# MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF *Rhizoctonia* ISOLATES COLLECTED IN FLORIDA AND SCREENING OF ST. AUGUSTINEGRASS GERMPLASM FOR BROWN PATCH AND LARGE PATCH RESISTANCE

By

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To Isabella and Natalia, my two little plant pathologists, and the best things ever happened to my life, with all my love for you

To my parents, Carlos and Nelly, for their love, inconditional support and guidance throughout this process and in every day of my life

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To my family and all my friends

To my God

To that beautiful place of the world called Colombia

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Fungi in the genus *Rhizoctonia* make up a complex taxonomical group of plant pathogens. Multinucleate species such as *Rhizoctonia solani* (*Thanatephorus cucumeris*), *Rhizoctonia zeae*, *Rhizoctonia oryzae* (*Waitea circinata* var. *zeae* and var. oryzae), *Rhizoctonia circinata* (not defined anamorph) and binucleate species such as *Rhizoctonia cerealis* (*Ceratobasidium cerealis*) are known for causing major and minor diseases on cool and warm season turfgrasses. *Rhizoctonia solani* causes brown patch and large patch diseases. Brown patch is a disease of cool season grasses caused by the AG 2-2 III B during summer. Large patch on warm season grasses caused by AG 2-2 LP is the most economically important disease that affects turf quality and development in fall and spring seasons. The present study was focused on describing the isolates present on the University of Florida collection that are associated mainly with warm season grasses. The description was based on the most important morphological features of the fungus and supported by molecular data obtained from amplification of Internal Transcribed Spacer regions, a gene phylogenetically informative. Screening of twenty St. Augustinegrass genotypes also was performed in order to evaluate the possible effect that ploidy level of the plants and morphotypes had on disease response.

# CHAPTER 1
# LITERATURE REVIEW

## *Rhizoctonia spp*

Fungi in the genus *Rhizoctonia* make up a complex taxonomical group of plant pathogens. In the turfgrass literature, four anamorph species of *Rhizoctonia* are widely recognized: *Rhizoctonia solani*, *Rhizoctonia zeae, Rhizoctonia oryzae,* and *Rhizoctonia cerealis* (27, 101, 110, 111, 113). With the discovery and naming of the teleomorphs for these anamorphic fungi, recent taxonomic revisions and reorganizations are still ongoing and have yet to be widely accepted in some circles, including turfgrass pathologists. For this reason, nomenclature is seldom consistent and can be difficult to follow.

Members of this genus have been found to be plant pathogens of many crops. The most widely studied species is *Rhizoctonia solani,* which is the most pathogenic and polyphagous species worldwide. It also has been reported as a saprophyte and having mycorrhizal behavior on orchids (101). It causes diseases on more than 200 species of plants, including corn, cotton, forest trees, fruits, ornamentals, potato, rice, soybeans, turfgrasses and wheat and shows extensive variation in characteristics such as geographical location, morphology, host specificity, pathogenicity (38, 108) and types of disease symptoms (97). The extent of this variation has been considered as evidence that this fungus is a species complex (35, 38).

### Morphology

The species concept for *Rhizoctonia solani* enhanced by Ogoshi 1975 and currently used, stipulates that isolates of *R. solani* possess the following characteristics: a) branching near the distal septum of cells in young vegetative hyphae, b) constriction of hyphae and formation of septa a short distance from the point of origin of hyphal branches, c) presence of dolipore septa,

and d) absence of clamp connections with conidia, rhizomorphs and sclerotia differentiated into rind and medulla (101).

Isolates of *Rhizoctonia* spp. produce simple or branched chains of cells called moniliod cells. These cells may be hyaline or brown and vary in shape (lobate, pyriform, irregular or barrel-shaped). They arise as buds or blown-out ends of pre-existing cells. Chains of moniliod cells are formed on or above the surface of a host or a substrate, but also within host tissue. They may be few and scattered or form loose to semi-compact masses varying in sizes, or they may be aggregated to form sclerotia. Sclerotia are considered resting or dispersal structures. Color, shape, size and location on culture media are often features to identify species in a wide sense. Sclerotial color of *R. solani* ranges between different shades of brown. Those of *Rhizoctonia zeae* are reddish, small, regular and ball-shaped, while those of *R. oryzae* are salmon colored and irregular (101).

Isolates of *Rhizoctonia* spp. may sporulate when exposed to specific environmental conditions, but sporulation is not commonly observed in nature or the lab. Short hyphal cells branch frequently and produce dense interwoven mats on which basidia are formed. Basidiospores (4) are spherical, oval or-pyriform (101). The teleomorphs of the *Rhizoctonia* spp. include: *Ceratobasidium cerealis* (anamorph is *Rhizoctonia cerealis*), *Waitea circinata* (anamorphs include *R. zeae*, *R. oryzae* and *R. circinata*), and *Thanatephorus cucumeris* (anamorph is *R. solani*).

**Taxonomic Classification**

*Rhizoctonia* has been placed within division Amastigota, subdivision Basidiomycotina, class Basidiomycetes, order Tulasnellales, subclass: Holobasidiomycetidae III-Hymenomycetes III (2, 3, 101).

The classic taxonomical scheme divides species into groups based on nuclear condition: multi nucleate (MNR), bi-nucleate (BNR) and uni-nucleate (UNR) and has been essentially based on hyphal fusion (vegetative incompatibility) (98). Thus, species are either binucleate (*R. cerealis*) or multinucleate (*R. solani*, *R. oryzae*, and *R. zeae*). The number of nuclei in multinucleate species may vary from 3 to 28 in young cells (101).

The first helpful scheme to classify isolates was the anastomosis reaction, which groups isolates into different Anastomosis Groups (AG's). Basically, isolates that form in AG have the ability of fusing their hyphae in a perfect reaction, showing a high level of cell compatibility, during this process, an exchange of cytoplasmatic content and nuclei may occur. Authors cite Matsumoto et al., 1932 and/or Schultz ,H., 1937 as the pioneers of this scheme of classification. In the United States, Parmeter, J.R, et al., 1969 was the first researcher to apply this concept to *Thanatephorus cucumeris*. This reaction is still used to classify some isolates. In recent years, AG's were also genetically supported by the use of DNA-DNA hybridization (98, 99).

**Hyphal Anastomosis**

Four different types of anastomosis are described: C0 (no reaction= incompatibility), C1(contact fusion), C2 (killing reaction= somatic fusion) and C3 (perfect fusion). AG are determined when a C3 reaction is observed between a pair of isolates. Commonly, isolates that share an AG, also have similar morphology and pathogenicity profiles, as well as similar physiological, serological and ecological features or responses. However, recent studies also have shown considerable variability within and between AG's. Some have proposed they are different evolutionary units and even distinct species. Moreover, this reaction has been considered insufficient for accurate identification and classification of the isolates, because some isolates lose the capability to self-anastomose, some will not anastomose with all isolates of the same AG and some anastomose with isolates of more than one AG (99). Mechanisms and

biological relevance of this reaction are still not completely understood. Cubeta and Vilgalys (1997) suggested the presence of a heterothallic mating system that may also undergo recombination through heterokaryon-homokaryon-mating under artificial conditions. Interestingly, further studies have shown that AG's have levels of host specificity.

Currently, fourteen different AG's of *R. solani* are recognized (AG-1 to AG-13 and AG-BI) including isolates from many crops. Seven AG's (AG-1, AG-2, AG-3, AG-4, AG-6, AG-8 and AG-9) have been further divided into subgroups to reflect differences observed in frequency of anastomosis, fatty acid and isozyme patterns, pathogenicity, thiamine requirement and cultural appearance among isolates (36, 80, 108).

The term intraspecific group (ISG), introduced initially by Ogoshi (1987) was later defined in 1992 and 1993 by Liu and Sinclair, to describe the molecular variability present in AG 2. Therefore, AG 2-1, AG 2-2 IIIB (rush type), AG 2-2 IV (sugar beet root rot type), AG 2-2 LP (warm seasons turfgrasses), AG 2-E and AG 2-F were recognized also as AG 2 subsets. AG 2-1 was further been divided intoAG-2t and AG-Nt (47, 54, 59, 101).

**Epidemiology and Disease Cycle**

*Rhizoctonia* spp can survive unfavorable environmental conditions over seasons, on plant debris, thatch or in soil in the form of mycelia or sclerotia (58). Most *Rhizoctonia* diseases are initiated by mycelium and/or sclerotia; however, several important diseases of beans, sugar beet and tobacco result from basidiospore infection (77). Basidiospores also serve as a source for rapid and long distance dispersal of the fungus (77). When the right conditions are present, spores germinate producing hyphae that when come into contact with healthy host plant tissue produces a  structure known as an infection cushion (6, 77). Lobate appresoria may be formed to penetrate after which the fungus produces penetration hyphae. New mycelia and sclerotia can continue the infection process in healthy tissue or rest until more tissue is available, or until

conducive environmental conditions return. The pathogen also can enter into the plant through wounds and stomata, and even colonize dead tissue (2, 58, 67, 77).

**Genetics of Infection**

Chemical stimulants released by actively growing plant cells and/or decomposing plant residues attract the fungus, which initiates the infection process by the production of many different extracellular enzymes that degrade cellulose, cutin, pectin and others components of plant cells walls. It has been hypothesized that each AG has a distinct process of infection. H factor, consists of a single gene with multiple alleles and may be is the outbreeding mechanism that AG 1 and AG 4 share (6). On susceptible sugar beet cultivars, the infection progressed to the periderm or outer secondary cortex in roots, while in resistant cultivars, the fungus is only able to produce hyphae and infection cushions with penetration pegs (6, 77).

Certain preexisting structural plant defenses, like the amount and quality of wax and cuticle that cover the epidermal cells, the structure of epidermal cell walls, the size, location, and shapes of stomata and lenticels, as well as the presence of thick-walled cells (2), may have a role in resistance to *Rhizoctonia spp.*

**Population Biology and Genetic Diversity**

Several molecular techniques have been used to accurately identify isolates of *Rhizoctonia* spp. and to investigate the genetic variation within and between the AG's and to support the identification of additional subgroups. The various molecular methods used include: isozyme analysis, total cellular fatty acid analysis, electrophoretic karyotyping, DNA-DNA hybridization, DNA fingerprinting based on random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), repetitive probe, AT-rich DNA restriction fragment length polymorphism (RFLP), single-copy nuclear RFLP, rDNA sequence analysis, the use of microsatellite, inter simple sequence repeats (ISSR) and the use of universal rice primers

(URPs). Unique zymogram-pattern groups are also a good system to identify MNR AGs and subgroups within each AG (97, 98). However, each of these methods have advantages and disadvantages when studying *Rhizoctonia* populations.

Hyphal anastomosis reaction can sometimes be subjective and difficult to interpretate. Sequence analysis of ribosomal DNA genes and ITS sequences has become useful, objective, and informative technique to make phylogenetics studies in this fungus.

**rDNA Sequence Analysis Using Internal Transcribed Spacer (ITS) Sequences**

In most fungi, three rRNA genes (28, 5.8 and 18 s rDNA) are present as repeated units separated by nontranscribed spacers with different evolutionary rates (98). The 5.8s rDNA gene is flanked by the internal transcribed spacer regions (ITS1 and ITS2). These two regions have been very useful for evaluating phylogenetic and taxonomic relationships (15). ITS regions have been particularly useful for identifying genetic groups at the species level because of their higher rate of molecular evolution and proximity to highly conserved rDNA regions (35).

Among the various molecular classification methods used for *Rhizoctonia* spp., the rDNA-ITS sequence analysis seems to offer the most accurate way for establishing taxonomic and phylogenetic relationships behind previously identified AG's (53, 98).

**St. Augustinegrass**

St. Augustinegrass, *Stenotaphrum secundatum* (Walter) Kuntze , is a stoloniferous and perennial species that belongs to the Class: Monocotyledons, Subclass: Commelinidae, Order: Cyperales and Family: Poaceae. A dense turf is formed when regularly mowed or grazed. It is propagated vegetatively using well-rooted sprigs, broadcasting stolons, plugs and sod (23, 70). Efforts to develop seeded cultivars have not been successful (23). It is native to some countries in Africa, Central America, Mexico, North America, South America and the Caribbean (23).

St. Augustinegrass grows on infertile to moderately fertile soils as well as a wide range of well or poorly drained soils, from sandy loams to light clays. This grass has good soil salinity tolerance, but moderate drought and shade tolerance. It can tolerate extreme shade and responds well to N fertilization (70). The species is commonly found in the humid tropics (rainfall from 1,000 – 2000 mm/yr) and subtropics and grows best between about $20^0$C and $30^0$C (23).

**Uses and Economic Importance**

St. Augustinegrass mostly occurs in natural swards or is planted as turfgrass and plays a role as a pasture or as a soil conservation groundcover under trees or near the sea where salt spray may damage other grasses. St. Augustinegrass is commonly used in homelawns, parks, roads, and recreation sports fields. In addition, Cook, B.G and his colleagues (2005) reported its palatability for small and large ruminants. Yields on the order of 5 t/ha/yr of dry matter have been reported when used in animal production, in humid-tropical locations (23).

**History of Breeding and Population Improvement**

Commercial and organized breeding development of St. Augustinegrass has been limited because it is primarily a clonal grass, not used on golf courses, which limits the potential market (21). Breeding efforts by public scientists have involved discovery of clonal types such as ‘Floratine’ and ‘Raleigh’, and the discovery of seedlings of partially unknown pedigree, for example, ‘Floratam’ and ‘Floralawn’. Before the UF breeding program was assigned to Dr. Russell Nagata (UF-Belle-Glade), only large-scale population improvement was done (from 1977-1996) (21). Since that time, two varieties, ‘Captiva’ and ‘FloraVerde’ have been released, displaying slow leaf extension and superior shade tolerance (75, 76).

**Genetics, Breeding and Selection Techniques**

*Stenotaphrum secundatum* evolved as a fertile diploid from *S. dimidiatum*. There are two distinct variants; a sterile, triploid originating at the Cape of Good Hope that produces little seed

and a fertile diploid variant emanating from the Natal region of South Africa. Mutation in the triploids is common, hence considerable diversity. Tetraploid types are completely sterile (23, 70). Most cultivars are diploids (2n = 18) and are subdivided into the Breviflorus and the Longicaudatus Races. Plants with elongated leaves and stolons are classified into the Longicaudatus category, also named “long-stemmed’ (13), and those with dwarf or short habit growth (narrow and short leaf blades and highly branching pattern) belong to the Breviflorus category, also named “short flowered” (13, 21). Polyploids identified in 1961, came from sterile triploids (2n=27) with irregular meiosis (21). Adaptive and morphological variations are associated with chromosome differences; diploids cultivars have narrower, thinner, more translucent, brighter green leaf blades while polyploids have coarser, thicker leaf blades which are more opaque and darker green color (13).

St. Augustinegrass is easy to hybridize artificially (13). Seed is set and easily produced within ploidy levels; however, crosses between different ploidy levels have not been successful (Busey 1982,Cook 2005). High rates of somatic mutations with adequate survival can be induced in sprigs using gamma rays (3000-4500 rads). The biggest challenge in breeding this crop is its perennial condition and long-term field evaluation (12, 23).

**Varietal Resistance**

Host plant resistance has potential for managing diseases caused by *R. solani*, but there are few reports on St. Augustinegrass genotypes with regard to their resistance to brown patch and large patch diseases. Host resistance could help reduce the cost of producing and maintaining lawns as well as provide a more environmentally friendly method of controlling the disease (58).

Use of host-plant resistance has been complicated by discrepancies between laboratory disease ratings and greenhouses observations for St. Augustinegrass selections when challenged inoculated by *R. solani* (46).

Liddell, D. E., et al, 2001, indicated that no resistance response in 15 St. Augustinegrass lines tested. However, Captiva and Texas Common seemed consistently susceptible, while Rosalawn and Floralawn seemed more resistant to BP.

## Brown and Large Patch Diseases

*Rhizoctonia* diseases of turf grasses were first recorded in the United States more than 80 years ago (Piper and Coe 1919). There are two *Rhizoctonia* related diseases of major importance: brown patch and large patch. In general, brown patch is caused by isolates of AG 2, subgroup 2, strain IIIB (AG 2-2 IIIB) on cool season grasses. Large patch disease (LP) is caused by isolates of AG 2, subgroup 2, strain Large Patch (AG 2-2 LP) on warm season grasses. Awareness of the distinctions between the two diseases is important because there are major differences in their occurrence and in effective means of control (7, 16, 50). Isolates of AG-1 have also been found causing diseases as well on cool-season turfgrasses (100). St. Augustinegrass, zoysiagrass and centipedegrass are most susceptible to this disease; common and hybrid bermudagrasses are rarely damaged (41).

