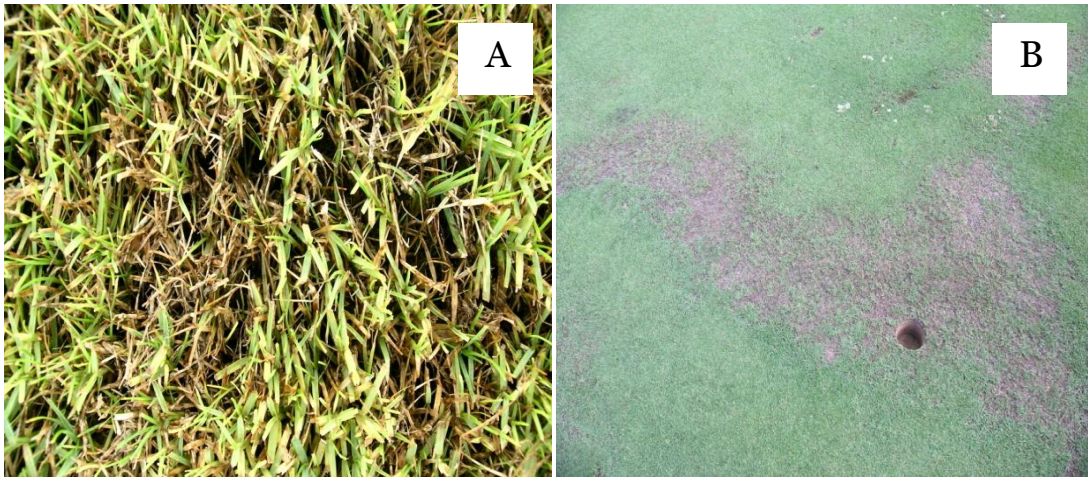

Figure 3-1. Symptoms of basal leaf blight of SP in south Florida. A) cultivar 'SeaIsle Supreme'. B) cultivar 'SeaDwarf'.

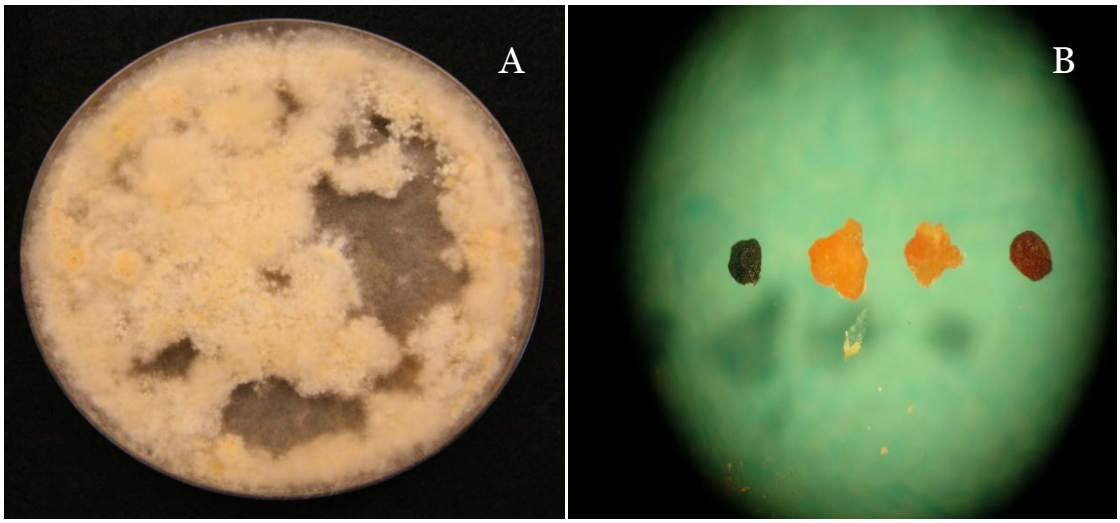

Figure 3-2. A) Characteristics of UWC isolate (SK-PSA-TM4) colony on PDA. B) Sclerotial characteristics left to right of *W*. *circinata* var. *circinata*, UWC, *W*. *circinata* var. *oryzae*, and *W*. *circinata* var. *zeae*.

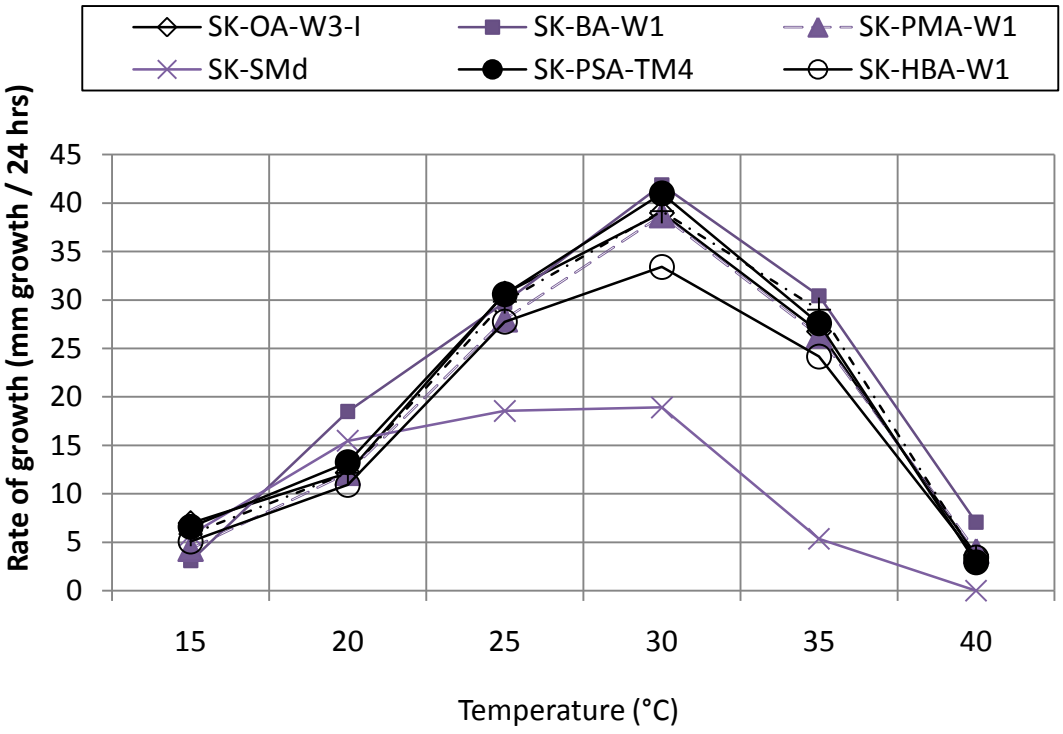

Figure 3-3. Comparison of mycelial growth rates (mm per day) of *Waitea circinata* varieties on potato dextrose agar at 15, 20, 25, 30, 35, and 40°C.