### Brown Patch

Brown patch is mainly a foliar disease that blights the blades of all cool-season turfgrass species (50, 55, 100). The disease most often occurs in summer, during hot humid weather when surface moisture and humidity are high and night temperatures are above 16-20$^0$C (7, 55, 100, 119). Infection is triggered by rainfall, excessive irrigation, or extended periods of leaf wetnesss on the leaf canopy (48 hours or more) (30). BP may start in any region of a lawn, but initial foci are located away from lawn borders (58). Damaged turf usually recovers when conditions no longer favor the spread of the disease (41). If light infections of BP occur, the affected turf generally recovers in 2 or 3 weeks. The disease may be spread longer distances by mycelium clinging to wet lawn mower wheels (58). The type IIIB strain also affects warm-season grasses

like bahiagrass, bermudagrass, centipedegrass, St. Augustinegrass and zoysiagrass and is also known to cause sheath blight of rice, damping-off and crown and root rot on a variety of other hosts (7, 41).

**Large Patch**

*Rhizoctonia* large patch (LP) is the most significant infectious disease of zoysiagrass species (*Zoysia japonica*). LP can appear as very large patches, sometimes exceeding 6 to 8 feet (1.8-2.4 meters) in diameter, with a yellow margin (50). Ideal environmental conditions for infection exist mostly from fall to spring in the United States when temperatures range from 77 F ($25^0$ C) to 83 F ($28^0$ C) within the turfgrass canopy (50). Isolates from AG 2-2 LP can be recovered from sheath tissues, thatch, and rhizosphere soil, regardless of whether the disease occurred or not and from the crowns of host plants, where it overwinters (7).

**Horticultural Management Practices for Large Patch and Brown Patch Diseases**

**General Recommendations**

Management recommendations included reducing thatch, limiting dew duration, and improving drainage, mowing, fertilization and irrigation practices. Fertility may also play a role. Lowering Nitrogen fertilizer and the use of a slow-release Nitrogen sources as well as maintaining Phosphorus and Potassium fertility levels according to soils test recommendations are helpful practices to reduce the risk of these diseases. (30, 41, 58).

Periodic mechanical dethatching or core aerification of turf is helpful in preventing thatch buildup that may harbor the fungus when conditions are conducive to disease (41, 58). Since moisture plays such an important role in disease development, irrigation practices that minimize foliar wetness and promote good drainage are also recommended (41).

**Control for Brown Patch**

Applications of fungicides are recommended once the first symptoms appear and weather conditions favor the spread of disease. Fungicidal sprays should continue until the turf starts to recover and/or until weather conditions no longer favor disease development (41). Fungicides containing myclobutanil, propiconazole, thiophanate-methyl, and triadimefon are recommended for disease control (30). *Azoxystrobin*, flutolanil, Iprodione, mancozeb, pyrasclostrobin, thiophanate-methyl, trifloxystrobin and vinclozolin are effective as preventive applications for management of brown patch (30).

**Control for Large Patch**

Prevention is the best management strategy to control the disease; but when fungicides sprays are needed. Strobilurins and Flutolanil provide exceptional control; however, timing of applying fungicides is critical for optimum levels of disease control (85).

# CHAPTER 2
# SCREENING ST. AUGUSTINEGRASS GERMPLASM FOR BROWN PATCH AND LARGE PATCH RESISTANCE

## Introduction

St. Augustinegrass (*Stenotaphrum secundatum* Walt. Kuntze) is the most popular lawn grass in Florida. The popularity of St. Augustinegrass (SAG) is due to its many favorable characteristics. Its has good performance on infertile soils, in soils with poor drainage and/or with high salinity conditions, it is moderately drought and shade tolerant and requires low conditions of maintaince. Approximately 1.5 million acres of SAG are managed in Florida's commercial and residential landscapes (120).

Large patch (LP) disease caused by the fungus *Rhizoctonia solani* anastomosis group (AG) 2-2 LP is the most important fungal disease of SAG. Brown patch (BP) is an important disease that primarily affects cool season grasses such as creeping bentgrass and is caused by the same anastamosis group AG 2-2, but different strain III B (AG 2-2 IIIB). Brown patch occasionally affects SAG and other warm season grasses but is rarely damaging enough to be of management concern. However, given favorable conditions for disease, LP and BP can both result in major turfgrass losses.

BP and LP typically occur at different times of the year, produce distinct symptoms, require very different control strategies, and primarily affect different turfgrasses species (115). The diseases can be managed through adjustments in cultural practices, fungicides sprays, or a combination of both. The use of resistant cultivars may reduce the reliance on pesticides and would be more environmentally friendly, although no commercially available cultivars are completely resistant to these diseases.

There are few reports addressing the resistance of St.Augustinegrass genotypes to LP. Assessments of resistance to BP in tall fescue (*Festuca arundinaceae*) have been conducted

using area under disease progress curves (AUDPC) to show differences in susceptibility between cultivars (39). Observations of turfgrass managers and researchers suggest that differences in susceptibility of SAG cultivars exist in the wide variety of cultivars that were principally developed for improved turf quality. Preliminary studies conducted by Datnoff, et al (2005) suggested a possible ploidy effect on LP resistance (L. Datnoff, personal communication). Ploidy level varies between SAG cultivars. Most cultivars are diploid (2X=18), but some are triploid (3X=27) and polyploid (3X=32) as well (12, 14). Ploidy level has been shown to play an important role in disease resistance in other crops, including red clover (*Trifolium pretense* L.) hybrids, *Musa* spp, and bentgrass (*Agrostis* spp) (22, 24, 81). The objective of this study was to evaluate 20 different cultivars of St. Augustinegrass with various ploidy levels and morphological characteristics for their reaction to isolates of *R. solani* that cause BP and LP using three quantitative measurements of disease (final severity, AUDPC, and intrinsic rate of infection development).

## Materials and Methods

### Plant Material

Ploidy level, morphotype (semi dwarf-dwarf or long and genotypes names of St. Augustinegrass selections used for this research are given in table 2.1. Forty-three-day-old plants were received in 3’’growing trays (Growing, Systems, Inc, WS, USA) from Dr. Russell Nagata,UF Everglades Research and Education Center (EREC). Plants were repoted into 4’’diameter plastic pots (KORD products, Toronto, Ontario, Canada) using approximately 92 g of Professional 4P potting mix (Fafard company, Agawam, MA, USA) and grown in the greenhouse for approximately 80 d or until plants began to fill the pots. Temperatures in the air-conditioned greenhouse ranged between 23 and 28°C. Plants were watered by drip irrigation, and pots sat in trays that held water. Watering was done twice a week or as needed. Plants were

fertilized every two weeks or as needed with 20 ml of 2.63 g Miracle Grow (The Scott's® company, LLC, Marysville, Ohio, USA) per liter of distilled water.

**Fungal Isolates**

Isolates UF0465 and UF0714 of *Rhizoctonia solani* were used in all inoculation experiments. The isolates were maintained in oat seed storage culture at 4°C. Colonies were transferred on oat seed from storage onto potato dextrose agar (PDA, Difco) plates. Isolate UF0465 was isolated from St. Augustinegrass exhibiting brown patch symptoms near Belle Glade, FL. The time of collection and anastomosis group of this isolate were unknown; however, the isolate was negative for the AG 2-2 LP PCR test (111). Isolate UF0714 was isolated in late April of 2007 from 'Palmetto' St. Augustinegrass exhibiting large patch symptoms on a sod farm in Belle Glade, FL. The isolate was confirmed as *R. solani* anastomosis group 2-2 LP with specific PCR primers (111).

**Inoculation Experiments**

Genotypes of SAG were inoculated with 5 mm-diameter mycelial plugs from three-day-old colonies of the aforementioned isolates. Agar plugs of sterile PDA were used to inoculate control plants. Agar plugs were placed on the leaf sheath of the terminal shoot and were secured to the plant with strips of parafilm "M" (Pechiney plastic packaging, Illinois, USA).

Inoculated plants were enclosed in 1 gallon sealable plastic bags (Glad company, USA) with one saturated wet paper towel placed at the bottom of the bag. Inoculated plants were placed in an incubator or controlled-climate growth room at 23 to 26°C. Photoperiod was maintained between 12 h light and 12 h dark. Levels of artificial lighting ranged between 32.63 – 49.0 $\mu mol/m^{-2}/s^{-1}$ (Li-Cor, Quatum/Radiometer/Photometer, model LI-250, light meter, USA). Relative humidity inside the bags was estimated to be near 100 % based on visible high condensation.

The experimental design was completely randomized. Each replication contained one sprig per pot. The number of replications varied among isolates, due to the number of available plants. Three (BP) and two (LP) experiments were conducted. Four (BP) and three (LP) replications of SAG were used for a total of 240 and 114 inoculated plants, respectively. Ratings of disease severity were recorded by visually estimating the percent disease daily until day six after the inoculation, when some cultivars were highly infected bu the disease.

**Statistical Analysis**

The logistic disease model was used to characterize and compare epidemics for each genotype. Intrinsic rate of infection "r", area under disease progress curve "AUDPC", and maximum disease severity "Y" were calculated. The differential equation is: $dy / dt = r_L y (1 - y)$, in which $r_L$ is the rate parameter (apparent infection rate) and y is disease severity. AUDPC was calculated according to:

$$AUDPC = \Sigma_{i=1}^{n-1} = \left( y_i + y_{i+1} / 2 \right) \left( t_{i+1} - t_I \right) (64).$$

Where i is the order index for the times, n is the number of times, y is the disease severity, and t is time. Maximum severity (Y) was the mean final disease rating of the total number of replications of each cultivar. Calculations were made using Statistical Analysis System (SAS Institute, Inc., Cary, NC).

Univariate procedure in SAS was used to test the equality of variance and the normality of the residuals of each variable by isolate. Residuals were normal; therefore, data of the experiments were pooled and analyzed together by isolate. Differences in cultivar means were obtained using the Mixed procedure in SAS with a random effect and least square means *t* test with Tukey- Kramer adjustment. Contrast statement was used to observe the effect of ploidy level and morphotype on each disease variable. Means were compared using the least significant

difference as appropriate. For all the comparisons, a significance level of $P \leq 0.05$ was used unless otherwise stated. SAS codes are included in Appendix A and B.

## Results

### Brown Patch Susceptibility

The inoculation method was successful in inducing disease. One day after inoculation, the fungus started to produce abundant aerial mycelia on most of the genotypes. All genotypes showed typical symptoms of the disease following inoculation. The first symptom observed was water soaking followed by brown irregular lesions. By day 2 (48 h), differences in percent severity ranging from 2.25 vs 14.7% (low to high) were observed among genotypes (data not shown). As the disease progressed, new lesions appeared and spread over the plant (Figs. 2.1 and Fig. 2.2).

All genotypes showed dark brown or black lesions on stems (canker-like lesions), especially below the internodes. Perceivable differences were observed in how fast disease spread and the amount of disease that occurred between genotypes. Sclerotia were produced on many, but not all genotypes. All the control plants remained free of BP disease. Normal growth and presence of new leaves were observed on them.

Mean AUDPC, apparent infection rate, and final severity by genotype for the combined BP experiments are given in Table 2.2. Statistically significant differences between genotypes are indicated in Table 2.3, 2.4, and 2.5 for final severity, apparent infection rate, and AUDPC, respectively.

### Final disease severity

Palmetto (94%), FX-313 (88%), Floratine (80%), FX-10, Delmar and FloraVerde (79%) and Classic (76%) had the highest final disease severity. FHSA-115 and Raleigh had the lowest final disease severity values.

**Apparent infection rate**

By the end of the experiment (6 days after inoculation), there were statistically significant differences between genotypes. Palmetto (1.55) and FX-10 (1.51) had the highest rate of infection. Delmar and FloraVerde (1.3), FX-313 and Classic (1.2) and Floratine (1.17) also exhibited high rates of infection. FHSA 115 (0.83), Mercedes (0.73) and Raleigh (0.68) had the lowest calculated rates of infection.

**Area under disease progress curve**

Palmetto (290), FX-313 (280), Floratine (230), Jade (220) and FX-10 (210) showed the highest values of AUDPC. Floratam (110), FHSA-115 (99), and Raleigh (86) had the lowest values of AUDPC.

**Effect of ploidy level and morphotypes on disease response variables**

Ploidy level did not have any significant effect on disease response variables. Means of diploid and triploid cultivars were not statistically different (Table 2.6). Morphotype did have a significant effect on all disease response variables ($P < 0.05$). Dwarf cultivars had statistically higher overall disease severity values on average than long-bladed cultivars.

**Large Patch Susceptibility**

Aerial mycelia were produced on the plants after 5 days. The first symptom observed was water-soaked lesions on leaves that became brown and irregular with time. By day 8, differences in severity percent ranging from 2.5 to 26 % (low to high), were observed among genotypes (data not shown). In advanced stages of infection (between 11 and 15 days), yellowing became a symptom evaluated. Production of new healthy tissue (recovery) was observed on Captiva, FX-313, FX-33, FX-10, Palmetto, Floratam, FH SA-115, Classic, Winchester, and Jade during the experiments. Sclerotia production was not observed in any of the cultivars. All control plants

remained free of LP symptoms by the end of the experiment. Normal growth and development of new leaves were observed on them.

Mean AUDPC, apparent infection rate, and final severity by genotype for the combined LP experiments are given in Table 2.7. Statistically significant differences between genotypes are indicated in Tables 2.8, 2.9, and 2.10 for final severity, apparent infection rate, and AUDPC, respectively.

**Final disease severity**

FX-10 (72%), Raleigh (59%) and FloraVerde (59%) had the highest final disease severity on average. Winchester (20%), T. Common (19%), Mercedes (16%) and Sunclipse (8,6%) had the lowest values.

**Apparent infection rate**

There were statistically significant differences between genotypes. FX-10 (0.63), FX-33 (0.37), Flora Verde and Jade (0.35) and Raleigh (0.32) showed the highest rate of infection. T. Common, Sunclipse (0.17) and Winchester (0.13) had the lowest rates. Palmetto, Delmar, Classic, Bitterblue, Floratine, Mercedes, Seville, and Floralawn had similar rates of disease progress.

**Area under disease progress curve**

Raleigh (310), FX-10 (280), FloraVerde (260), Jade (210) and FX-313 (200) had the highest AUDPC values while T. Common and Floratine (99), Mercedes (97) and Sunclipse (52) had the lowest values. The rest of the genotypes showed intermediate values between these two groups.

**Effect of ploidy level and morphotype on disease response variables**

Ploidy level had no significant effect on measured disease variables. Means of diploid and triploid genotypes were not statistically different (Table 2.11). Morphotype had a significant

effect on final severity ($P < 0.0110$). Dwarf genotypes had higher maximum disease severity values than long-bladed cultivars.

## Discussion

Results of this study indicated that some genotypes could be differentiated for delay in severity as early as day 2 for BP and at day 8 for LP. The optimal time to evaluate genotypes for their resistance or susceptibility appears to be 5 to 6 days after the inoculation for BP and 14 days after the inoculation for LP. At this particular point for these two diseases, susceptible materials are highly infected, especially for the BP isolate. Ratings too early or too late could lead to inappropriate assumptions. In these experiments, AUDPC was the most informative variable calculated for the genotypes. AUDPC gave the best means separation between genotypes for both diseases. However, values of rate parameter and maximum severity were useful to compare disease susceptibility of the different genotypes.

### Screening for Brown Patch Disease

Palmetto, FX-313, Floratine, Jade and FX-10 were considered highly susceptible genotypes based on AUDPC, followed by Delmar, FloraVerde and Classic. Captiva, Mercedes and Floratam, but principally, FHSA-115 and Raleigh were considered genotypes with possible resistance.The delayed response in severity in Raleigh may corroborate the presence of some resistance. Overall, Raleigh was considered as the most resistant genotypes. This finding contradicts previous reports that Raleigh is quite susceptible to large patch under greenhouse conditions and suggests cultivar response may differ between large patch and brown patch isolates (46). Further studies need to be addressed to elucidate the possible components of tolerance in this cultivar, if present.

Sclerotia production was observed on 10 genotypes. It was most noticeable in FX-10 (3 sclerotia), Sunclipse (4), and FX-33 (4). It is unclear if susceptibility of the cultivar played a role

or if the absence of healthy plant material triggered production of sclerotia. Few studies have reported the production of sclerotia by *Rhizoctonia solani* on turfgrass. This may be further evidence that the brown patch disease and isolates that cause it are distinct from large patch isolates previously studied.

**Effect of ploidy level and morphotype on disease response variables:** There was no effect of ploidy level and the response of genotypes. Similar reactions were observed on the triploid and diploid genotypes. Morphotypes had an effect on AUDPC, final severity, but mainly on rate parameter; dwarf genotypes developed disease much faster than long ones. The colonization process might have been faster because of the small, compacted leaves and the short internodes of the dwarf genotypes. Additional work is needed to elucidate these differences and in the underlying causes.

**Screening for Large Patch Disease**

Raleigh, FX-10, FloraVerde, Jade and FX-313 were considered highly susceptible genotypes, followed by Captiva and FX-33, whereas T. Common, Floratine, Mercedes and mainly Sunclipse were considered to be possibly resistant.

Plant growth was observed on 13 genotypes after inoculation with the fungus. The concept of crop recovery can be an estimate of post epidemic vegetative regrowth of foliage or other plant parts, and it can be used to evaluate genotypes. Selection of genotyes that show enhanced crop recovery may reveal a tolerance to *R. solani* that would be difficult to detect in a crop such as turfgrass where biomass reduction is not considered an important result of disease (16).

**Effect of ploidy level and morphotype on disease response variables:** Host ploidy level did not significantly affect AUDPC, maximum severity, or rate parameter. Morphotypes had an effect only on final severity. Long-bladed plants had lower disease severity values than dwarf

genotypes. Wider and longer leaves, and longer internodes may confer structural defense to the fungus.

**Comparison of St. Augustinegrass Reaction for Brown Patch and Large Patch Isolates**

Flentje, N. T., 1957 suggested that there are several stages during infection by *R. solani* when the process could be affected. These stages are: failure of the hyphae to attach to the plant surface; failure to form infection structures; failure of penetration pegs to penetrate and failure of penetrating hyphae. Infection, colonization and even sclerotia production of the fungus were observed on most of the cultivars in these trials. Therefore, the term “tolerance” is the most adequate for those materials with less or reduced damage. However, results of this study indicated that some components of resistance may be present on long-bladed cultivars. Infection on these genotypes occurred at lower proportions, especially on early stages of infection (48 h). Constitutive factors such as cuticle tickness, may have had an effect on conferring resistance to *Rhizoctonia* on the genotypes evaluated. Morphological features such as leaf width and length of tall fescue cultivars, reported by Green II. et al, 1999 could have an effect on delaying disease response and reducing percent severity on those SAG genotypes less affected by the isolate.

The *Rhizoctonia*-SAG pathosystem is likely governed by partial or horizontal resistance, since varying different levels of susceptibility were observed on the genotypes. No material has been reported as immune, suggesting the absence of dominant resistance genes. This suggests the presence of many genes to confer effective resistance against these diseases. However, not all commercial SAG cultivars were included in this study. Future screening projects should include additional cultivars and breeding lines to corroborate the polygenic nature of the resistance. Additional research is needed to elucidate these mechanisms and to corroborate responses obtained in controlled environment experiments with those observed in the field.

One of the objectives of this research was to develop a screening protocol for St. Augustinegrass that could evaluate differences in susceptibility between cultivars to diseases caused by *R. solani.* Methods outlined did result in good and discernable amounts of disease with both isolates. Results corroborated the efficacy of the methodology proposed by Datnoff et al 2005. The data from experiments with different isolates were not combined, because a significant interaction effect between isolate and cultivar was noted. Although LP and BP are caused by the same species of fungus, there were differences in the response of cultivars to the two isolates. This was unexpected but suggests that screening efforts should rely on an isolate that causes the most economically important disease for the particular turfgrass species. Discerning the differences in cultivar response to the different isolates was not the objective of this research, and experiments were not designed to elucidate those differences. Future studies should take into account that these results suggest that screening efforts with an aggressive isolate that causes a relatively unimportant disease (brown patch) may not produce usable and valuable selection for resistance to even closely related diseases (large patch). Finally, for future and established disease screening projects, it would be desirable to have additional information about the effect of plant age, pathogenicity of different isolates and effect of different environmental conditions to understand better this *Rhizoctonia solani* pathosystem and to define the components associated with disease response against this pathogen.

Table 2-1. Morphotypes and ploidy level of St. Augustinegrass germplasm.

| GENOTYPE | MORPHOTYPE | Ploidy level *** |
|---|---|---|
| Captiva | Dwarf- semi dwarf * | 2 X |
| Classic | Dwarf- semi dwarf | 2 X |
| Delmar | Dwarf- semi dwarf | 2 X |
| FloraVerde | Dwarf- semi dwarf | 2 X |
| FX-313 | Dwarf- semi dwarf | 2 X |
| Jade | Dwarf- semi dwarf | 2 X |
| Seville | Dwarf- semi dwarf | 2 X |
| Texas Common | Dwarf- semi dwarf | 2 X |
| FX-10 | Dwarf- semi dwarf | 3 X |
| Mercedes | Long ** | 2 X |
| Palmetto | Long | 2 X |
| Raleigh | Long | 2 X |
| Sunclipse | Long | 2 X |
| Winchester | Long | 2 X |
| Bitterblue | Long | 3 X |
| FH SA-115 | Long | 3 X |
| Floralawn | Long | 3 X |
| Floratam | Long | 3 X |
| Floratine | Long | 3 X |
| FX-33 | Long | 3 X |

* Dwarf-semi dwarf genotypes: plants with short habit growth, narrow and short leaf blades.

** Long :plants with elongated and wider leaves and stolons.

*** Diploid (2X)= 9 chromosomes/nuclei. Triploid (3X)=27 chromosomes/nuclei.

Table 2-2. AUDPC, apparent infection rate, and final severity of St. Augustinegrass genotypes inoculated with the brown patch isolate. Means represent combined data from three experiments.

| Cultivar | AUDPC * | Apparent infection rate (units/day) | Final severity (%) |
|---|---|---|---|
| Palmetto | 290 | 1,55 | 94 |
| FX-313 | 280 | 1,2 | 88 |
| Floratine | 230 | 1,17 | 80 |
| Jade | 220 | 0,91 | 70 |
| FX-10 | 210 | 1,51 | 79 |
| Sunclipse | 180 | 0,98 | 72 |
| Delmar | 180 | 1,3 | 79 |
| Winchester | 180 | 1,13 | 71 |
| FloraVerde | 180 | 1,3 | 79 |
| Classic | 170 | 1,2 | 76 |
| FX-33 | 160 | 1 | 66 |
| Bitterblue | 150 | 0,91 | 57 |
| T.Common | 140 | 1,06 | 63 |
| Seville | 140 | 1,02 | 58 |
| Floralawn | 130 | 0,98 | 55 |
| Captiva | 120 | 1,15 | 60 |
| Mercedes | 120 | 0,73 | 50 |
| Floratam | 110 | 0,88 | 50 |
| FHSA-115 | 99 | 0,83 | 46 |
| Raleigh | 86 | 0,68 | 35 |

Means obtained using Proc Mixed code with random effect in SAS. Means were significantly different at $P \leq 0.05$ according to pairwise t-test with Tukey adjustment.
* AUDPC: Area Under Disease Progress Curve

Table 2-3. T-test results of comparisons between final disease severity of St. Augustinegrass genoytpes inoculated with the brown patch isolate of *R. solani*.

| | Sunclipse | Mercedes | Floralawn | Bitterblue | Captiva | FX-313 | FX-33 | FX-10 | Palmetto | Raleigh | Floratam | Floratine | T. Common | FHSA-115 | Classic | Winchester | Seville | Jade | Delmar | FloraVerde |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sunclipse | | | | | | | | | | * | | | | | | | | | | |
| Mercedes | | | | | | * | | | * | | | | | | | | | | | |
| Floralawn | | | | | | * | | | * | | | | | | | | | | | |
| Bitterblue | | | | | | | | | * | | | | | | | | | | | |
| Captiva | | | | | | | | | * | | | | | | | | | | | |
| FX-313 | | | | | | | | | | | * | | | * | | | | | | |
| FX-33 | | | | | | | | | | | | | | | | | | | | |
| FX-10 | | | | | | | | | | * | | | | | | | | | | |
| Palmetto | | | | | | | | | | | * | | | | | | * | | | |
| Raleigh | | | | | | | | | | | | * | | | * | * | | * | * | * |
| Floratam | | | | | | | | | | | | | | | | | | | | |
| Floratine | | | | | | | | | | | | | | * | | | | | | |
| T. Common | | | | | | | | | | | | | | | | | | | | |
| FHSA-115 | | | | | | | | | | | | | | | | | | | * | * |
| Classic | | | | | | | | | | | | | | | | | | | | |
| Winchester | | | | | | | | | | | | | | | | | | | | |
| Seville | | | | | | | | | | | | | | | | | | | | |
| Jade | | | | | | | | | | | | | | | | | | | | |
| Delmar | | | | | | | | | | | | | | | | | | | | |
| FloraVerde | | | | | | | | | | | | | | | | | | | | |

* Means were significantly different at $P \leq 0.05$ according to pairwise t-tests with Tukey adjustment.

Table 2-4. T-test results of comparisons between apparent infection rates calculated for St. Augustinegrass genotypes inoculated with the brown patch isolate of *R. solani*.

| | Sunclipse | Mercedes | Floralawn | Bitterblue | Captiva | FX-313 | FX-33 | FX-10 | Palmetto | Raleigh | Floratam | Floratine | T. Common | FHSA-115 | Classic | Winchester | Seville | Jade | Delmar | FloraVerde |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sunclipse | | | | | | | | * | * | | | | | | | | | | | |
| Mercedes | | | | | | * | | | | | | | | | * | | | | * | * |
| Floralawn | | | | | | | | * | * | | | | | | | | | | | |
| Bitterblue | | | | | | | | * | * | | | | | | | | | | | |
| Captiva | | | | | | | | | | * | | | | | | | | | | |
| FX-313 | | | | | | | | | | * | | | | | | | | | | |
| FX-33 | | | | | | | | * | * | | | | | | | | | | | |
| FX-10 | | | | | | | | | | | * | | | | | | * | * | | |
| Palmetto | | | | | | | | | | | | | * | | | | * | * | | |
| Raleigh | | | | | | | | | | | | * | | | * | | | | * | * |
| Floratam | | | | | | | | | | | | | | | | | | | | |
| Floratine | | | | | | | | | | | | | | | | | | | | |
| T. Common | | | | | | | | | | | | | | | | | | | | |
| FHSA-115 | | | | | | | | | | | | | | | | | | | * | * |
| Classic | | | | | | | | | | | | | | | | | | | | |
| Winchester | | | | | | | | | | | | | | | | | | | | |
| Seville | | | | | | | | | | | | | | | | | | | | |
| Jade | | | | | | | | | | | | | | | | | | | | |
| Delmar | | | | | | | | | | | | | | | | | | | | |
| FloraVerde | | | | | | | | | | | | | | | | | | | | |

* Means were significantly different at $P \leq 0.05$ according to pairwise t-tests with Tukey adjustment.

Table 2-5. T-test results of comparisons between area under disease progress curves calculated for St. Augustinegrass genotypes inoculated with the brown patch isolate of *R. solani*.

| | Sunclipse | Mercedes | Floralawn | Bitterblue | Captiva | FX-313 | FX-33 | FX-10 | Palmetto | Raleigh | Floratam | Floratine | T. Common | FHSA-115 | Classic | Winchester | Seville | Jade | Delmar | FloraVerde |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sunclipse | | | | | | * | | | | | | * | | | | | | | | |
| Mercedes | | | | | | * | | | * | | | | | | | | | | | |
| Floralawn | | | | | | * | | | * | | | | | | | | | | | |
| Bitterblue | | | | | | * | | | | | | * | | | | | | | | |
| Captiva | | | | | | | * | | | | | | * | | | | * | | | |
| FX-313 | | | | | | | | | * | | | | | | | | | | | |
| FX-33 | | | | | | | | | | * | | | | | | | | | | |
| FX-10 | | | | | | | | | | | | * | * | | * | | * | | | |
| Palmetto | | | | | | | | | | | | * | | | | | | * | | |
| Raleigh | | | | | | | | | | | | * | | * | | | | * | | |
| Floratam | | | | | | | | | | | | | | | | | | | | |
| Floratine | | | | | | | | | | | | | | | | | | | | |
| T. Common | | | | | | | | | | | | | | | | | | | | |
| FHSA-115 | | | | | | | | | | | | | | | | | | | | |
| Classic | | | | | | | | | | | | | | | | | | | | |
| Winchester | | | | | | | | | | | | | | | | | | | | |
| Seville | | | | | | | | | | | | | | | | | | | | |
| Jade | | | | | | | | | | | | | | | | | | | | |
| Delmar | | | | | | | | | | | | | | | | | | | | |
| FloraVerde | | | | | | | | | | | | | | | | | | | | |

* Means were significantly different at $P \leq 0.05$ according to pairwise t-tests with Tukey adjustment.

Table 2-6. Effects of ploidy level and morphotype on disease response variables for brown patch disease experiments.

| | | Pr > F ** | Ploidy means | Ploidy | Morph- means | Morphotype |
|---|---|---|---|---|---|---|
| AUDPC | Ploidy | 0.1132 | 175.25 A | 2X | 181.66 A | Dwarf |
| | Morphotype | 0.0385 | 155.41 A | 3X | 157.64 B | Long |
| YMAX | Ploidy | 0.0922 | 68.894 A | 2X | 72.308 A | Dwarf |
| | Morphotype | 0.0033 | 62.642 A | 3X | 62.188 B | Long |
| r parameter | Ploidy | 0.5686 | 0.5686 A | 2X | 1.18432 A | Dwarf |
| | Morphotype | 0.0005 | 0.0005 A | 3X | 1.01308 B | Long |

* Values obtained using contrast procedure in SAS to evaluate effect of ploidy and morphotype on disease response. Means of genotypes were analyzed using Least Significant Difference.
** Means followed by the same letter are not significantly different according to t-test comparison at $P \leq 0.05$.
AUDPC: Area Under Disease Progress Curve
YMAX: Final severity
r parameter: apparent infection rate

Table 2-7. AUDPC, apparent infection rate and final severity of St Augustinegrass genotypes inoculated with large patch isolate. Means represent combined data from two experiments.

| Cultivar | AUDPC | Apparent infection rate (units/day) | Final severity (%) |
|---|---|---|---|
| Raleigh | 310 | 0.32 | 59 |
| FX-10 | 280 | 0.63 | 72 |
| FloraVerde | 260 | 0.35 | 59 |
| Jade | 210 | 0.35 | 39 |
| FX-313 | 200 | 0.30 | 45 |
| Captiva | 190 | 0.29 | 40 |
| FX-33 | 190 | 0.37 | 40 |
| Floralawn | 180 | 0.20 | 30 |
| Palmetto | 170 | 0.21 | 28 |
| Seville | 170 | 0.24 | 38 |
| FHSA-115 | 160 | 0.31 | 31 |
| Classic | 150 | 0.22 | 30 |
| Bitterblue | 140 | 0.22 | 28 |
| Delmar | 140 | 0.22 | 25 |
| Winchester | 120 | 0.13 | 20 |
| Texas Common | 99 | 0.17 | 19 |
| Floratine | 99 | 0.22 | 23 |
| Mercedes | 97 | 0.23 | 16 |

* Means obtained using Proc Mixed code with random effect in SAS. Means were significantly different at $P \leq 0.05$ according to pairwise t-test with Tukey adjustment.

Table 2-8. T-test results of comparisons between final disease sverity of St. Augustinegrass genotypes inoculated with the large patch isolate of *R. solani*.

| | Sunclipse | Mercedes | Floralawn | Bitterblue | Captiva | FX-313 | FX-33 | FX-10 | Palmetto | Raleigh | Floratam | Floratine | T. Common | FHSA-115 | Classic | Winchester | Seville | Jade | Delmar | FloraVerde |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sunclipse | | | | | | | | * | | | | | | | | | | | | |
| Mercedes | | | | | | | | * | | | | | | | | | | | | |
| Floralawn | | | | | | | | | | | | | | | | | | | | |
| Bitterblue | | | | | | | | | | | | | | | | | | | | |
| Captiva | | | | | | | | | | | | | | | | | | | | |
| FX-313 | | | | | | | | | | | | | | | | | | | | |
| FX-33 | | | | | | | | | | | | | | | | | | | | |
| FX-10 | | | | | | | | | | | | | * | | | | | | | |
| Palmetto | | | | | | | | | | | | | | | | | | | | |
| Raleigh | | | | | | | | | | | | | | | | | | | | |
| Floratam | | | | | | | | | | | | | | | | | | | | |
| Floratine | | | | | | | | | | | | | | | | | | | | |
| T. Common | | | | | | | | | | | | | | | | | | | | |
| FHSA-115 | | | | | | | | | | | | | | | | | | | | |
| Classic | | | | | | | | | | | | | | | | | | | | |
| Winchester | | | | | | | | | | | | | | | | | | | | |
| Seville | | | | | | | | | | | | | | | | | | | | |
| Jade | | | | | | | | | | | | | | | | | | | | |
| Delmar | | | | | | | | | | | | | | | | | | | | |
| FloraVerde | | | | | | | | | | | | | | | | | | | | |

* Means were significantly different at $P \leq 0.05$ according to pairwise t-tests with Tukey adjustment.