	1*	5	9	14	36	39	42	44	45	46	59	60	61	66	69	70	74	76	90	91	92	93	94
[1]																							
[5]	0.038																						
[9]	0.038	0.000																					
[14]	0.040	0.004	0.004																				
[36]	0.053	0.040	0.040	0.045																			
[39]	0.053	0.054	0.054	0.056	0.076																		
[42]	0.048	0.054	0.054	0.057	0.076	0.009																	
[44]	0.055	0.042	0.042	0.047	0.002	0.079	0.078																
[45]	0.053	0.040	0.040	0.045	0.000	0.076	0.076	0.002															
[46]	0.053	0.040	0.040	0.045	0.000	0.076	0.076	0.002	0.000														
[59]	0.048	0.054	0.054	0.057	0.076	0.009	0.000	0.078	0.076	0.076													
[60]	0.048	0.054	0.054	0.057	0.076	0.009	0.000	0.078	0.076	0.076	0.000												
[61]	0.937	0.895	0.895	0.904	0.878	0.935	0.923	0.883	0.878	0.878	0.923	0.923											
[66]	0.053	0.040	0.040	0.045	0.000	0.076	0.076	0.002	0.000	0.000	0.076	0.076	0.878										
[69]	0.912	0.883	0.883	0.892	0.852	0.895	0.892	0.857	0.852	0.852	0.892	0.892	0.163	0.852									
[70]	0.048	0.054	0.054	0.057	0.076	0.009	0.000	0.078	0.076	0.076	0.000	0.000	0.923	0.076	0.892								
[74]	0.035	0.009	0.009	0.009	0.042	0.056	0.056	0.045	0.042	0.042	0.056	0.056	0.890	0.042	0.863	0.056							
[76]	0.919	0.899	0.899	0.909	0.868	0.912	0.909	0.873	0.868	0.868	0.909	0.909	0.152	0.868	0.020	0.909	0.880						
[90]	1.658	1.635	1.635	1.646	1.604	1.695	1.697	1.607	1.604	1.604	1.697	1.697	1.484	1.604	1.383	1.697	1.646	1.447					
[91]	0.053	0.040	0.040	0.045	0.013	0.081	0.075	0.016	0.013	0.013	0.075	0.075	0.855	0.013	0.838	0.075	0.047	0.854	1.578				
[92]	0.055	0.042	0.042	0.047	0.016	0.078	0.072	0.018	0.016	0.016	0.072	0.072	0.855	0.016	0.838	0.072	0.050	0.854	1.592	0.002			
[93]	0.050	0.037	0.037	0.042	0.020	0.072	0.067	0.023	0.020	0.020	0.067	0.067	0.869	0.020	0.852	0.067	0.045	0.868	1.607	0.007	0.004		
[94]	0.048	0.035	0.035	0.040	0.023	0.070	0.064	0.025	0.023	0.023	0.064	0.064	0.869	0.023	0.852	0.064	0.042	0.868	1.607	0.009	0.007	0.002	

Figure 3-4. Genetic distance matrix based on sequence data utilizing rDNA internal transcribed spacer (ITS)1, 5.8S rRNA, and ITS2 regions of the rDNA locus from isolates of *Waitea circinata* and related fungi.

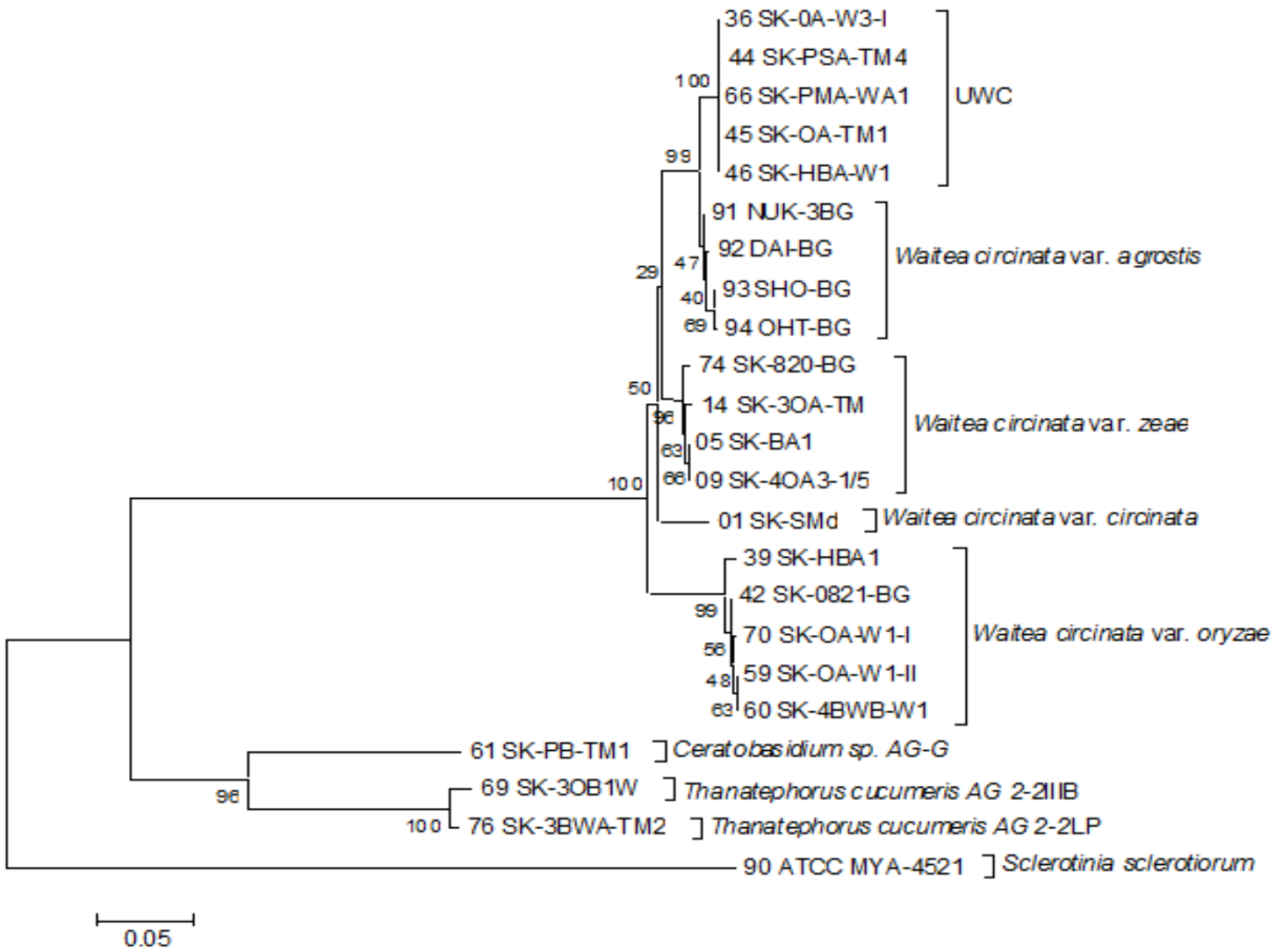

Figure 3-5. Phylogenetic distance tree comparing nucleotide sequences from the rDNA of *Waitea circinata* isolates and related fungi.