Table 2-9. T-test results of comparisons between apparent infection rates calculated for St. Augustinegrass genotypes inoculated with the large patch isolate of *R. solani*.

| | Sunclipse | Mercedes | Floralawn | Bitterblue | Captiva | FX-313 | FX-33 | FX-10 | Palmetto | Raleigh | Floratam | Floratine | T. Common | FHSA-115 | Classic | Winchester | Seville | Jade | Delmar | FloraVerde |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sunclipse | | | | | | | | * | | | | | | | | | | | | |
| Mercedes | | | | | | | | | | | | | | | | | | | | |
| Floralawn | | | | | | | | | | | | | | | | | | | | |
| Bitterblue | | | | | | | | | | | | | | | | | | | | |
| Captiva | | | | | | | | | | | | | | | | | | | | |
| FX-313 | | | | | | | | | | | | | | | | | | | | |
| FX-33 | | | | | | | | | | | | | | | | | | | | |
| FX-10 | | | | | | | | | | | | | * | | | * | | | | |
| Palmetto | | | | | | | | | | | | | | | | | | | | |
| Raleigh | | | | | | | | | | | | | | | | | | | | |
| Floratam | | | | | | | | | | | | | | | | | | | | |
| Floratine | | | | | | | | | | | | | | | | | | | | |
| T. Common | | | | | | | | | | | | | | | | | | | | |
| FHSA-115 | | | | | | | | | | | | | | | | | | | | |
| Classic | | | | | | | | | | | | | | | | | | | | |
| Winchester | | | | | | | | | | | | | | | | | | | | |
| Seville | | | | | | | | | | | | | | | | | | | | |
| Jade | | | | | | | | | | | | | | | | | | | | |
| Delmar | | | | | | | | | | | | | | | | | | | | |
| FloraVerde | | | | | | | | | | | | | | | | | | | | |

* Means were significantly different at $P \leq 0.05$ according to pairwise t-tests with Tukey adjustment.

Table 2-10. T-test results of comparisons between area under disease progress curves calculated for St. Augustinegrass genotypes inoculated with the large patch isolate of *R. solani*.

| | Sunclipse | Mercedes | Floralawn | Bitterblue | Captiva | FX-313 | FX-33 | FX-10 | Palmetto | Raleigh | Floratam | Floratine | T. Common | FHSA-115 | Classic | Winchester | Seville | Jade | Delmar | FloraVerde |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sunclipse | | | | | * | | | * | | * | | | | | | | | * | | * |
| Mercedes | | | | | | | | * | | * | | | | | | | | | | * |
| Floralawn | | | | | | | | | | | | | | | | | | | | |
| Bitterblue | | | | | | | | * | | * | | | | | | | | | | |
| Captiva | | | | | | | | | | | | | | | | | | | | |
| FX-313 | | | | | | | | | | | | | | | | | | | | |
| FX-33 | | | | | | | | | | | | | | | | | | | | |
| FX-10 | | | | | | | | | | | | * | * | | * | * | | | * | |
| Palmetto | | | | | | | | | | * | | | | | | | | | | |
| Raleigh | | | | | | | | | | | | * | * | * | * | * | * | | * | |
| Floratam | | | | | | | | | | | | | | | | | | | | |
| Floratine | | | | | | | | | | | | | | | | | | | | * |
| T. Common | | | | | | | | | | | | | | | | | | | | * |
| FHSA-115 | | | | | | | | | | | | | | | | | | | | |
| Classic | | | | | | | | | | | | | | | | | | | | |
| Winchester | | | | | | | | | | | | | | | | | | | | * |
| Seville | | | | | | | | | | | | | | | | | | | | |
| Jade | | | | | | | | | | | | | | | | | | | | |
| Delmar | | | | | | | | | | | | | | | | | | | | |
| FloraVerde | | | | | | | | | | | | | | | | | | | | |

* Means were significantly different at $P \leq 0.05$ according to pairwise t-tests with Tukey adjustment.

Table 2-11. Effects of ploidy level and morphotype on disease response variables for large patch disease experiments.

| | | Pr < F ** | Ploidy means | Ploidy | Morph-means | Morphotype |
|---|---|---|---|---|---|---|
| AUDPC | Ploidy | 0.7232 | 177.20 A | 3X | 190.41 A | Dwarf |
| | Morpho | 0.1043 | 170.44 A | 2X | 155.67 A | Long |
| YMAX | Ploidy | 0.3999 | 37.278 A | 3X | 40.815 A | Dwarf |
| | Morpho | 0.0110 | 32.955 A | 2X | 28.475 B | Long |
| r parameter | Ploidy | 0.0763 | 0.33070 A | 3X | 0.30862 A | Dwarf |
| | Morpho | 0.1011 | 0.24613 A | 2X | 0.23933 A | Long |

*Values obtained using contrast procedure in SAS to evaluate effect of ploidy and morphotype on disease response. Means of genotypes were analyzed using Least Significant Difference.
** Means followed by the same letter are not significantly different according to t-test comparison at $P \leq 0.05$.
AUDPC: Area Under Disease Progress Curve
YMAX: Final severity
r parameter: apparent infection rate

Figure 2-1. Brown patch disease on genotype FX-313 six days after inoculation. Abundant aerial mycelia associated with severe foliar blight symptoms on leaves and sheaths.


Figure 2-2. Brown patch symptoms on the genotype Delmar six days after inoculation. Lesions show water soaking and general necrosis of leaf tissue.

Figure 2-3. Large patch symptoms. Lesions, water soaking, general necrosis of sheath and crown tissue and yellowing of newest leaves

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# CHAPTER 3
# PHYLOGENETIC ANALYSIS OF *RHIZOCTONIA* ISOLATES COLLECTED FROM TURFGRASSES IN FLORIDA

## Introduction

Historically, taxonomy of *Rhizoctonia* spp. has been confusing and variable, since the classical species concept is so wide, many have considered it a species complex (26, 47, 94, 89). *Rhizoctonia* taxonomy is further complicated by the reportedly wide host range and uncommon occurrences of sexual structures. The classical hyphal anastomosis reaction has been a fundamental technique to classify isolates into distinct groups (26). Currently, 14 different anastomosis groups or independent evolutionary groupings exist (35, 37). However, anastomosis reaction can be open to subjective interpretation (26). Additionally morphological features of hyphae also have been used to differentiate species. However, these features can vary with specific environmental conditions. Most recently, the use of phylogenetic and molecular analyses have had a big impact in the classification of these fungi.

Ribosomal RNA genes are conserved genes that contain sequence components possessing different evolutionary rates. Some of the sequence regions are phylogenetically and taxonomically informative for fungi (15). Internal transcribed spacer regions (ITS 1 and ITS 2) are found between the 18 S and 5.8 S, and the 5.8 S and 28 S genes respectively. The ITS regions have become essential for population studies with fungi.

Molecular genetic analyses support some AG's, such as AG4, AG 5, AG6, AG7, AG8, AG 11, AGBI and some subgroups (AG 1-IA, IB, IC) (36). The publication of the rDNA-ITS sequences in the GenBank also has facilitated direct comparison of data obtained by different research groups and has led to progress in the *Rhizoctonia* spp. systematics.

However, some sequences deposited in GenBank lack information either at the start or the end of the informative sequence. Missing sequence data is problematic for phylogenetic

analyses. Recently, some *Rhizoctonia* researchers (99) reported for inaccuracies in the currently accepted classification system, citing the need for better quality data to make concrete conclusions. The ladder phenomenon observed in some *R. solani* AG 2 isolates, reported by Salazar et al 1999 might explain the number of ambiguous and unresolved nucleotides which affect phylogenetic analyses.

The principal objective of this study was to characterize *Rhizoctonia* isolates from turfgrass in Florida by using DNA cloning and sequencing protocols to obtain good quality sequence data. Secondary objectives were:1) to standardize the protocol to obtain data with the minimum number of missing or ambiguous nucleotides, 2) classify isolates to AG or species level by comparison with known sequence data in GenBank, and 3) evaluate specific primers for some AGs reported in literature.

## Materials and Methods

### Fungal Isolates Collection

Fungal isolations were made from small pieces of infected leaves and sheaths (2-5 mm). Tissue was soaked in 10% household bleach for 30 seconds, rinsed 3 consecutive times in de-ionized water, dried with a paper towel, and transferred to water agar media without antibiotics. Presence of *Rhizoctonia* mycelia was confirmed 2-3 days after plating. Efforts resulted in 32 isolates of *Rhizoctonia* spp. obtained from warm season grasses collected from various turfgrass fields in Florida. A single isolate was obtained from a cool season grass (*Poa trivialis*) while 9 isolates were collected from vegetable crops (bean, corn, and rice) grown in the Everglades agricultural area for comparison.

To recover isolates from storage, 4 infested oat seeds were aseptically transferred to PDA medium. Additional transfer of hyphae were made onto 9 cm petri dishes containing

approximately 20 mL of Potato Dextrose Agar medium (PDA, 33 g/L) and incubated at 25°C in the dark. Cultures were transferred every 2-3 weeks.

**Sequence Data from GenBank**

Sequences of partial rDNA coding regions from 34 isolates with reported anastomosis groups (AG) were included as reference sequences. One sequence of an *Athelia rolfsii* isolate was included as an outgroup (98), for a total of 76 taxa. All the external sequences were downloaded from GenBank through the National Center of Biotechnology Information website. A complete list of isolates used in this study, describing the plant host, source, year of collection and the genbank accession number is presented in Table 3-1 and 3-2.

**Genomic DNA Extraction**

Genomic DNA from 42 isolates maintained on PDA culture plates was extracted using Extract-N-Amp (Sigma-Aldrich) and/or Dneasy Plant Mini Kit (Qiagen, Valencia, CA). Threads of mycelia (aerial or prostrate) from each isolate were used for DNA extraction. Isolates with no aerial mycelia were grown on 100 mL of Luria Bertani broth (LB) (10.0 g. of Tryptone, 5.0 g. of Yeast extract and 5.0 g. of NaCl), covered by aluminum foil under agitation until adequate mycelia growth was observed (5-6 days). Mycelia was collected with vacuum filtration and ground in liquid Nitrogen by using mortar and pestle to obtain a fine powder. When needed, fungal mycelia were crushed using a bead beater and tungsten carbide beads. DNA was eluted in deionized-distilled sterile water.

**Polymerase Chain Reaction Amplifications and Primer Sets Used**

Primer sets specific to AG 2-2LP, AG 2-2 IIIB, and AG 2-2 IV (20, 111) were used to determine if some isolates were related to this group of AG's. The primer set ITS1 and ITS4 (121) were used to amplify the internal transcribed spacer regions and the 5.8s rDNA sequence. All primers were synthesized by Integrated DNA Technologies, Inc. Table 3-3 shows the

sequences, PCR reaction mix conditions and the profile used for the amplification of each pair of primers. The temperature cycles were controlled with MyCycler™ thermal cycler (Bio-Rad, California, USA). PCR products were visualized by running 5 μL of the reaction product on a standard 1% agarose gel with the addition 1 μL of ethidium bromide stock solution (20mg/ml). Products were electrophoresed at 110 volts; amplified products were visualized using a UV transilluminator. Products from amplification with ITS primers were purified using QIA prep spin Miniprep kit (QIAGEN) following manufacturer's instructions.

**Bacterial Cloning of Amplicons**

The amplicons were submitted to an additional extension cycle with dATP (100 mM; 2'-deoxyadenosine-5'-triphosphate solution, Bioline, Boston, USA) to be cloned in the pCR II vector (plasmid) (pCR 2.1 TOPO TA cloning kit, Invitrogen, Inc.) through ligase reaction and chemical transformation into competent cells of *E. coli* (One Shot™ TOP 10, Invitrogen, Inc.). Transformed colonies were obtained after the incubation of the ligated products at 37°C, and 16 were confirmed to have the plasmid through the presence of blue-white colonies using LB media (same composition mentioned above + 16 g.of agar) amended with X-GAL (2%; 5-Bromo-4-Chloro-3-indolyl-ß-D-galactopyranoside, Bioline), IPTG (100 mM; Isopropyl- ß-D-thiogalactopyranoside, Bioline) and ampicillin salt solution (50 mg/ml). Bacterial DNA of positive clones (=plasmid + fungal DNA) was cleaned using QIA prep spin Miniprep kit (QIAGEN) according to manufacturer's instructions. Restriction analysis with EcoRI (strain RY13, Invitrogen, Inc.) was done to verify the presence and fragment size of the insert. Fragments were visualized on 2% agarose gel amended with with 2 μL of ethidium bromide stock solution. Electrophoresis was carried out at 120 volts for 45 minutes.

**Sequencing**

An average of three-four clones/ isolate were sequenced bidirectionally. Sequencing of the clones was done at the University of Florida DNA Sequencing Core Laboratory using a Perkin Elmer Applied Biosystems ABI PRISM model 3130 automated DNA sequencer ABI (Applied Biosystems, Foster City, CA, USA) that uses four-dye fluorescent labeling methods and a real time scanning detector.

**Sequence Alignment, Molecular and Phylogenetic Analyses**

Forward and reverse sequence data of each bacterial clone were aligned to get a consensus sequence. Consensus sequences of all clones for each isolate were obtained by automated multiple alignment using ClustalW (109) software package available in MEGA version 4 software (107). For gap opening and gap extension, default options were set up. No more than 7 ambiguous sites (as maximum) were found between strains of the same clone. Ambiguous sites between strains were solved by majority rule of any particular nucleotide at the specific site. A final alignment of all consensus sequences using ClustalW was done, using the same options described above. Alignment was later adjusted manually under MEGA version 4.

To observe results from different phylogenetic methods and to support congruency of the tree topology, data were analyzed using the most common phylogenetics packages. Neighbor-Joining (NJ) with two Kimura parameter model (52) was run using MEGA version 4 (107) and maximum parsimony (MP) with Phylogenetic Analysis Under Parsimony -PAUP 4.0 b10 (105). Heuristic searches were performed with random stepwise addition (1000 replications) and branch swapping algorithm using tree bisection-reconnection (TBR). Clade stability was evaluated using 1000 replicates with arrangement limited to 1,000,000 per replicate. For Bayesian inference and Maximum Likelihood a model of nucleotide substitution was used with the program Modeltest v3.4 (90). The best fit model of sequence evolution was chosen based on the Akaike Information

Criterion (AIC) (44). The model selected was TVM + G, transversion model with variable base frequencies, variable transversions and equal transitions.Bayesian inference (BI) method was estimated with MrBayes v3.1.2 (92). The Markov Chain Monte Carlo (MCMC) was run with four chains for 10,000,000 generations. Sampling was done every 100 generations starting with a random tree. A 90% majority rule consensus tree was obtained. Maximum Likelihood (ML) was analyzed using GARLI software version 0.96 (124). A 50% majority rule consensus tree was obtained.

## Results

A total of thirty-three isolates were tested to determine if they were AG 2-2 LP, AG 2-2 III B or AG 2-2 IV, and only 12 were positive for AG 2-2 LP. Isolate 07-06 (from Bermudagrass) was positive for AG 2-2 IIIB. No isolate amplified with the AG 2-2 IV primers.

Tree topology of the four phylogenetic analyses was very similar (trees not shown). Basically, the four analyses were consistent in differentiating isolates of *Rhizoctonia solani*, *Waitea circinata*, *Athelia rolfsii*, and *Marasmius oreades* with boostrap values of 100%. The ML analysis showed bootstrap values of 88 % for the *R. solani* clade. Because of this, results are analyzed based on Bayesian analysis (90% majority rule) and MP.

Regarding Bayesian analysis twelve different clades with more than one isolate were differentiated and seventeen isolates were located into single clades (Figure 3-1). Twelve isolates of the *W. circinata* group (11 of var. *circinata* and 1 of var. *zeae*) were weakly supported as clades when compared to clades formed by isolates of var. *oryzae*, *zeae*, and *agrostis*. Anastomosis groups were shown as independent evolutionary units with sufficient levels of heterogeneity (20, 25, 26, 35, 61, 53, 93, 94, 95). Most of the isolates within the *W. circ.* clade are not clearly identified at the species level when they are compared to tester isolates. Only 0705, rice 3 and 0538 appear to be similar (91%) to var. *oryzae* (Table 3-1 and figure 3-1).

Isolates 0544A, 0709, and 0631 were identical but different from the other *W. circinata*-like isolates.

In relation to MP analysis, 787 characters were used, 241 were constant, 113 variable characters were parsimony-uninformative and 433 parsimony informative. Fourteen different clades with more than one isolate were conformed and four isolates were located as single clades (Figure 3-2). Various isolates representing different anastomosis groups formed a clade but with weak support. Varieties within *W. circ*. group were better differentiated with high bootstrap values. *W. circinata*-like isolates used in this study formed three clades and were not clearly grouped with the tester isolates; this was especially so in the clade formed by 0544A, 0709 and 0631. Isolates of *W. circ.* var. *circinata* formed a defined clade.

Isolates from vegetables crops were located within the *R. solani* clade. Two AG were identified (AG 4-HG-III and AG 1-IA). Other isolates seem to be similar to AG 2-2 isolates and one isolate (Beans 8) could not be accurately classified.

**ITS regions and 5.8s rDNA gene length variation:** Table 3-4 shows the length of the sequenced amplicons for each defined clade. AG 2-2 LP and AG 1-IA clades had the longest amplicons. Isolates of *W. circ*. var. *agrostis* had the shortest. The length of the 5.8s rDNA gene sequence corresponded to 143 bp and was highly conserved among all the isolates. Fifteen bp were found to be variable among *W. circ*. group and *R. solani*. Within *W. circ*. clade, 0544A, 0709 and 0631 showed differences in three nucleotides in the 5.8 s rDNA gen. These three sites are: base pair 81 (T/C), 92 bp (C/T), 103 bp (G/A).

## Discussion

Primers specific for AG groups differentiated the AG 2-2 LP subgroup; however, diagnostic should also be supported by other additional features, due to the high similarity among AG 2-2 subgroups. The fragment size of this region was about 400 bp. AG 2-2 subgroup

primers are different by a few nucleotides. Although, these primers are used to amplify a highly conserved region, molecular diagnosis should be done carefully, because the ladder phenomenon of *Rhizoctonia* may cause changes in nucleotides that result in a misidentification of isolates. Isolate 0706 was positive for AG 2-2 IIIB (Brown patch primer), but in the tree appeared not to be highly similar to the tester isolate.

In this study, some host specialization by AG's was observed. Warm season grasses like St. Augustinegrass, *Seashore paspalum* and *zoysia* grass were infected by isolates of the subgroup AG 2-2 LP. A high level of sequence variation was observed in the rDNA ITS regions of *Rhizoctonia* spp; however 5.8s rDNA gene appears to be highly conserved, mainly within species. *Rhizoctonia.solani* and *Waitea* species have fifteen distinct informative nucleotides in this gene that could be used to differentiated them at the species level. Lengths of amplicons support the species classification, since they are smaller in *Waitea circinata* ITS regions. Furthermore, the variety classification in the *Waitea* group was well supported by high bootstrap values.

Isolates in the AG 2-2 LP clade were highly similar and clearly different than the rest of the AG's included in the study. Surprisingly, isolates from beans showed very similar ITS sequence data, may indicating a possible gene flow between isolates under natural conditions, and the possibility of finding alternative hosts to certain AG groups or subgroups.

Clade organization of bean 1, 2 and 4 isolates were consistent with pathogenicity previously reported for the AG 4- HG II, but mainly for AG 4-HG III. Although this AG and their subgroups have been suggested as different species, the phylogenetic analysis only allowed observing the differences between HGI-II and III. Pathogenicity reported by AG 4-HG III on common, snap, lima, dry edible beans is consistent with their isolation from field beans.

Bean 5 and 7 show high similarity between them, but dissimilarity with Bean 8, which is found as a single clade. This may indicates of some level of population diversity, since isolates were taken from the same field at the same season of the year. Isolates 0465, 0650 and 0649, collected in different years and sites in Alachua-Co (Florida) from St. Augustinegrass and the corn isolate were identical and thus, placed in a very distinct cluster, along with the AG 1-IA tester (100 % similarity). Isolate 0465 was chosen based on preliminary studies of its pathogenicity on St. Augustinegrass, and results of the screening with this isolate on different genotypes of this host showed a high level of virulence in many cultivars.

Isolates from this AG have been reported causing diseases on rice, corn and cool season turfgrasses (49, 53, 86, 93, 100, 106), but no report was found of this AG causing brown patch symptoms on warm season grasses. Therefore, it may constitute a unique finding not previously reported. Applications of rDNA-ITS sequence analysis have been very useful, especially to clarify *Rhizoctonia* taxonomy and to develop specific sets of primers that have allowed AG identification or confirmation based on accurate methodology. Results of this research constitute are novel information for Florida, because previous phylogenetic studies of *Rhizoctonia* were from other hosts or were conducted in different countries or USA states. However, diagnosis using AG 2-2 LP primers and phylogenetic trees were consistent on detecting this AG on warm season grasses.

Isolates 0408 (bermudagrass) and 0658 (*S. paspalum*) were highly similar with respect to ITS sequences, although collected from two different warm season grasses at different seasons of the year. They were located apart from any of the other clades formed and were found to be consistent with results obtained using the homology search in BLAST. They showed high homology with *Ceratobasidium* sp AG-G, a binucleate *Rhizoctonia* sp. A similar observation

was found with 0515A and 0515B (from bermudagrass); their homology search revealed them as *Rhizoctonia cerealis*. The use of *A. rolfsii* as an outgroup was appropriate (Sharon et al 2006, Sharon et al 2008). Isolate 0545 was classified as member of *Tricholomataceae* sp or *Marasmius oreades* sp*;* two taxa as far related as the isolate proposed as an outgroup. It could be suggested as outgroup for future phylogenetic analyses.

Cloning protocol became an adequate methodology to obtain sequence data of excellent quality. Only three ambiguous sites were unresolved within 115 clones. The few ambiguities present (7 as maximum) in a few clones were easily solved by majority rule. Thus, the sequence data obtained during this study are considered an excellent contribution for future phylogenetic analyses of this fungal pathogen. Complete sequence data for ITS 1 and 2 regions, and the 5.8 s rDNA gene were obtained with this methodology. However, with all of the molecular and phylogenetic analyses performed by *Rhizoctonia* researchers, only Pope and Carter 2001, Ming et al 2003, and Tewoldemedhin et al 2006 reported a cloning protocol for obtaining sequence data. Briefly, de la Cerda, et al 2007, mentioned two heterozygous positions on *Waitea circinata* var. c*ircinata* isolates. Tree topology with high bootstrap values clearly supported *Rhizoctonia* species differentiation. Maximum parsimony and Bayesian analysis seemed to be the most appropriate analysis that biologically explain the data; however, some differences were found between both analyses.

The access to sequences deposited in GenBank has allowed many researchers to perform phylogenetic analyses based on direct comparison of sequence data. It has been found that many sequences published in the database are incomplete. According to Gonzales et al 2001, “the accumulation of a larger and high quality rDNA sequence database should establish a foundation

for the development of a species concept in *Rhizoctonia* for testing hypotheses related to geographic subdivision, host and ecological specialization".

Currently, the rDNA-ITS sequences deposited in GenBank vary significantly; from the longest rDNA-ITS sequences of AG 2-2IV (isolate BC10) of about 678 bp to the shorter sequences of *W. circinata* of about 570bp (98). Sequences obtained through cloning in this study varied from 703 bp (longest) to 583 bp (shortest). This may indicate that the use of the direct sequencing method is not highly reliable for obtaining good quality data with this pathogen, especially when the sequence data obtained is used to elucidate the presence of new species or to re-name others (including AG's groups).

The ladder phenomenon or heterozygous condition in a multinucleate fungus, has been little considered by *Rhizoctonia* researchers. Salazar, et al.,1999 reported the existence of more than one sequencing the ITS 1 and ITS 2 regions in AG 2-2 isolates. The condition was named "the ladder phenomenon"due to the visualization of the different bands in a gel. Pope and Carter 2001 also reported "the existence of more than one ITS sequences in isolates of AG-6, AG-8 and AG-12. This phenomenon in *Rhizoctonia* could be explained by: i) the existence of different sequences in the same ribosomal region, ii) different sequences in the same chromosome or different chromosomes from the same nucleus, or iii) different sequences in different nuclei of this multinucleate heterokaryotic fungus (94). Boysen et al 1996 and O'Donnell et al 1998, observed sequence ladders in *R. solani* AG 4 isolates and in *Fusarium* species, respectively. Results of this study demonstrated the existence of this phenomenon in the isolates selected, through the existence of nucleotides ambiguities in clones of the same isolate. According to Salazar et al 1999, it seems that the third hypothesis is the most appropriate.

Table 3-1. Fungal isolates used in this study for phylogenetic analysis

| Isolate | Host | Date | Size | BLAST search | BLAST search |
|---|---|---|---|---|---|
| 0502 | *Seashore paspalum* | 01//2005 | 702 | *R.solani (99%)- T. cucumeris* (98%) | *T. cucumeris* AG 2-2IV, 2-2 IIIB (96%) |
| 0501 | *Seashore paspalum* | Jan-05 | 700 | *R.solani (99%)- T. cucumeris* (98%) | *T. cucumeris* AG 2-2IV, 2-2 IIIB (96%) |
| 0470-B | St. Augustinegrass | 11/10/2004 | 700 | *R. solani (98%)* | *T. cucumeris* AG 2-2 IV (97%), 2-2 IIIB (96%) |
| 0508-A | *Seashore paspalum* | pending | 697 | *R. solani (99%)- T. cucumeris (98%)* | *T. cucumeris* AG 2-2 IV (97%), 2-2 IIIB (96%) |
| 0544-B | *Seashore paspalum* | 9/12/2005 | 697 | *R.solani (99%)- T. cucumeris* (98%) | *T. cucumeris* AG 2-2IV, 2-2 IIIB (96%) |
| 0714 | St. Augustinegrass | 4/24/2007 | 699 | *R. solani (98%)- T. cucumeris (98%)* | *T. cucumeris* AG 2-2 IV, 2-2 IIIB (96%) |
| 0508-B | *Seashore paspalum* | pending | 698 | *R.solani (99%)* | *T. cucumeris* AG 2-2IV, 2-2 IIIB (96%) |
| 0508-C | *Seashore paspalum* | pending | 698 | *R. solani (98%)* | *T. cucumeris* AG 2-2 IV, 2-2 IIIB (96%) |
| 0702-B | *Seashore paspalum* | 1/16/2007 | 698 | *R.solani (99%)- T. cucumeris* (98%) | *T. cucumeris* AG 2-2IV, 2-2 IIIB (96%) |
| 0472 | *Zoysia grass* | 9/11/2004 | 701 | *R. solani (98%)* | *T.* cucumeris *AG 2-2IV (96%)* |
| 0470-A | *St. Augustinegrass* | 11/10/2004 | 697 | *R.solani (99%)- T. cucumeris* (98%) | *T. cucumeris* AG 2-2IV, 2-2 IIIB (96%) |
| 0481 | *St. Augustinegrass* | 9/12/2004 | 697 | *R. solani (99%)- T. cucumeris (98%)* | *T. cucumeris* AG 2-2 IV, 2-2 IIIB (96%) |
| 0706 | Bermudagrass | 12/2/2007 | 697 | *R.solani (98%)- T. cucumeris* (97%) | *T. cucumeris*AG 2-2 IIIB(97%)-AG 2-2 IV (96%) |
| 0650 | *St. Augustinegrass* | 8/29/2006 | 673 | *T. cucumeris (99%). C. oryzae-sativae(99%)* | *T. cucumeris* AG I-1A (99%) |
| 0649 | *St. Augustinegrass* | 8/28/2006 | 673 | *T. cucumeris (99%). C. oryzae-sativae(99%)* | *T. cucumeris* AG I-1A (99%) |
| 0465 | *St. Augustinegrass* | unknown | 673 | *T. cucumeris (99%). C. oryzae-sativae(99%)* | *T. cucumeris* AG I-1A (99%) |
| 0408 | *Bermudagrass* | 1/30/2004 | 616 | *Ceratobasidium sp* AG-G (99%) | *R. solani* (99%) |
| 0658 | *Seashore paspalum* | 8/24/2006 | 617 | *Rhizoctonia sp (99%)* | *Ceratobasidium* sp AG-G (99%) |
| 0515-A | Bermudagrass | 4/20/2005 | 647 | *R. cerealis (99%)* | *Ceratobasidium* sp CAG-1-AG-D (98%) |
| 0515-B | Bermudagrass | 4/20/2005 | 648 | *R. cerealis (98%)* | *Ceratobasidium* sp CAG-1-AG-D (98%) |
| 0544-A | *Seashore paspalum* | 9/12/2005 | 587 | *W. circinata (94 %)* | *R. zeae* (88 %) |
| 0709 | Bermudagrass | 4/17/2007 | 588 | *W. circinata (98%)* | *R. zeae* (87%) |
| 0631 | Bermudagrass | 04/27/2006 | 589 | *W. circinata (94%)* | *R. zeae* (88%) |
| 0503 | Bermudagrass | Jan-05 | 605 | *R. zeae (98%)* | *W. circinata* (98%) |
| 0705 | *Seashore paspalum* | 5/1/2007 | 614 | *W. circinata (99%)* | *R. zeae* (92%) |
| 0538 | Bermudagrass | 4/8/2005 | 621 | *W. circinata (97%)* | *R. zeae* (93%) |
| 0528-A | *Poa trivialis* | 10/6/2005 | 602 | *W. circinata var circinata (99%)* | *Thanatephorus* sp AG I-1DA (100%) |
| 0707-AB | Bermudagrass | 1/29/2007 | 605 | *R. zeae (99%)* | *W. circinata* (98%) |
| 0633 | Bermudagrass | 04/19/2006 | 605 | *R. zeae (99%)- W. circinata (98%)* | *W. circinata* var *circinata* (93%) |
| 0524 | St. Augustinegrass | Jul-05 | 606 | *R. zeae (99%)* | *W. circinata* (98%) |
| 0647 | *Seashore paspalum* | 08/15/2006 | 607 | *R. zeae (99%)* | *W. circinata* (98%) |
| 0503-A | Bermudagrass | Jan-06 | 605 | *R. zeae (99 %)* | *W. circinata* (98%) |
| 0545 | St. Augustinegrass | 9/12/2005 | 630 | *Tricholomataceae* sp (98%) | *Marasmius oreades* (97%) |
| Bean 1, 2 ,4 | Snap beans "Bronco" | 10/12/2004 | 686 | *T.cucumeris ,T .cucumeris* AG 4-HG-III | |
| Bean 5 | Kidney bean (C. Harmon) | Jun-06 | 701 | *T.cucumeris*, *R.solani* | |

Table 3-1. Continued.

| Isolate | Host | Date | Size | BLAST search | BLAST search |
|---|---|---|---|---|---|
| Bean 7 | Lima beans (C. Harmon) | Jun-06 | 700 | *T. cucumeris, R. solani* | |
| Bean 8 | Snap bean (C. Harmon) | Jun-06 | 627 | *Ceratobasidium sp* AG-B (o), *Rhizoctonia sp* | |
| Rice 1 | Unknown variety | 8/14/2004 | 683 | *T. cucumeris*, *T. cucumeris* AG 4-HG III | |
| Rice 3 | Unknown variety | 12/8/2004 | 615 | *R.oryzae, W.circinata* | |
| Corn 2 | Sweet corn | 10/12/2004 | 673 | *T. cucumeris*, *T. cucumeris* AG 4-HG III | |

Table 3-2. Sequences obtained from the GenBank used in this study for phylogenetic analysis.

| Species-AG | GenBank accession | Number of bp |
|---|---|---|
| *Athelia rolfsii* | AY684917 | 646 |
| *W. circinata var. agrostis* | AB213590 | 570 |
| *W. circinata var. agrostis* | AB213575 | 571 |
| *W. circinata var. circinata* | AB213582 | 565 |
| *W. circinata var. circinata* | AB213580 | 564 |
| *W. circinata var. circinata* | AB213585 | 565 |
| *W. circinata var. circinata* | AF222799 | 568 |
| *W. circinata var. circinata* | AB213579 | 565 |
| *W. circinata var. circinata* | AB213583 | 563 |
| *W. circinata var. circinata* | AB213584 | 565 |
| *W. circinata var. circinata* | AB213586 | 564 |
| *W. circinata var. circinata* | AB213587 | 564 |
| *W. circinata var. circinata* | AB213581 | 565 |
| *W. circinata var. oryzae* | AB213591 | 576 |
| *W. circinata var. oryzae* | AB213589 | 575 |
| *W. circinata var. oryzae* | AB213590 | 575 |
| *W. circinata var. zeae* | AB213597 | 571 |
| *W. circinata var. zeae* | AB213594 | 571 |
| *W. circinata var. zeae* | AB213593 | 571 |
| AG 1-IA | AB122133 | 672 |
| AG 1-IB | AB122139 | 678 |
| AG 1-IC | AB122142 | 651 |
| AG 1-ID | AB122130 | 693 |
| AG 2-2 IIIB | AF354116 | 689 |
| AG 2-2 IV | AB000014 | 684 |
| AG 2-2 LP | AB054866 | 678 |
| AG 4-HGI | AB000007 | 659 |
| AG 4-HGII | AB000006 | 661 |
| AG 4-HGIII | AY154659 | 667 |
| AG 5 | AF153778 | 650 |
| AG 6 GVI | AF153780 | 625 |
| AG 7 | AB000003 | 636 |
| AG 8 | AF153797 | 624 |
| AG 9 | AF354065 | 652 |
| AG 10 | AF153800 | 649 |
| AG 11 | AF153802 | 633 |
| AG 12 | AF153804 | 658 |
| AG 2-BI | AB054873 | 674 |