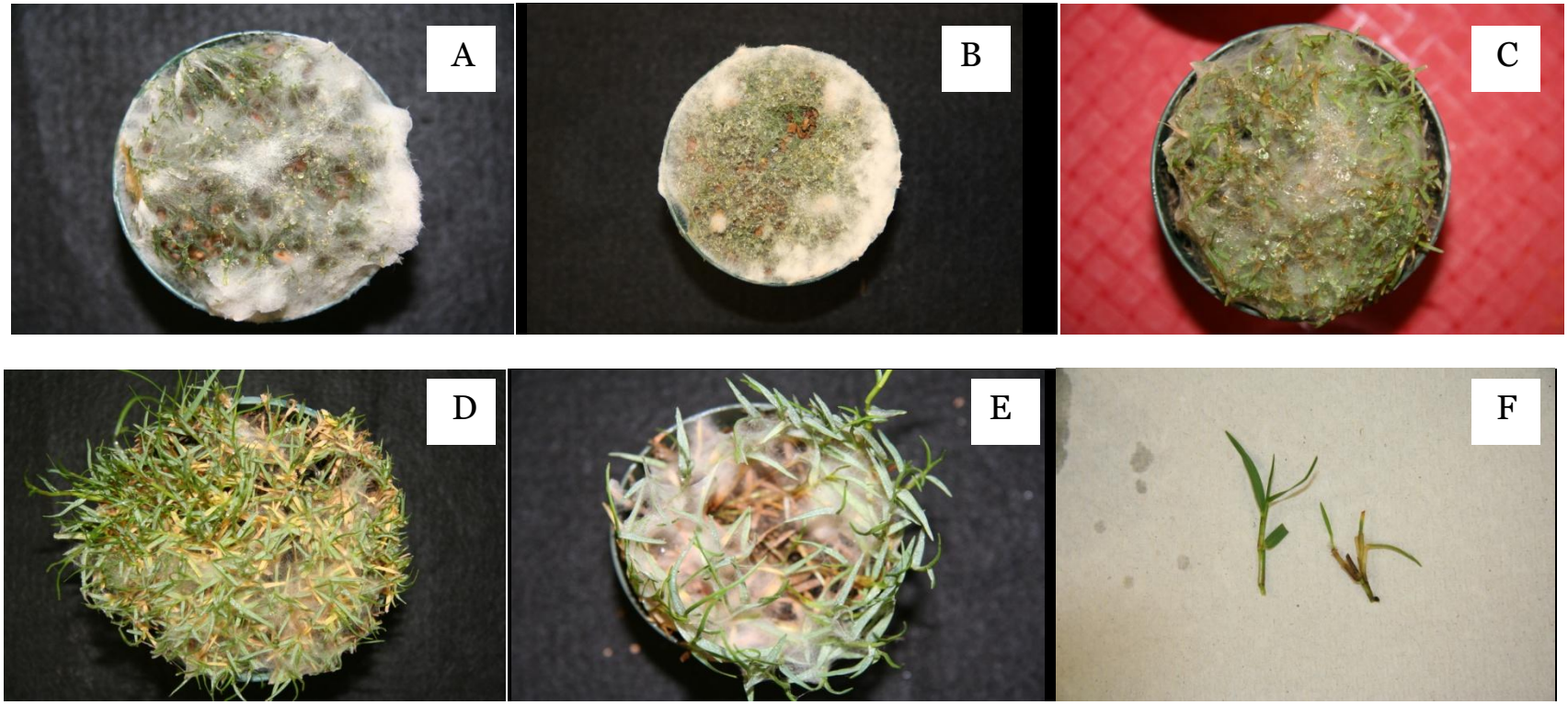

Figure 3-6. Basal leaf blight symptoms 3 d after inoculation with *W. c*. var. *prodigus*: A) 'Penncross' creeping bentgrass. B) 'Dark Horse' Poa trivialis. C) 'Sonesta' bermudagrass. D) 'SeaDwarf' SP. E) 'SeaIsle Supreme' SP. F). shoots of 'SeaDwarf' SP non-inoculated (left) and inoculated (right).

CHAPTER 4
THE INTERACTION OF *CHRYSORHIZA ZEAE*, *RHIZOCTONIA SOLANI* AG 2-2LP AND SALINE WATER ON *PASPALUM VAGINATUM*

Introduction

Seashore paspalum (SP), *Paspalum vaginatum* Swartz, is a turfgrass species widely recognized for its quality and tolerance to salts. Turfgrass roots can be negatively impacted in their ability to take up water due to high soluble salts. Irrigation water containing high salt levels is becoming a limiting factor in development of golf courses around the world and in Florida (Neylan, 2007). Seashore paspalum is noted as a salt tolerant warm season turfgrass species (Allar, 2001, Duncan and Carrow, 2000b). Soil salinity can be a major environmental constraint limiting the use of some of the other primary, high quality turfgrass species for golf courses, including but not limited to hybrid bermudagrass, *Cynodon dactylon* (L.) Pers., and zoysiagrass, *Zoysia* spp. Sodium, Na^+ is the most hazardous component of high EC irrigation water and can accumulate in root zones, resulting in precipitation of other essential nutrients with bicarbonates or carbonates or directly by displacement (Richards, 1954). Continued reliance on highly saline irrigation water irrigation can adversely affect turf quality of SP if salts accumulate in the root zone (Duncan and Carrow 2005a, Berndt 2005). The salt tolerance of SP differs among varieties with significant damage at varying levels (Berndt, 2005; Raymer and Braman, 2005). Certain varieties of SP such as ‘Adalayd’ are more sensitive to salt than ‘Tifway’ bermudagrass (Carrow and Duncan, 1998).

Environmental and cultural stresses are well documented as increasing susceptibility of turfgrass to fungal infection and subsequent disease symptom expression. High salinity soils or continued irrigation with highly saline water is an added stress to most turfgrass species. Tests with saline water irrigation to ‘SeaDwarf’

SP reduced shoot growth resulting in total suppression of stolons at EC_w levels about 19.1 ds m^{-1} (Berndt 2005). Certain varieties of SP such as 'FSP-1' SP exhibited enhanced rooting while exhibiting 50% reduction in top growth in response to irrigation with an 18.4 dsm-1 high salt water mixtures (Peacock and Dudeck, 1985).