Table 3-3. Nucleotide sequence, cycle profile, and PCR reaction mix for amplification of AG 2-2LP, IIIB, and IV and ITS primers.

| Primers | Sequence | PCR reaction mix | Profile | Cycles | Product size (bp) expected |
|---|---|---|---|---|---|
| AG 2-2 LP | 5'-AGGCAGAGAAACATGGATGGGC- 3' | 10 µL of REDE-Extract Amp | Initial denaturation for 2 min at $94^0$C | | |
| | 5'-CCTCCAATACCAAAGTGAAACCAAATC- 3' | 0.5 µL of each primer (1X stock = 20 nmol) | 40 s at $94^0$C | 30 | 400 |
| | | 5 µL of deionized-distilled water | 1 min at $62^0$C | | |
| | | 4 µL of fungal DNA | 1 min at $72^0$C | | |
| | | | 5 min at $72^0$C | | |
| AG 2-2 IIIB | 5'-AGGCAGAG(A/G)CATGGATGGGAG-3' | Same as AG 2-2 LP. | Same as AG 2-2 LP. | 30 | 500 |
| | 5'-ACTTGGCCA(A/C)CCTTTTTATC-3' | | | | |
| AG 2-2 IV | 5'-AGGCAGAGACATGGATGGGAA-3' | Same as AG 2-2 LP. | Same as AG 2-2 LP. | 30 | 500 |
| | 5'-CTTGGCCACCC(A/C)TTTTTTAC-3' | | | | |
| ITS 1 | 5'-TCCGTAGGTGAACCTGCGC-3' | 25 µL of REDE-Extract Amp | Initial denaturation for 3 min at $94^0$C | 30 | 700 |
| ITS 4 | 5'-TCCTCCGCTTATTGATATGC-3' | 2 µL of each primer (1X stock = 20 nmol) | 1 min at $94^0$C | | |
| | | 11 µL of deionized-distilled water | 1 min at 55 $^0$C | | |
| | | 10 µL of fungal DNA | 2 min at $72^0$C | | |
| | | | 10 min at $72^0$C | | |

Table 3- 4. Length variation within ITS 1 and ITS 2 regions and 5.8s r DNA gene within isolates.
* Values from minimum to maximum number of base pair

| Clade | ITS 1 lengh (bp)* | 5.8 s r gen (bp) | ITS 2 lengh (bp)* |
|---|---|---|---|
| AG 2-2 LP | 287-292 | 143 | 267 |
| Binucleate | 222-253 | 143 | 251-252 |
| AG 1-IA | 263-264 | 143 | 266 |
| 0647, 0503A, etc | 249-251 | 143 | 213 |
| 0705, rice 3, 0538 | 252-253 | 143 | 219-221 |
| 0544A, 0709, 0631 | 234-235 | 143 | 210-211 |
| *W. circ*. var. *oryzae* | 238-239 | 143 | 193-194 |
| *W. circ*. var. *zeae* | 239-240 | 143 | 188 |
| *W. circ*. var. *circinata* | 232-245 | 143 | 187-214 |
| *W. circ.* var. *agrostis* | 239 | 143 | 188-189 |

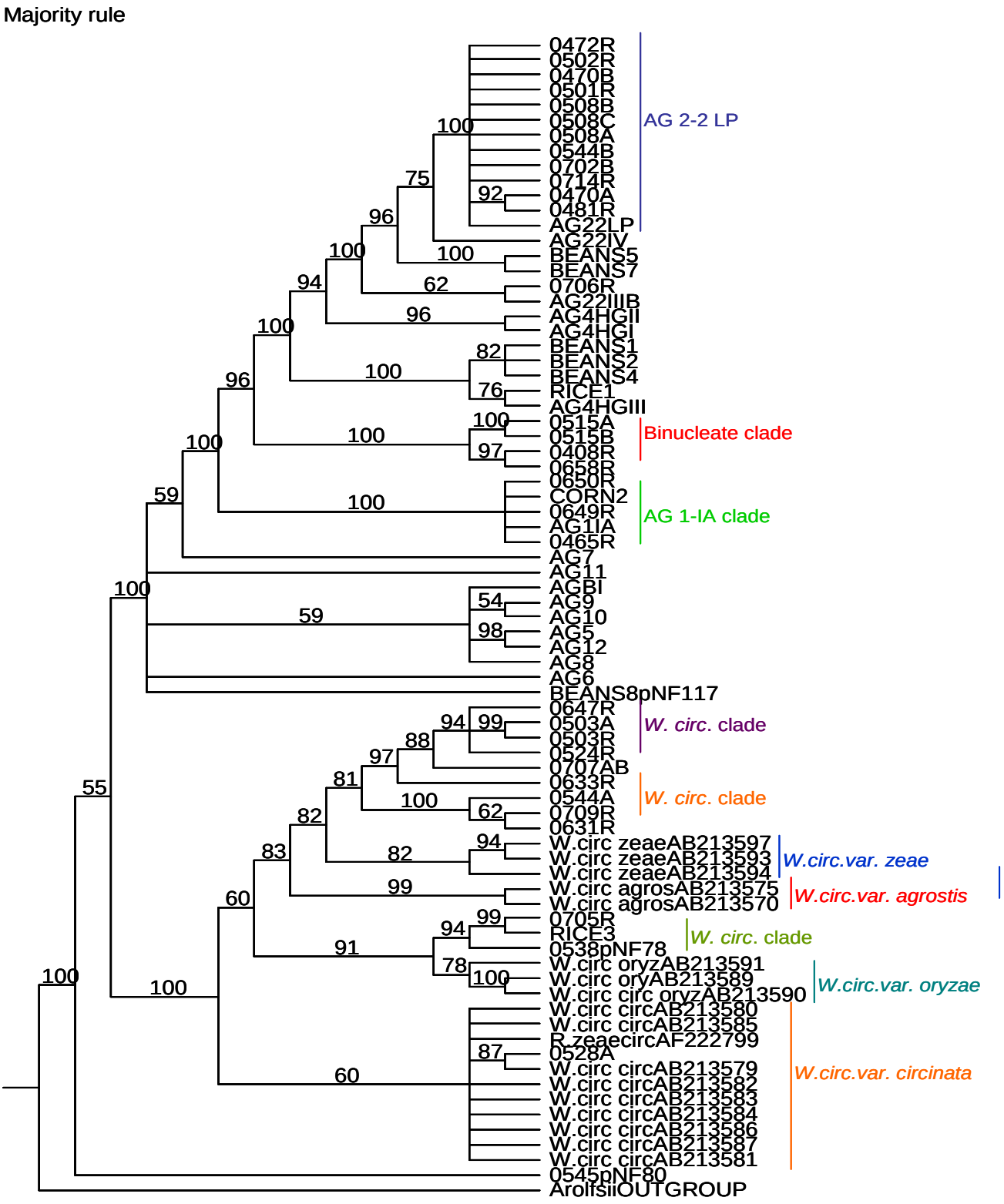


Figure 3-1. Fifty percent majority rule consensus tree from Bayesian inference analysis of rDNA ITS gen sequences. Number above branches indicates posterior probability values.

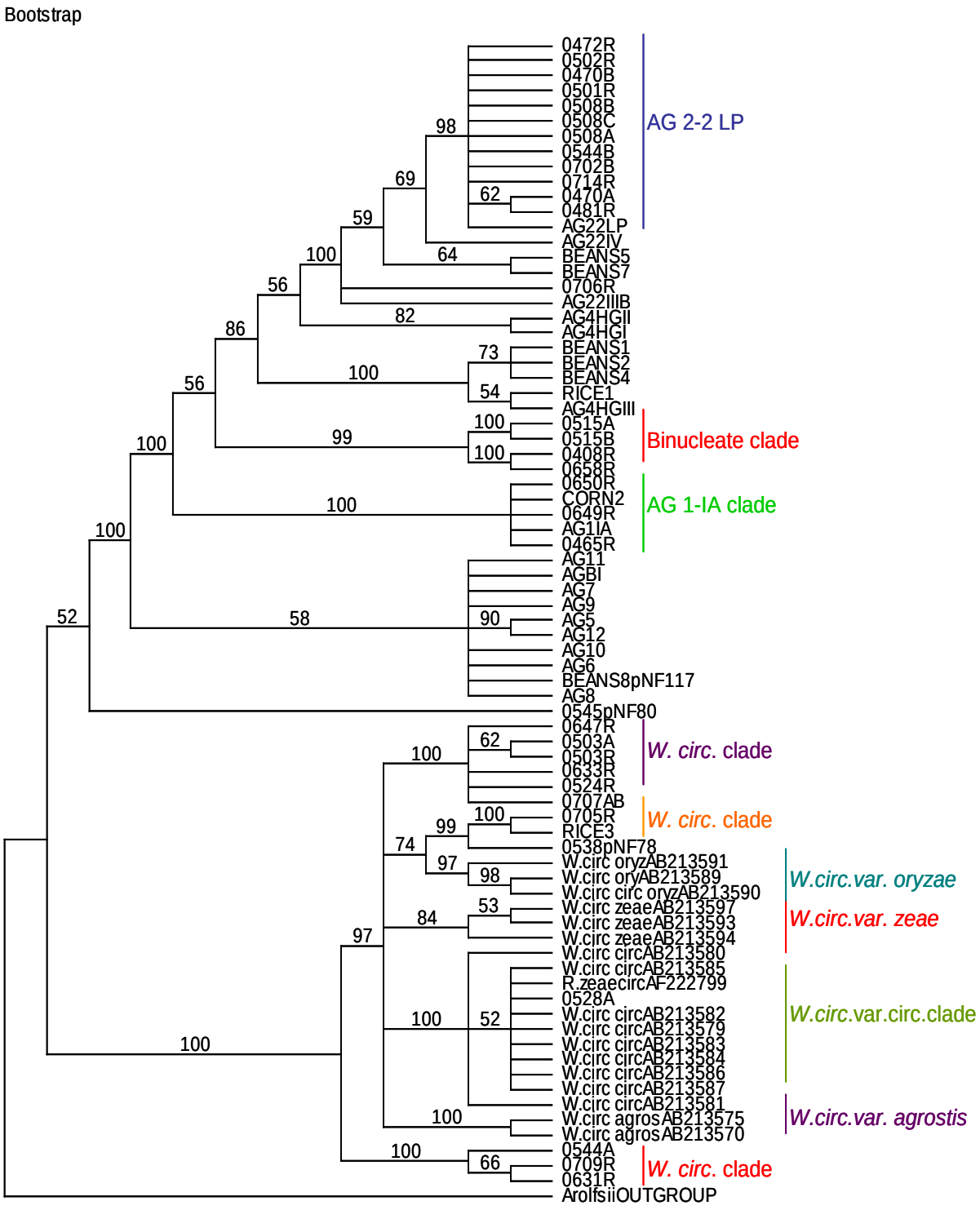


Figure 3-2. Phylogenetic tree of rDNA ITS gen sequence from Maximum Parsimony analysis. Numbers above branches indicate bootstrap value. Tree Length:1740 Consistency index (CI) :0.573Retention index (RI) : 0.915 Rescaled consistency index (RC)(RC=CI*RI): 0.524.

# CHAPTER 4
# MORPHOLOGICAL CHARACTERIZATION OF *RHIZOCTONIA* ISOLATES FROM TURFGRASSES AND OTHER HOSTS IN FLORIDA

## Introduction

Members of the genus *Rhizoctonia* include a wide collection of pathogenic, saprophytic, and endophytic species with varying morphological features, host ranges, and geographical distributions (101). Many groups within the genus have been considered species complexes (19, 35, 36, 47, 61) with overlapping and variable taxonomy and morphology. Taxonomy of the genus has undergone significant revision in recent years based on sequence data of DNA regions of phylogenetic importance, such as internal transcribed spacer (ITS) regions and recently, beta tubulin genes (20, 27, 34, 35, 38, 47, 53, 60, 61, 91, 94, 95, 110, 111, 113, 121).

Species of *Rhizoctonia* were historically differentiated primary by morphological characters (101) including affinity for hyphal fusion (anastomosis reaction) (6, 26, 53, 91, 95, 100, 101). Currently 14 anastamosis groups (AG's) have been described. However, since the genetic basis of this phenomena are not fully understood and because method (s) of determining AG's is not always accurate (5, 19, 26, 53, 95, 97, 117), the use of DNA sequence divergence has become important for additional clarification in classifying isolates in anastomosis groups.

In order to clarify taxonomy, many attempts have been made to associate morphological features and sequence data (95). As a result, knowledge about this fungus has improved. But, many taxonomic questions remain, however.

The primary objective of this study was to characterize the morphological features of *Rhizoctonia*-like isolates collected from turfgrass and some vegetable hosts in Florida. A secondary objective was to compare these morphological features with molecular data through the use of ITS sequences.

## Materials and Methods

### Fungal Isolates Collection

The methodology for collecting isolates was described in the phylogenetics chapter three. In summary, thirty one isolates were collected from warm season grasses and one isolate was collected from a cool season grass, all from different fields in Florida, and thirteen isolates were from vegetables crops (corn, rice and snap beans).

A complete list of isolates used in this study, describing the plant host, source, year of collection is presented in Table 3-1 and 3-2 of chapter III.

Isolates were recovered from short term storage and were maintained at 25°C in the dark, and were transferred to PDA every 2 to 3 weeks. During all evaluations, isolates were maintained in Petri dishes (9 cm) containing 20 mL of Potato Dextrose Agar (PDA) medium.

### Cultural Morphology

Isolates were incubated at 5 different temperatures (23, 26, 28, 33 and 38°C) on 20 mL of PDA medium in the dark, and their morphological features were recorded at different times during the incubation period, starting 1 week after transfer. Isolates were described on the basis of the following cultural characteristics such as kind and color of colony and mycelia (aerial, flat, abundance, color), zonation (radial growth), sclerotia formation and features (color, size, location, time to production and estimated number) and finally the determination of nuclear condition. Fifteen isolates were chosen to estimate the size of sclerotial structures produced at 23, 28 and 33°C. The length and width of nine sclerotia randomly chosen from a petri dish were measured for each isolate and temperature, for a total of 27 sclerotia/isolate. Sclerotial size was estimated using the average of these measurements.

**Nuclear Condition**

A small of agar plug of each isolate from an active culture grown in PDA was placed on one side of a sterilized glass slide coated with a thin layer of water agar (1.5 %). Two slides were prepared for each isolate. Slides were located in a moist chamber and incubated at 26°C in the dark and humidity was maintained using wet paper towels. After 2 or 3 days, mycelia were stained using safranin O in distilled water (0.5 %) and destained with KOH (3%) (9). Young hyphal cells tips were observed microscopically under oil immersion (100X). Ten hyphal tips were selected from each slide and the number of nuclei per cell was recorded. A total of twenty hyphal tips per isolate were observed. Nuclei data were analyzed using Statistical Analysis System (SAS, Institute, Inc., Cary, NC) analysis of variance. Means separation by isolate was done using Fisher Least Significant Difference at $P = 0.05$ level.

**Mycelia Growth Rate**

Mycelia agar disks of 5 mm diameter were taken from the 3 day-old pure culture (grown on PDA), and were transferred to the center of 9 cm petri dishes containing 20 mL of the same medium. Petri dishes were incubated at 26, 28, 33 and 38°C in the dark. Three replications were done for each isolate and for each temperature. Each experiment was replicated 4 times. Colony diameter was measured every 24 hours, until the colony of any isolate reached the edge of the petri dish. Colony growth rate per day was calculated as the difference of radial growth between ratings. Data obtained were analyzed using Statistical Analysis System (SAS, Institute, Inc., Cary, NC) through analysis of variance for mean separation using Least Significant Difference at $P = 0.05$ level.

## Results

### Morphological Characterization

Morphological characters are summarized in (Table 4-1). Sclerotial features varied among isolates and are summarized in Table 4-2. Figure 4-4 shows the sclerotia features of the different *Rhizoctonia* spp. Sixteen isolates produced aerial mycelia with light to dark brown colonies. Ten isolates formed a group with buff appearance (Fig. 4-1 (A, B and C). Isolates 0465, 0649 and 0650 shared similarity mainly in colony color (light to dark brown) (Fig. 4-1-D and E), but isolates 0408 and 0658 were slightly different from the others. Both showed light brown colonies, with sandy appearance (Fig. 4-1-G, H and I). Isolate 0658 produced little aerial mycelia, and 0408 produced few dense aerial mycelia strands and had zonate growth at 33°C. Eleven isolates had a flat growth, feathery appearance, without or with aerial hyphae and with colony colors ranging from beige to medium yellow, orange and pink (Fig.4-2 (A-I). These isolates were further divided into three additional groups, using data from mycelial growth rate. Isolates 0647, 0503A (Fig.4-2-B), 0524, 0633(Fig. 4-2-D), 0707AB had colonies that were white or a light yellow mixed with light pink and orange pigmentation. Isolates 0705 (Fig.4-2-C) and 0538 had similar colony morphology but had more aerial mycelia that became denser with extended incubation. Isolates 0544A (Fig.4-2-I), 0709, 0631(Fig.4-2-A) and 0528A were mainly light yellow to beige without aerial mycelia. Zonation was only observed for 0633 and 0707. Colonies of 0515A, 0515B (Fig.4-2-E) and 0545 were considered unique due to their distinct morphology and were classified as *Rhizoctonia*-like isolates. Colonies were white to dark beige without aerial mycelia and with zonate growth (Table 4-1). At $38^0$C isolates were affected morphologically. Isolate 0538 produced a white and crinkled dense mycelia with cottony appearance and isolates 0503A and 0633 showed similar features, but without the crinkled growth.

**Mycelial Growth Rate**

The mycelial growth rates of the isolates are summarized in Table 4.3. Significant differences between isolates in each temperature were observed; however the growth at $25^0$C and $28^0$C was similar for most of the isolates. At 28 $^0$C , isolates had higher rates of growth, specially the *Waitea circinata* group. At 33 $^0$C, the growth of isolates from *R. solani* group decreased dramatically; only *W. circinata* isolates continue growing. Most isolates were not able to grow at $38^0$C.

**Nuclear Condition**

Number of nuclei per hyphae differed between isolates and are summarized in table 4.1. Isolates were binucleate (Fig. 28) and multinucleate (Figs. 30-32). The number of nuclei ranged from two to eleven. Other features of *Rhizoctonia solani* such dolipore septa were observed (Fig. 29).

## Discussion

Two main groups of isolates were clearly distinguished and related to suspected species. They were the *Rhizoctonia solani* group with aerial mycelia production and brown pigmentations and the *Waitea circinata* group with different colony colors and little aerial mycelia. Isolate 0545, which had very distinct features is most likely not a *Rhizoctonia* species but *Marasmius oreades*. Sclerotial features also were considered important for the differentiation of these species. Some *Rhizoctonia solani* isolates produced large sclerotial structures on the agar surface and some isolates of *W. circinata* produced small sclerotia with orange, pink, and red pigmentation. These were commonly submerged in the medium.These sclerotial observations agree with those previously reported (56), in addition, red pigmentation of sclerotia also observed for *R. zeae* isolates in this study. The presence of the three variants of *W. circinata* is supported by slight differences in colony morphology and sclerotia features. However, the

classification of three isolates (0544A, 0709 and 0631) remains uncertain. These isolates shared sclerotial features and colony morphology with the *R. zeae* isolates and with the isolate of *W. circ*.var *circinata.* However, sclerotia were produced on the agar surface and their mycelial growth rate decreased at 33°C.

Some isolates produced no real sclerotia as it has been reported previously by Sneh et al 1991 and Aoyagi et al 1998. Morphological features described in this study for that group agree with those reported for AG 2-2IIIB-IV and LP. AG's commonly associated with brown patch and large patch diseases on warm-season (IV-LP) and cool-season grasses (IIIB). At higher temperatures (33°C), mycelial growth decreased which also agrees with Hyakumachi et al 1998.

Differences in morphology clearly indicate that a number distinct species of *Rhizoctonia* are present in the UF collection from turfgrasses. All of them, except for isolate 0528A were collected from warm season grasses. Among them are: *Rhizoctonia solani* (AG 2-2 IIIB-IV and LP), *Rhizoctonia solani* (AG 1-IA), *Waitea circinata* var. *zeae*, *Waitea circinata* var. *oryzae*, *Waitea circinata* var. *circinata*, *Ceratobasidium* sp. (AG-D- *R. cerealis*), *Ceratobasidium* sp. (AG-G) and *Tricholomataceae* sp. Previous reports have indicated that *R. zeae* and *R. oryzae* occurs as pathogens of cool and warm season grasses, mostly grown in warm humid regions.

Isolate 0706, collected from bermudagrass, had similar morphology to AG 2-2 IIIB-IV isolates and tested negative for AG 2-2 LP primer (111). This isolate may be something different.

Three isolates with morphology similar to *R. solani* AG I-1A were collected from St. Augustinegrass on lawns showing brown patch (not large patch) symptoms at the end of the summer. The presence of real sclerotia and the absence of buff mycelia placed them in a different morphology group in this study. This AG is reported to cause sheath blight of rice and

sheath blight on creeping bentgrass but has not been reported to cause brown patch disease on warm season grasses. This is the first report of brown patch presumably caused by *R. solani* AG 1-IA on warm season grasses. Isolates of *W. circ*. group (var. *zeae* and *oryzae*) were collected mainly from bermudagrass, but also from *seashore paspalum* and St. Augustinegrass. Binucleate isolates were collected from bermudagrass and *W. circ. var circinata* was isolated from *Poa trivialis.*

Colony features were not substantially different between temperatures of 23 to 33°C within groups of isolates. Changes in morphology were evident for some isolates at 33°C. Only several isolates of *W. circ*. group were able to grow at 38°C, but their growth rates were not significantly higher (11 mm/day) than at lower temperatures. Growth at this temperature could help preliminarily determine if isolates may be in this group.

Growth rate of the isolates were similar between 25 and 28°C, but 23 isolates had increased growth rates at 28°C which was optimal for growth. Growth of 0515A and 0515B (*R. cerealis*) was reduced at 28°C, suggesting that the optimal temperature for these particular isolates is less than 25°C.

At 33°C, 23 of the isolates had a significant decrease in growth rate. Isolates in *W. circ.* group had similar rates at 28°C. Four isolates (0515A, 0515B, 0709 and 0631) were not able to grow at this temperature. Isolates 0709 and 0631 are similar to *W. circ*. var. *zeae* in morphology, so they were expected to grow at this temperature (113). However, growth rates of these two isolates were similar to 0528A (var. *circinata*), which may indicate that they are closer to *circinata* variety than *zeae* and *oryzae*. At 38 C isolates were not able to growth.

Nuclear condition was not a determinant for isolate identification. Thirty-three isolates were multinucleate and four were binucleate. This finding determined the presence of binucleate

species within the collection. Isolates with multinucleate condition showed variation between isolates. The higher numbers of nuclei were seen in isolates of *R. solani*. There was no consistent difference between young and old hyphae (data not shown). Although hyphal diameter was not measured, it was observed during nuclei counts that hyphal cells of *R. solani* isolates were noticeably wider than those of *W. circ*. group.

Although, morphological features were relatively stable at different temperatures, they should only be considered a step in correct identification of the pathogen. Many authors report that they are not totally stable. Additional information, like molecular data (gene sequencing or PCR tests) and/or morphology under different culture media should be combined to support observations of morphological features under specific conditions to approach a correct isolate identification.

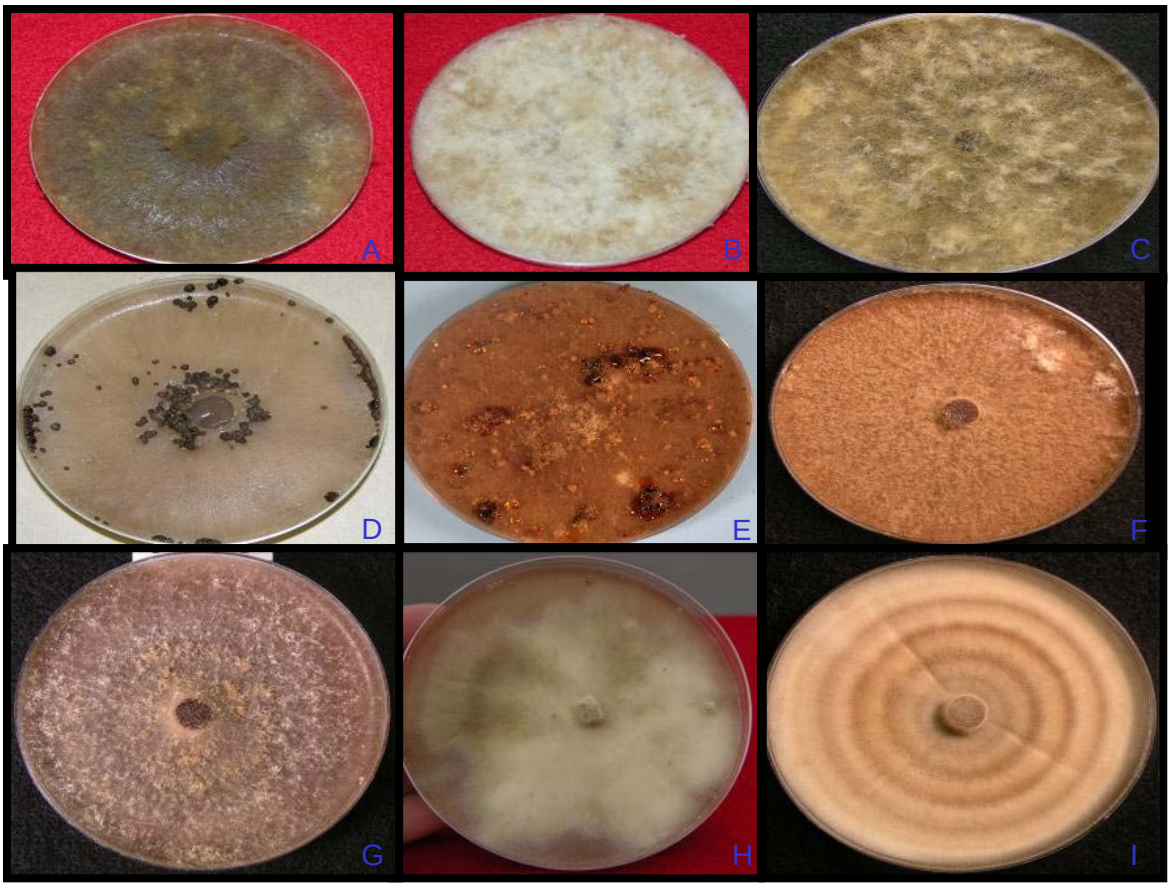


Figure 4-1. Colony features of *Rhizoctonia* isolates.A) to C). Isolates of AG 2- 2IIIB-IV: 0481, 0502, 0544B. D)-E). Isolates of AG I-1A: 0465 and 0650. F). 0706. G) to I). *Ceratobasidium* sp AG-G: 0658 and 0408 at 28 and 33 $^{0}$C.


Figure 4-2. Colony features of *Rhizoctonia* isolates. A). 0631 (*R. zeae- W. circinata*). B). 0503A (*W. circinata* var. circinata). C). 0705 (*R. oryzae- W. circ*.). D). 0633 (*R. zeae- W. circinata*). E). 0515A-B (*R. cerealis- Ceratobasidium* AG-D). F)-G). AG HG-III: Beans 2 and 4. H). Beans 7 (*T. cucumeris- R. solani*). I). 0544A (*W. circinata*)

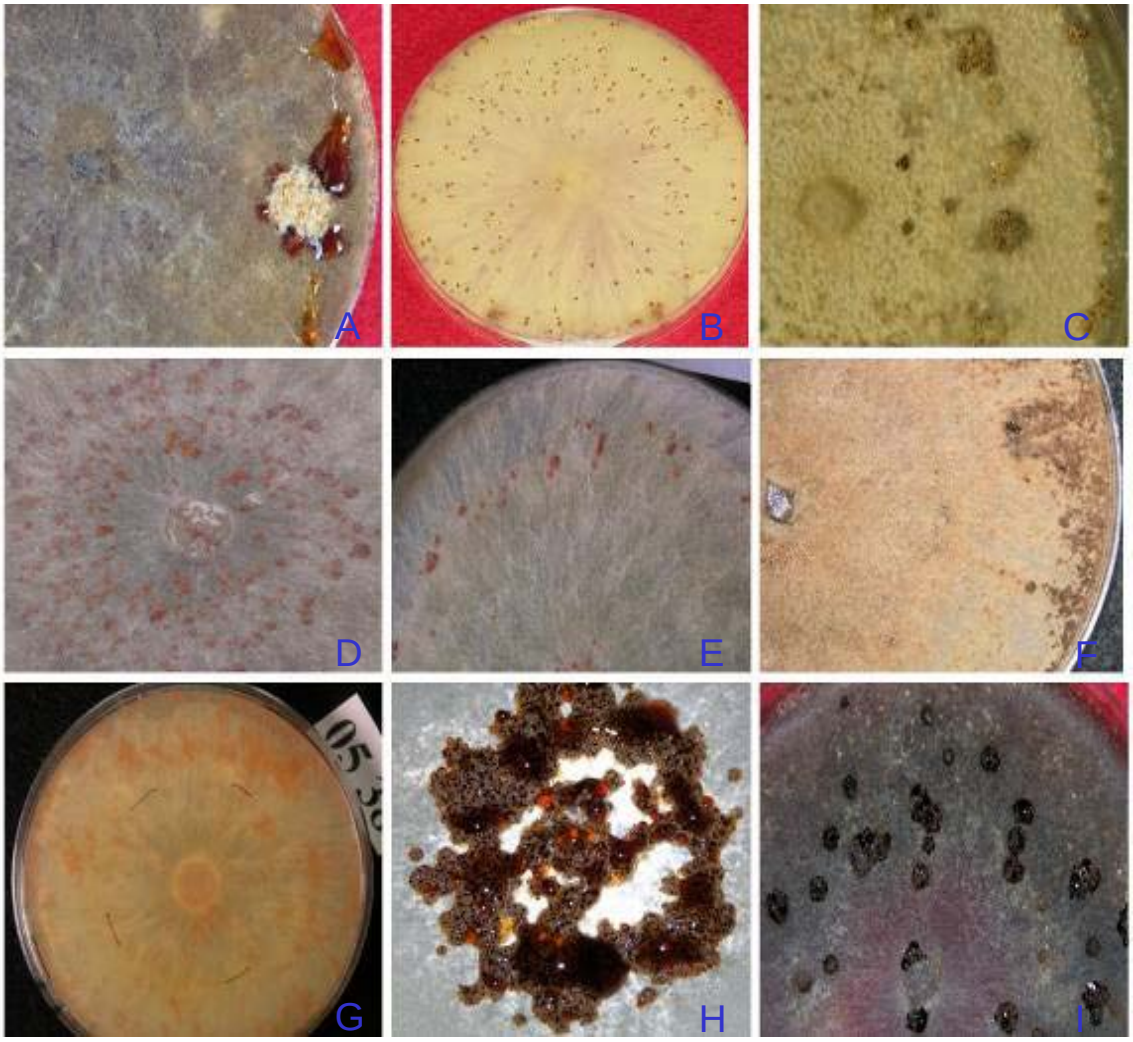


Figure 4-3. Different classes of sclerotia of *Rhizoctonia* species. A). 0470A. B). 0503A. C). 0515A-B. D). 0524. E). 0647. F). Beans 7. G). 0538. H). Rice 1. I). Rice 4.