Upon its introduction as a high quality warm season turfgrass alternative to bermudagrass, SP was promoted as having fewer disease issues especially in areas of high soil or irrigation salt levels based on the negative effects of salt on fungi (Duncan and Carrow, 2000a). Stress due to salt injury and disease management should be jointly investigated for interactions of one with the other (Yenny, 1994). Leaf and sheath spot caused by *Chrysorhiza zeae,* and large patch, caused by *Rhizoctonia solani* AG 2-2LP, have been identified as prominent diseases of seashore paspalum (SP) (Haygood and Martin; 1998; Martin et al., 1983b). *Chrysorhiza zeae* has been isolated from SP exhibiting symptoms of leaf and sheath spot (Stiles et al., 2008). Large patch disease on several cultivars of SP in coastal South Carolina was identified as being caused by *Rhizoctonia solani* AG 2-2LP (Canegallo and Martin, 2006). Leaf and sheath spot is favored by temperatures approaching and exceeding 30^{o}C (Martin and Lucas, 1984a; Burpee and Martin, 1992; Elliottt, 1999) which is higher than what is optimal for large patch development (Couch, 1995). Limited research results have been published on the interaction of saline water and disease caused by known warm season turfgrass pathogens such as *C. zeae* and *R. solani* AG 2-2LP.

Materials and Methods

Pathogen Isolations and Identification

Isolates of *C. zeae* and *R. solani* AG 2-2LP were obtained from a scouting program conducted every two months from August 2007 to January 2009, on eight SP

golf courses in South Florida. Turfgrass samples exhibiting foliar blight or thinning of the foliar canopy were removed from golf course fairways or roughs.

Above-ground and below-ground surface sterilized chlorotic SP tissue pieces and the use of selective media were utilized to identify isolates of *Rhizoctonia*-like fungi. These fungi exhibited uniform hyphal diameter with right- and acute angled branching and no spore production. The isolates were transferred via hyphal tips to obtain pure cultures in sterilized potato dextrose agar (PDA). Isolates were maintained in the dark at 25°C.

Rhizoctonia solani AG 2-2LP and *C. zeae* isolates were confirmed via ITS region DNA sequencing of ribosomal DNA including ITS1, the 5.8S ribosomal subunit, and ITS2 utilizing polymerase chain reaction. Specifically, the PCR product from extracted DNA from the fungal isolates was cleaned with a QuickLyse Miniprep 250 kit according to manufacturer's directions (Qiagen Inc., Valencia, CA). The PCR products for each isolate were inserted into *E. coli* and cloned with a Topo TA Kit (Invitrogen, Carlsbad, CA). The transformed bacterial clones were lysed and cleaned with a miniprep kit according to manufactures directions (QIAGEN QuickLyse Miniprep kit, Qiagen Inc., Valencia, CA). The purified plasmids were submitted for sequencing to the Interdisciplinary Center for Biotechnology Research at the University of Florida in Gainesville, FL. The resulting sequences confirmed the identity of the isolates utilized in these experiments as *Chrysrohiza zeae* or *Rhizoctonia solani* AG 2-2LP.

Inoculum Preparation

Fifty grams of oat seed were soaked in 50 mL of distilled water for 24 h in a glass flask with a foam plug. The imbibed oat seed were autoclaved once a day for three sequential days. Using one-day-old PDA cultures of each *C. zeae* and *R. solani* AG 2-

2LP isolate, 5 agar plugs from the leading edge of growth were transferred to the sterilized seed. The flasks were vigorously shaken approximately every 2 days to mix the mycelia with the seed and were maintained at 25º C for 2 to 3 weeks in a dark incubator. The oat seed colonized with *C. zeae* and *R. solani* AG 2-2LP were dried for 2 weeks under a fume hood in autoclaved paper bags. Inoculum was stored at 5º C in sterilized 100 mL glass vials sealed with parafilm.

Turfgrass Establishment and Maintenance

'SeaDwarf' SP was sprigged into 10 cm pots by placing 5 to 8 stolon pieces of SP on the surface of each pot filled with calcine clay (Turface MVP; Profile Products LLC, Buffalo Grove, IL). The pots of SP were irrigated twice daily on an automated irrigation system and maintained in a greenhouse for 3 to 4 months. The SP was fertilized twice at 0.5 grams per 10 cm pot one month after sprigging (6-12-8 starter fertilizer, Harrell's LLC, Lakeland, FL). The SP was trimmed up to three times weekly until the turfgrass canopy completely closed over the surface of the calcine clay for each pot. Approximately four months after sprigging, saline water treatments and inoculations were applied.

Salt Treatments

Two experiments were conducted to determine the effects of saline water applications and *C. zeae* and *R. solani* AG 2-2LP inoculations on SP. It was determined that 150 mL of water resulted in approximately 15% drainage of water through each pot of SP. For all four experiments, saline water treatments were poured topically twice daily, once in the morning and once in the afternoon for three subsequent days, to the pots of established 'SeaDwarf' SP at concentrations of 0, 10,000, 15,000 and 20,000 ppm sodium chloride (NaCl) (Morton pool salt). The salt was dissolved in

distilled water to the appropriate concentrations. There were three replications of each treatment. Treatments were arranged in a completely randomized design.

***Chrysorhiza zeae* inoculations**

Directly following the last saline water treatment, the SP was inoculated by uniformly placing six colonized oat seeds of isolate SK-BA-1 or six sterile oat seed, as a mock-inoculated untreated check, within the foliar canopy of each pot. Pots were placed in polyethylene bags containing a moist paper towel to maintain high humidity. For the first study, the bags were sealed and placed in an incubator for 3 d at 30°C with 12 h of light and 12 h of darkness per 24 h cycles. The SP pots were removed from the bags and transferred to a greenhouse for 4 additional days, 7 d after inoculation, and rated for turf quality and severity of symptoms reflected as chlorosis and necrosis. Turf quality ratings were made on a scale of 1 to 9, with 9 being the best possible turfgrass quality (dark green, no chlorosis or necrosis apparent). Turfgrass severity ratings were made utilizing the Horsfall-Barratt scale (Horsfall and Barratt, 1945) then transformed using logit transformation to percent turf damage prior to conducting statistical analysis. Following the last ratings, the SP was removed from the pots and thoroughly flushed with water to dislodge all calcine clay from the roots and rhizomes. Four pieces of stem and leaf tissue were excised from each treatment, surface sterilized in 70% ethanol for 30 seconds, then twice rinsed with sterile water prior to transferring to 2% water agar media and 1/5th strength PDA + rifampicin and ampicillin for observance of the presence of *Chrysorhiza* mycelial growth. For both studies, the “washed” SP was placed in labeled paper bags and dried at 65°C for 7 d in a GS Blue M Electric Constant Temperature Cabinet, model OV-51OA-3 (General Signal Co., Blue Island, IL). Dried SP was weighed and dry weights recorded.

For the second trial, the methodology as previously described was repeated but modified in that the potted SP turfgrass plants were placed in the polyethylene bags for 7 d at 30°C then rated for turf quality and severity of chlorosis and necrosis. Following the 7 d incubation period, the SP pots were removed from the bags and transferred to a greenhouse for 20 additional days, 27 d after inoculation, and rated again for turf quality and turf severity as described above. Following completion of the study, colonization of the SP tissue was explored, and dry weights determined in the same manner as was performed in the first trial.

All turfgrass quality, severity ratings and dry weight data were subjected to variance general linear model (GLM) and the means separated using Fischer's LSD (α = 0.05) utilizing SAS v. 9.0 (SAS Institute Inc., Cary, NC).

***Rhizoctonia solani* AG 2-2LP inoculations**

Saline water applications at the same concentrations and inoculations with six colonized oat seeds by *R. solani* AG 2-2LP (isolate SK-VBB4-1/5-I-2), or six sterile oat seed were made using the same methodology as described for the *C. zeae* inoculation studies. Pots were placed in polyethylene bags containing a moist paper towel to maintain high humidity for 7 d at 25°C. After 7 d, the potted SP was transferred to a greenhouse and rated 14 d post inoculation. Host tissue was was plated after surface sterilization and the presence of fungi was noted in the same manner as was performed for the *C. zeae* inoculation studies.

The first inoculation trial with isolate SK-VBB4-1/5-I-2 resulted in low disease severity, and there was no recovery of the isolate from surface sterilized tissue 14 d after inoculation. In order to enhance disease symptoms, a different *R. solani* AG 2-2LP isolate (isolate 07-14 recovered from 'Floratam' St. Augustinegrass at Belle-Glade, FL

24-Apr., 2007) was used for the second saline water pathogenicity trial, and the incubation period in sealed polyethylene bags at 25°C was extended from 7 to 14 d.

After 7 and 14 d post incubation, the potted SP removed from the bags were rated for turf quality and for severity of symptoms using the methodology and rating scales utilized with the *C. zeae* inoculation and saline studies. For the second trial, the SP treatments were rated at 7 d and immediately returned to the sealed bags in the incubator for an additional 7 d. A second set of ratings were made and the studies were terminated at 14 d post inoculation.

Foliar chlorosis and necrosis ratings were transformed using logit transformation from the Horsfall-Barratt scale (Horsfall and Barratt 1945) to percent turf damage before conducting statistical analysis. All data were analyzed utilizing analysis of variance and means were separated according to Fisher's LSD ($\alpha = 0.05$) utilizing SAS v. 9.0 (SAS Institute Inc., Cary, NC).

Results

Chrysorhiza zeae Salinity Experiment 1

No significant differences were observed in this trial at the 95% significance level. Means are presented and general trends in the data will be discussed (Figure 4-1). Turf quality was reduced in SP inoculated with *C. zeae*, but the salt water treatments did not significantly limit or enhance turf quality in the inoculated turf. The salt water treatments as well as *C. zeae* inoculation had no obvious effect on the severity of foliar chlorosis and necrosis in SP (Figure 4-2). Compared to the non-treated check, dry weight of SP tended to be less in SP treated with 15,000 ppm sodium chloride, but not when treated with 10,000 or 20,000 ppm salt water. Inoculation with *C. zeae* had no obvious effect on dry weight of SP alone or in the presence of any of the saline treatments.

Seashore paspalum inoculated with *C. zeae* had low turf quality (Table 4-1). Saline treatments had no effect on turf quality or foliar symptoms. There was no interaction, positive or negative, associated between inoculation with *C. zeae* and salinity. *Chrysorhiza zeae* was recovered and colonization and infection confirmed from all inoculated SP treatments.

***Chrysorhiza zeae* Salinity Experiment 2**

The model was not significant at the 95% confidence level. Quality of the SP turf was not affected by any of the saline treatments (Figure 4-3). Seashore paspalum inoculated with *C. zeae* had numerically lower quality after incubation for 7 d when no saline water was applied. None of the salt water treatments of the same concentration differed in quality of the turf either 7 or 27 d after inoculation. Seashore paspalum had the numerically higher chlorosis and necrosis with the lowest dry weights where 20,000 pppm saline water was applied compared to all other treatments (Figure 4-4). Inoculation with *C. zeae* had no effect on foliar chlorosis and necrosis or the dry weight of SP with or without saline water.

Chrysorhiza zeae was recovered and confirmation of colonization and infection was obtained from all of the inoculated treatments.

***Rhizoctonia solani* AG 2-2LP Salinity Experiment 1**

No significant differences were detected for this experiment. For the first study using *R. solani* AG 2-2LP (isolate SK-VBB4-1/5-I-2), the SP quality treated with the 20,000 ppm saline water was numerically lowest compared to the non-salt water check at 7 d after inoculation (Table 4-3). SP quality was not affected by inoculation with *R. solani* AG 2-2LP compared to SP mock-inoculated with sterile oat seed at the same saline water concentrations 14 d after inoculation.

After incubating for 7 d, chlorosis and necrosis of the SP turfgrass was numerically greatest at the 20,000 ppm of sodium chloride compared to the water check. There were no differences in chlorosis or necrosis when comparing the same saline treatments 14 d after inoculation. Differences were not apparent with applications of salt at 14 d compared to the non-saline check.

The SP inoculated with *R. solani* AG2-2LP did not differ in chlorosis or necrosis 14 d after inoculation compared to the SP mock-inoculated with sterile seed indicating low virulence with this isolate (Table 4-4). There was no significant interaction between inoculation with *R. solani* AG 2-2LP and salt applications. *Rhizoctonia solani* was not recovered from any of the excised, surface-sterilized tissue in the study confirming the lack of colonization with this isolate.

***Rhizoctonia solani* AG 2-2LP Salinity Experiment 2**

Both inoculation and salinity variables contributed significantly to the differences in severity of turfgrass chlorosis and necrosis. Lower quality ratings resulted with the SP treated with 15,000 and 20,000 ppm sodium chloride concentrations compared to the non-saline and 10,000 ppm saline water applications (Figure 4-5). However, for *R. solani* AG 2-2LP-inoculated SP quality was significantly lower and levels of chlorosis and necrosis increased compared to uninoculated controls (data not shown). There were no significant differences between treatments for dry weight.

Significantly lower turf quality and higher chlorosis and necrosis was apparent 14 d after inoculation with *R. solani* AG 2-2LP compared to mock-inoculated SP and with increasing salt water concentrations (Table 4-5). There was no significant interaction between salt treatments and inoculation with *Rhizoctonia solani* AG 2-2LP. *Rhizoctonia*

solani was recovered in culture from excised, surface-sterilized tissue from all *R. solani* AG 2-2LP inoculated treatments.

Discussion

Pathogenicity comparisons between *C. zeae* and *R. solani* AG 2-2LP inoculated on the same turfgrass species under similar conditions is generally lacking. Most comparisons between these fungi are based on fungal morphology and growth characteristics in culture (Sanders et al., 1978; Martin et al., 1983a; Martin and Lucas 1984a; Moore 1987; Sneh 1998; and Hyakumachi et al., 1997). The two inoculation studies with *Chrysorhiza zeae* and *Rhizoctonia solani* AG 2-2LP were designed to determine the effect of saline irrigation on disease symptoms whether reflected by turf quality, chlorosis or necrosis symptoms or reductions in dry weights. There were some notable differences between the four studies. The disease symptoms could not be differentiated from salt damage.

Experimental error was high for three of the four trials conducted and resulted in not many significant differences being observed. The sources of experimental error were recorded as variation between turfgrass quality of pots of SP used for the experiments and underperformance of one *R. solani* and the *C. zeae* isolates in the chosen environmental parameters. Warm season turfgrass is difficult to grow for pot studies, and the general lack of data in the literature reflects this fact.

Disease symptoms can be confused with or exacerbated by salt injury. Symptoms in cool season turfgrass caused by the pathogens *Pythium aphanidermatum* (Pythium blight) and *Labyrinthula terrestris* (rapid blight) is enhanced due to high soluble salts (Camberato et al., 2005; Martin et al., 2002; Rasmussen 1988).

In the second large patch trial, saline water applications failed to suppress disease symptoms caused by *R. solani*. Turf quality tended to decrease with increasing concentrations of salt water. Salt water treated SP had lower turf quality and greater levels of chlorotic and necrotic tissue at every concentration greater than 0 ppm when inoculated with *R. solani* AG 2-2LP over the duration of the trial. General trends in the data from the other trials also suggest that given more uniform plant material and more replication, similar results could be expected. Extending the incubation period to 14 d and inoculating the SP with the *R. solani* AG 2-2LP isolate 07-14 resulted in more definitive differences between treatments as contrasted to the first trial.

Pathogenicity of the *C. zeae* isolate was reflected by decreased turfgrass quality ratings corresponding to the inoculated treatments versus pots that were not inoculated although the differences were not statistically significant. Leaf and sheath spot is a difficult disease to diagnose and causes non-uniform damage on greens and other turfgrass areas where it occurs. Symptoms in pots of inoculated grass were difficult to distinguish from other stresses brought on by the incubation and other factors.

Chrysorhiza zeae is routinely diagnosed at a high frequency from seashore paspalum and bermudagrass turfgrass samples submitted to the University of Florida's RapidTurf diagnostic clinic utilizing selective media to differentiate it from *R. solani* (Harmon personal communication). The high isolation frequency of *C. zeae* from turfgrass samples submitted from golf courses indicates either the symptomatic turfgrass was not easily identified as a known disease, and/or the turfgrass was not responding to actions taken by the turf manager to encourage recovery.

Compared to *C. zeae*, it was easier to visually observe *R. solani* colonization of SP tissue with the naked eye, in experiment 2, as readily visible brown “cob-webby” appearing mycelia was present in abundance on the turfgrass tissue. The *C. zeae* mycelium was not easily visible, even directly following removal from the 100% relative humidity environment. This may explain the difficulty golf course superintendents experience in accurately identifying leaf and sheath spot disease.

Salt concentrations applied up to or below 20,000 ppm did not prevent colonization by *C. zeae* or *R. solani* AG 2-2LP when SP was inoculated following saline water applications. Salt water applied post-infection could possibly enhance fungal suppression, but this was not tested. However, the negative effects that resulted from twice-daily saline applications for 3 sequential days on turf quality and turf damage of ‘SeaDwarf’ SP may counter any benefits in fungal pathogen suppression.

An important factor to consider in the environmentally controlled studies versus a field situation was that the saline water applications were made in high volumes resulting in a “flushing” of the root zone in each pot. Additionally the calcine clay that served as the rooting media is not a true reflection of a finer soil texture or higher cation exchange capacity of a native soil or constructed golf course green. Thus the accumulation of salts in the root zone or soil profile of SP from continued reliance on saline irrigation may be more detrimental to the turf at concentrations lower than were tested in this trial.

Table 4-1. Trial 1 - Interaction of *C. zeae* inoculation and saline water on 'SeaDwarf' SP

Treatment (salt as sodium chloride)	LSD (α = 0.05)	Results (trends)
inoculated	NS	No differences
salinity (10,000 ppm, 15,000 ppm & 20,000 ppm)	NS	No differences
inoculation X salinity	NS	No significant interaction

Table 4-2. Trial 2 - Interaction of *C. zeae* inoculation and saline water on 'SeaDwarf' SP

Treatment (salt as sodium chloride)	LSD (α = 0.05)	Results (trends)
inoculated	NS	No differences
salinity (10,000 ppm, 15,000 ppm & 20,000 ppm)	NS	No differences
inoculation X salinity	NS	No significant interaction

Table 4-3. Comparison of turf quality, turf damage and dry weight of 'SeaDwarf' SP with saline irrigation as affected by inoculation with *Rhizoctonia solani* AG 2-2LP

	Turf Quality[a]		Chlorosis/Necrosis (%)[b]		
Treatment[c]	7 d after inoc	14 d after inoc	7 d after inoc	14 d after inoc	Dry weight (grams)
0 ppm salt	5.8	6.7	11.1	7.4	11.4
0 ppm salt, inoc	6.2	5.8	5.6	12.8	9.8
10,000 ppm salt	3	4.8	40	24.2	10.8
10,000 ppm salt, inoc	3.7	5	20.2	22.7	10.9
15,000 ppm salt	3.5	4.5	38.7	26.3	10.3
15,000 ppm salt, inoc	4	4.7	26.3	20.2	10.7
20,000 ppm salt	3.8	3.7	46.2	40	10.7
20,000 ppm salt, inoc	2.8	3.5	61.3	46.2	10.3

[a] Turf Quality rated on 0 to 9 scale, 9 = dark, green, highest quality.

[b] Turf damage rated utilizing Horsfall-Barratt scale, data logit transformed to % chlorosis/necrosis.

[c] All treatments noted as inoc = inoculated with *C. zeae*, all other treatments mock-inoculated with sterile oat seed.

Table 4-4. Trial 1 - Interaction of *R. solani* AG 2-2LP inoculation and saline water on 'SeaDwarf' SP

Treatment (salt as sodium chloride)	LSD (α = 0.05)	Results (trends)
inoculated	NS	No differences
salinity (10,000 ppm, 15,000 ppm & 20,000 ppm)	NS	No differences
inoculation X salinity	NS	No significant interaction

Table 4-5. Trial 2 - Interaction of *R. solani* AG 2-2LP inoculation and saline water on 'SeaDwarf' SP

Treatment (salt as sodium chloride)	LSD (α = 0.05)	Results (trends)
inoculated	√	Low turf quality, high chlorosis/necrosis
salinity (10,000 ppm, 15,000 ppm & 20,000 ppm)	√	Decreasing turf quality, increasing chlorosis/necrosis with increasing salinity
inoculation X salinity	NS	No significant interaction

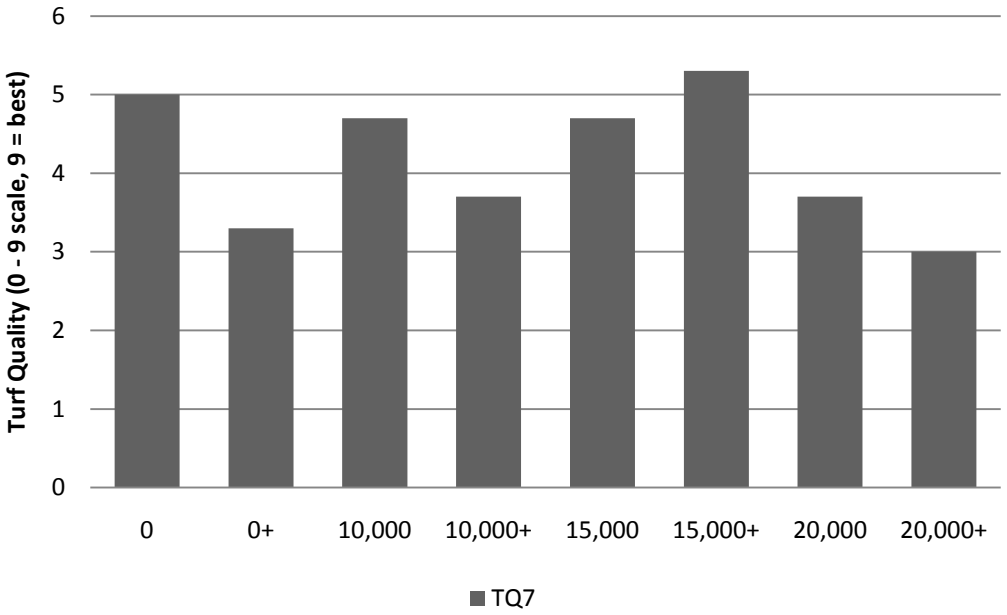

Figure 4-1. Turf Quality (TQ7) of 'SeaDwarf' SP 7 d after inoculation with (+) *C. zeae* coupled with saline water (0 to 20,000 ppm NaCl).

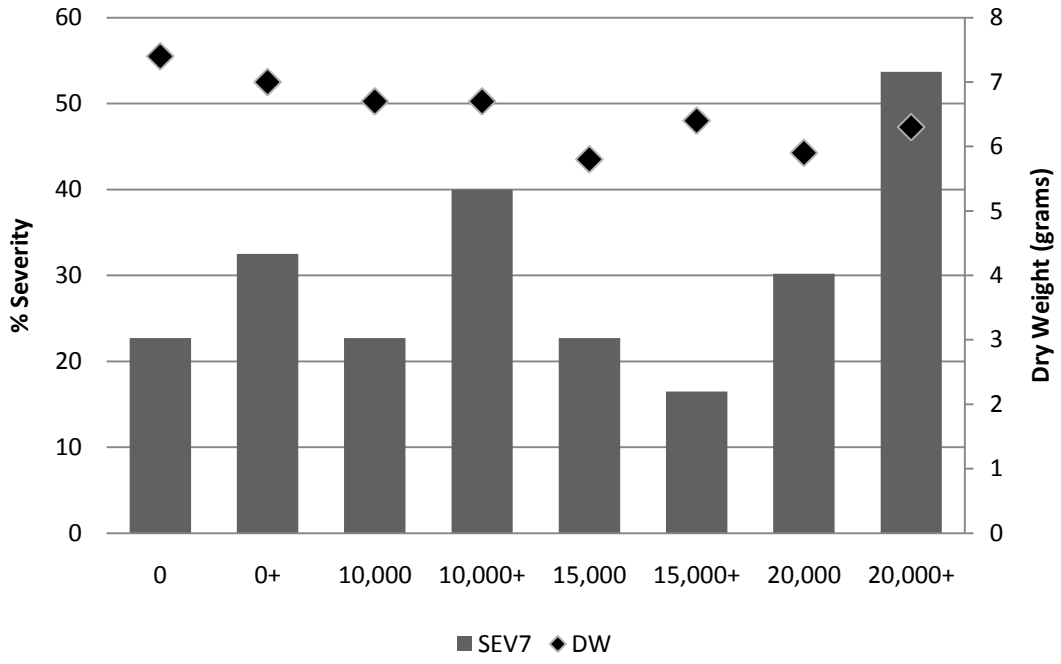

Figure 4-2. % Severity chlorosis/necrosis (SEV7) and dry weights (DW) of 'SeaDwarf' SP 7 d after inoculation with (+) *C. zeae* coupled with saline water (0 to 20,000 ppm NaCl).

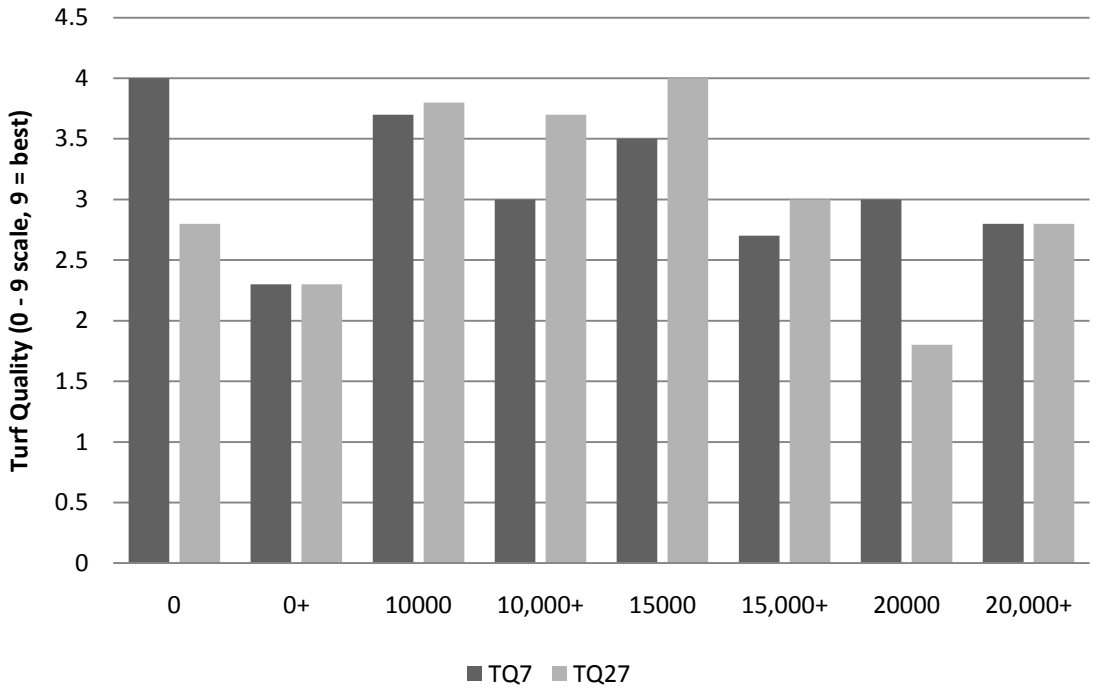

Figure 4-3. Turf Quality ratings 7 d (TQ7), 27 d (TQ27) of 'SeaDwarf' SP inoculated with (+) *C. zeae* coupled with saline water (0 to 20,000 ppm NaCl).

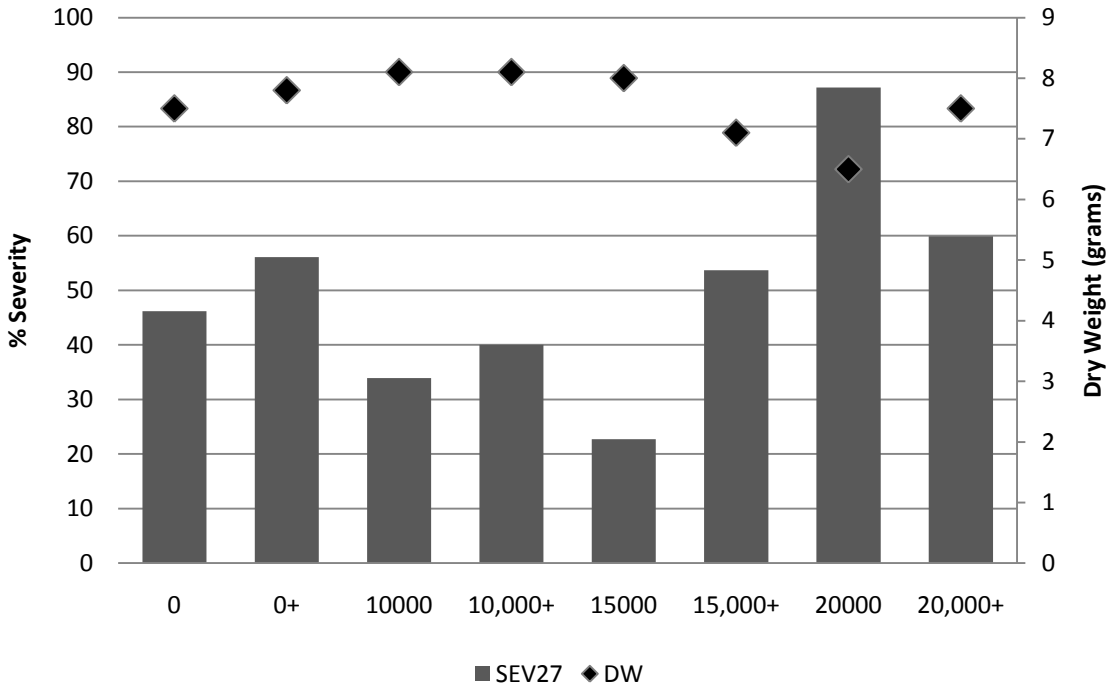

Figure 4-4. % Severity chlorosis/necrosis (SEV27) and dry weights (DW) of 'SeaDwarf' SP 27 d after inoculation with (+) *C. zeae* coupled with saline water (0 to 20,000 ppm NaCl).

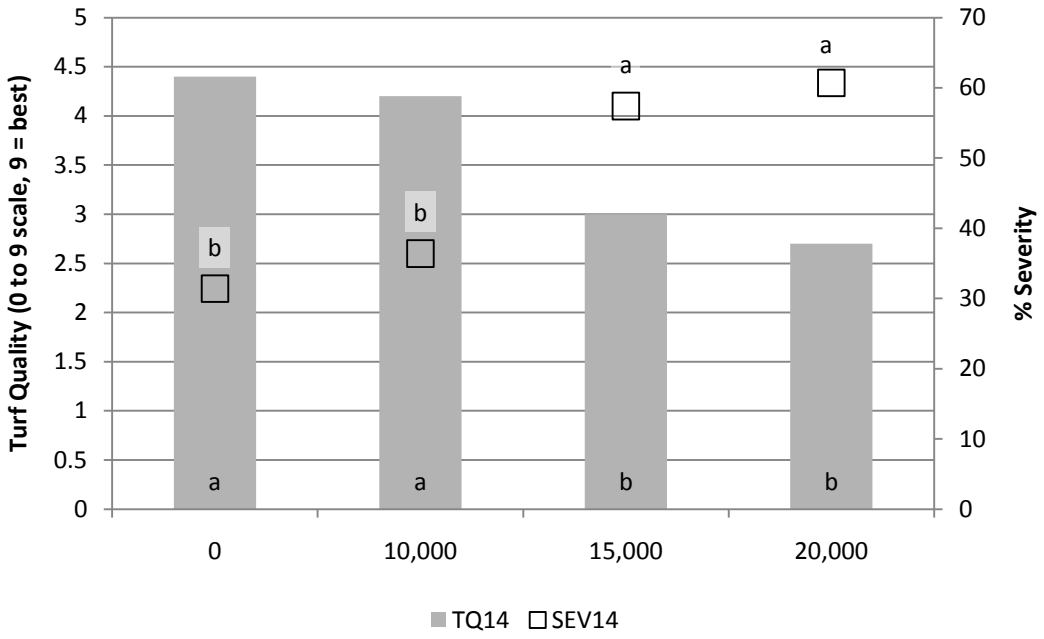

Figure 4-5. Turf quality (TQ) and % severity chlorosis/necrosis (SEV) ratings of 'SeaDwarf' SP 14 d after inoculations (+/- *R. solani)* coupled with saline water (0 to 20,000 ppm NaCl). Bars or boxes with same letter are statistically equivalent (t-tests using Fischer's LSD, α = 0.05).

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BIOGRAPHICAL SKETCH

Steven J. Kammerer was born to Richard and Dorothy Kammerer in Xenia, OH. Steve started working at the local orchard at the early age of 15 riding his bicycle 5+ miles back and forth from home after school and over the summer. Steve developed a love for being outside and working with plants.

Steve attended the Ohio State University with the intention of becoming an engineer at the urging of his father before changing majors to the field of Horticulture, a serious deviation from all the other occupations within his family. Prior to graduating in 1986 with a B.S. degree, Steve completed an internship conducting field agricultural pesticide research with Mobay Chemical Company in Howe, IN. Steve was accepted into the University of Georgia graduate school where he completed his M.S. degree in Plant Pathology working with peaches and nectarines under the guidance of Dr. Floyd Hendrix in 1989. Before graduation, Steve accepted and completed another internship with Fermenta Plant Protection as a field technical manager. After graduation, Steve accepted a full-time position with Fermenta. Steve married Rosemary in 1995, and they were blessed with two children, a boy and a girl, Christian and McKenna. From March 1989, Steve worked through multiple company changes to his current position with Syngenta Crop Protection as a field technical manager for turfgrass and aquatics.

Eighteen years after graduating from UGA, Steve resumed his education at UF in the pursuit of a doctoral degree in plant pathology as an aside to his full-time job with Syngenta. His degree requirements were completed in August 2011 with the approval and acceptance of this dissertation. He continues as a Syngenta employee currently seeking other positions of responsibility.