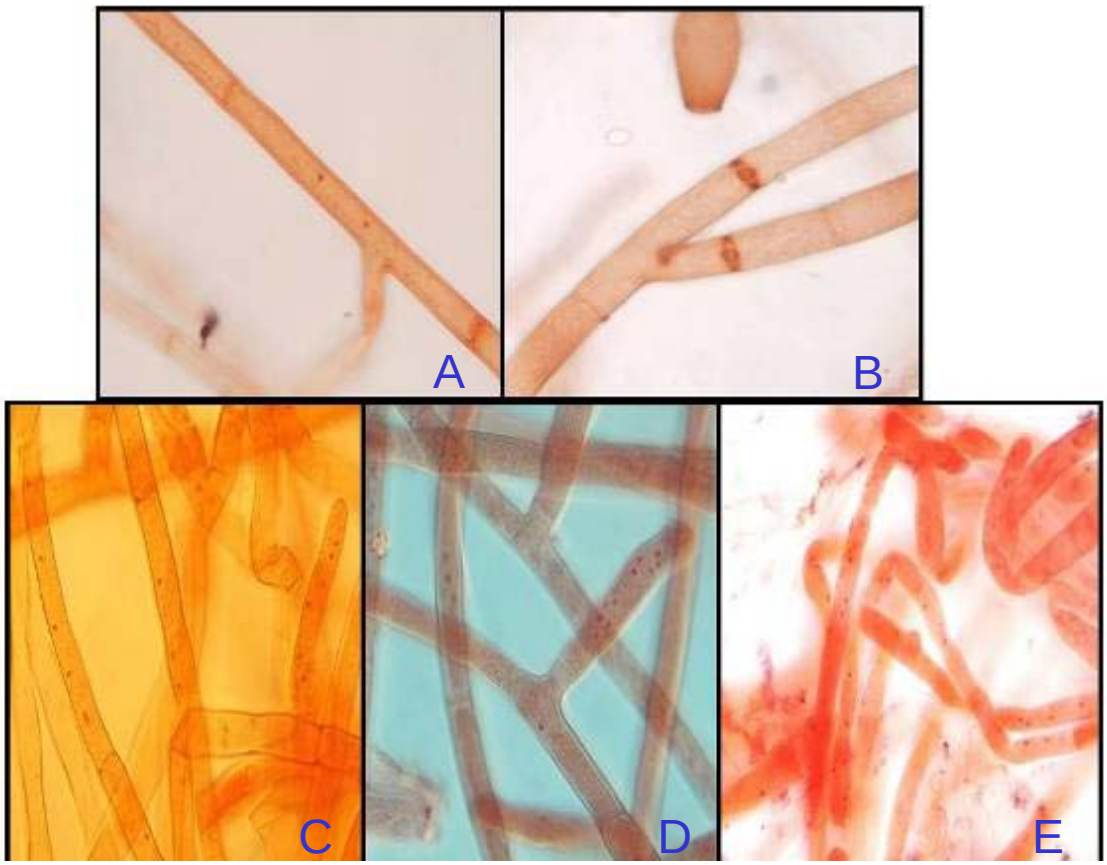


Figure 4-4. Nuclear condition of *Rhizoctonia* species A). Binucleate condition on isolate 0408. B). Dolipore septa of *Rhizoctonia solani* isolate. C) to E). Different nuclei number in multinucleate isolates.

Table 4-1. Morphological features of *Rhizoctonia* spp.

| Isolate | Nuclei/cell | Colony description 23-33 $^0$C | Color 23-33 $^0$C |
|---|---|---|---|
| 0502 | 6 | Light brown aerial mycelia. Buff appearance. | Light-dark brown colony |
| 0470B | 5.3 | Light brown thin aerial mycelia. Buff appearance. | Light -dark brown colony. |
| 0501 | 4.7 | Light brown dense aerial mycelia. Buff appearance. | Light -dark brown colony. |
| 0508A | 6.0 | Light brown thin aerial mycelia. Buff appearance. | Light -dark brown colony. |
| 0508B | 10.5 | Light brown thin aerial mycelia. Buff appearance. | Light -dark brown colony. |
| 0508C | 6.2 | Light brown thin aerial mycelia. Buff appearance. | Light -dark brown colony. |
| 0702B | 8.8 | Dark beige-dark brown dense aerial mycelia. | Dark beige-dark brown colony. |
| 0714 | 6.3 | Light brown dense aerial mycelia. Buff appearance. | Light -dark brown colony. |
| 0470A | 5.7 | Light brown thin aerial mycelia. Buff appearance. | Light -dark brown colony. |
| 0481 | 10.1 | Light brown thin aerial mycelia. Buff appearance. | Light -dark brown colony. |
| 0508B | 10.5 | Light brown thin aerial mycelia. Buff appearance. | Light -dark brown colony. |
| 0544B | 5.9 | Light brown dense aerial mycelia. Buff appearance. | Light-dark brown colony. |
| 0472 | 6.6 | Thin light brown aerial mycelia. | Light brown thin aerial mycelia. Buff appearance. |
| 0515A | 2.0 | Flat growth. Sandy appearance. No aerial mycelia. | White-dark beige colony |
| 0515B | 2.0 | Flat growth. Sandy appearance. No aerial mycelia. | White-dark beige colony |
| 0706 | 6.9 | Medium brown thin aerial mycelia. Zonation. | Dark beige-medium brown colony. |
| 0408 | 2.0 | Light brown aerial mycelia. Few but dense mycelia. Zonation at 28 0C * | Light-medium brown colony. |
| 0658 | 2.0 | Thin few light brown aerial mycelia. Sandy appearance. | Light brown colony. |
| 0650 | 3.6 | Medium brown aerial mycelia. | Medium-dark brown colony. |
| 0649 | 6.5 | Light brown colony and white few-thin aerial mycelia. | Light brown colony. |
| 0465 | 4.6 | Light brown thin-few aerial mycelia. | Light dark brown colony. |
| 0647 | 2.1 | Flat growth. Few thin aerial mycelia, color dark beige. | Dark beige- dark greyish colony. |
| 0503A | 5.1 | Flat and feather growth. No aerial mycelia. | White-light yellow-pink colony |
| 0524 | 4.6 | Flat growth. Few aerial mycelia. Crinkled growth. | Dark beige- white colony. |
| 0633 | 4.6 | Flat and feather growth. No aerial mycelia. Zonation | Beige-light orange or pink colony. |
| 0707AB | 5.6 | Flat growth. Beige- white thin aerial mycelia.Zonation | Medium pink- beige colony. |
| 0631 | 5.1 | Flat colony. Feather growth. No aerial mycelia. | Beige-light yellow. |
| 0705 | 7.9 | Flat and feather growth. Dark beige-yellow thin aerial mycelia. | Dark beige-yellow colony. |
| 0538 | 6.3 | Flat and feather growth. Light yellow-white few thin aerial mycelia. | Light yellow-pink colony. |
| 0544A | 5.1 | Flat colony. Feather growth. No aerial mycelia. | Light orange |
| 0709 | 5.9 | Flat colony. No aerial mycelia. | Light yellow-beige colony. |
| 0528A | 6.5 | Flat and feather growth | Light yellow-beige colony, thiny aerial mycelia. |
| 0545 | unknown | Flat and feather growth | Dark white-light yellow colony. |

*This feature was only observed in this isolate at 28 $^0$C.

**Not or weak growth was observed at 38 $^0$C.

Table 4-2. Sclerotial features of *Rhizoctonia* spp.

| Days | Color | Size | Location | Sclerotial number | Observation |
|---|---|---|---|---|---|
| 9 | Cream, light brown | Small, very small | Above | Many, NC | Cotton appearance, mainly old growth, aggregated, round. |
| 6, 9 | Cream-ligh medium brown, light orange, dark brown with time | Small-medium, VS Small-medium | Above | Many | Mainly in new growth, round. |
| 5,6 | Medium brown, cream-beige, white | Small, very small | Above | Many, NC | Aggregated, star formation. |
| 4,5,6 | Light-medium brown, white | Very small- S-M | Above | Many, NC | Star formation- scattered, aggregated, No real sclerotia. |
| 4, 6, 9 | Light-medium brown | Small-medium | Above | Many, NC | Aggregated circular, new growth, round. |
| 4, 5, 6 | White young, medium brown-brown | Small- medium-big | Above | Many, C (74-100 at 9 day) | Some with exudate,new-old growth. Some like tumors. |
| 9 | Orange, salmon | Small-medium | A-below | Many, NC | New growth. Aggregated more noticeable with time, round. |
| 6,9 | Dark brown, light brown | Small, very small | Above | Many, NC | Distributed in all media, aggregated circular. |
| 5, 6 | Cream, light brown, dark brown with time. | Small-medium Medium (15 days) | Above Below | Many, NC Few | Cotton balls when young, mainly in old growth. |
| 5,9 | White, beige | Small-medium | Above | Many, NC | Aggregated circular, old growth, cotton. |
| 5,9 | White, some light brown | Small-very small | Above | Many, NC | Aggregated circular, old growth, cotton, exudate, round,new growth. |
| 6, 9 | Cream, light pink, white,light brown | Very small,s-m, some B | Above | Many, NC | Aggregated, scattered, cotton, old growth, orange exudate. Round , mass appearance. |
| 3,4,5 | Brown, white young | Medium-big, big with time | Above | Many, C | Approximately 100-122 sclerotia at 9 days, new growth. |
| 12 | Dark orange, dark red with time | Very small | A-below | Few | Round, found in medium growth. 60 at 15 days. |
| 12 | Dark orange, dark red with time | Small | A-some B | Approx, 45 | Mainly in new growth, but also distributed, round. |
| 5 | Light brown | Very small | Below | Many, NC | Aggregated, star formation. |
| 15 | Light orange, salmon | Very small-medium | Above-B | Many, NC | Mainly new growth, round. |
| 19 | Light brown | Small | | | Star formation. |
| 19 | Cream | Medium | Above | | Mainly in new growth. |
| 19 | Light brown | Small | | | Star formation. |
| 19 | Light brown | Small | Above | | |

all, S: small, M: medium, B: big.
a: ≤ 1.3 mm, medium: 1.3 - 3.0 mm, big: > 3.0 mm.
elow.
ıtable, sclerotia-like, C:countable

Table 4-3. Mycelial growth rate of *Rhizoctonia* spp at different temperatures.

| | Colony growth rate (cm/day) | | | |
|---|---|---|---|---|
| Isolate | 25 | 28 | 33 | 38 |
| 0502 | 1.4 | 1.4 | 0.6 | 0.05 |
| 0470B | 1.7 | 1.9 | 0.5 | 0.12 |
| 0501 | 1.5 | 1.7 | 0.6 | 0.18 |
| 0508A | 1.9 | 2.3 | 0.96 | 0.07 |
| 0508B | 1.9 | 2.3 | 1.0 | 0.16 |
| 0508C | 2.0 | 2.2 | 1.3 | 0.17 |
| 0702B | 1.4 | 1.3 | 0.2 | 0 |
| 0714 | 1.9 | 2.2 | 0.8 | 0.13 |
| 0470A | 1.7 | 2.0 | 0.6 | 0.06 |
| 0481 | 1.6 | 1.6 | 0.8 | 0.07 |
| 0544B | - | - | - | - |
| 0472 | - | - | - | - |
| 0515A | 0.87 | 0.71 | 0 | 0 |
| 0515B | 0.84 | 0.62 | 0 | 0 |
| 0408 | 1.3 | 1.8 | 1.5 | 0.40 |
| 0658 | 1.2 | 1.6 | 1.4 | 0.77 |
| 0650 | 1.9 | 2.8 | 1.2 | 0 |
| 0649 | 2.1 | 2.5 | 1.7 | 0.18 |
| 0465 | 2.3 | 2.7 | 1.7 | 0.21 |
| 0647 | 1.8 | 2.5 | 2.1 | 0.58 |
| 0503A | 2.0 | 2.6 | 2.6 | 0 |
| 0524 | 2.0 | 2.8 | 2.7 | 1.0 |
| 0633 | 2.0 | 2.8 | 2.7 | 1.1 |
| 0707AB | 1.4 | 2.3 | 1.9 | 0 |
| 0631 | 2.3 | 2.4 | 0.2 | 0 |
| 0705 | 2.0 | 2.6 | 2.0 | 0.30 |
| 0538 | 2.2 | 2.8 | 2.4 | 0.70 |
| 0544A | 2.0 | 2.5 | 1.3 | 0 |
| 0709 | 1.8 | 1.5 | 0 | 0 |
| 0528A | 2.0 | 2.4 | 1.6 | 0.37 |
| 0545 | 1.0 | 1.7 | 1.8 | 0.27 |

* Means of mycelial growth rate were analyzed using Fisher Least Significant Difference at $P<0.05$ level.

# CHAPTER 5
# SUMMARY AND CONCLUSIONS

Growth chamber inoculations of diploid and triploid St. Augustinegrass (SAG) genotypes with different morphotypes (Longicaudatus=long bladed leaves and Breviflorus= dwarf-semi dwarf plants) using brown patch and large patch isolates resulted on successful infections for both diseases. Delay in disease response was observed on some genotypes at 48 h after the inoculation; however by the end of the experiments, no material was found to be immune to the isolates. Genotypes showed different levels of susceptibility, with long bladed genotypes less affected in terms of AUDPC, rate parameter and final severity. Ploidy level had no effect on disease response; indicating that additional factors associated with resistance are may be present besides ploidy.

Structural mechanisms of defense against Rhizoctonia suggested by many authors appeared not to play a role on SAG genotypes; however, experiments were not designed to elucidate resistance mechanisms. Brown patch (BP) isolate was confirmed as anastomosis group 1-IA, reported causing BP on different cool season grasses, but not previously reported on warm season grasses, which constitutes a possible first report of this AG in Florida.

AUDPC was the most informative disease measurement to evaluate genotypes for their disease response. Response of the genotypes was different for both isolates, therefore, future screening studies should be focused on evaluating materials against large patch disease, the most economical and serious disease on warm season grasses. Potential effects of environmental conditions and plant age should be studied to understand better this pathosystem better.

Effectiveness of the AG 2-2 LP primers and the protocol was observed through the presence of thirteen positive isolates from warm season grasses, confirming the reports of this AG made by different authors. Only one isolate was identified as AG 2-2 IIIB. Cloning

procedure, not commonly used for *Rhizoctonia* researchers was successful for the obtention of excellent quality of sequence data of the ITS rDNA gene. The heterozygous condition of the fungus was clearly observed through nucleotides differences on sequence data of different clones of the same isolate. For phylogenetic analysis of this species complex it is highly suggested to use molecular methodologies that produce unambiguous data. Thus, data from different research groups can be used confidently to perform different analyses.

Tree topology using the most common phylogenetic analyses was highly similar, supporting the results obtained. However, maximum parsimony and Bayesian analysis explained the data better. *R. solani, W. circinata, A. rolfsii* and *M. oreades* isolates were clearly differentiated in the trees. Within *R. solani* clade, some AG tester's isolates appeared to be as independent evolutionary units with possible polyphaletic origin.

Different varieties of *W. circinata* species were well differentiated in the clade. However, three isolates of the UF collection could not be easily identified as *W. circinata* varieties. A high level of sequence variation was observed in the rDNA ITS 1-2 regions of *Rhizoctonia* spp; however 5.8s rDNA gene appears to be highly conserved, mainly within species.

One isolate of *Poa trivialis*, a cool season grass, was identified as *Waitea circinata* var. *circinata* for first time in Florida. Morphological features and inoculations to observe its pathogenicity, corroborated these results.

AG 1-IA and AG 4-HG II and AG 4-HG III were isolated from vegetables crops. The analysis also confirmed the presence the AG 1-IA isolates collected on St. Augustinegrass, a warm season grass. Apparently, it may also constitute the first report of this AG on the host.

Morphology features of the isolates were highly correlated with phylogenetic clades.

Currently, phylogenetic and molecular systematics of *Rhizoctonia* species are one of the most principal subject of research. Thus, results of sequence data obtained with this study, are considered a great contribution for actual and future investigations.

Based on morphology, isolates were also clearly differentiated. Four groups were formed. The first one grouped isolates with dark brown colonies, aerial and dense mycelia production, without the presence of sclerotia. The second group showed brown colonies, with thiny aerial mycelia or absence of it and with production of evident a lot of units of sclerotia on surface. The third group, only had few isolates, showing brown colonies, with zonation, dense aerial mycelia, and without sclerotia formation. All of these isolates were classified within *Rhizoctonia solani* group.

The last group, was conformed by isolates with colonies showing: beige, light yellow, orange and pink colors, with flat, feather growth, and thiny aerial mycelia. The group was further divided based on sclerotia features. Some isolates produced these structures only on agar surface, others only submerged and some in both locations of the dish. They were smaller than *R.solani* isolates, and usually showed similar colony colors. These isolates were classified within *W. circinata* group.

Morphology was agree with description of the AG 2-2 LP, AG 1-IA, *R. oryzae, R. zeae* and *R. circinata* species, reported in the literature. Morphology and mycelial growth rate were similar in temperature ranges from 23 $^{0}$C to 33 $^{0}$C using PDA media and dark conditions. At higher temperatures such as 38 $^{0}$C, mycelial growth rate decreased, dramatically for *R. solani* isolates. *Waitea circinata* isolates were able to continue growing as reported in the literature. Nuclei condition was only informative to classify isolates on multinucleate and binucleate

condition. In multinucleate isolates, the nuclear number did not provide significant information to differentiate isolates.

APPENDIX A
SAS PROC MIXED CODE TO ANALYZE INTRINSEC RATE OF GROWTH

```
OPTIONS PS=65 LS=70;
DATA norma1;
input cultivar $ rep rpar exp;
cards;
NUF146      1     0.13516     1
NUF146      1     .           2
NUF146      2     0.1476      1
NUF146      2     0.20164     2
NUF146      3     0.65787     1
NUF146      3     0.08368     2
NUF152      1     0.26027     1
NUF152      1     0.21972     2
NUF152      2     0.23461     1
NUF152      2     0.29863     2
NUF152      3     0.51835     1
NUF152      3     0.41415     2
NUF157      1     0.16642     1
NUF157      1     0.11741     2
NUF157      2     0.16769     1
NUF157      2     0.24907     2
NUF157      3     0.43378     1
NUF157      3     0.20164     2
NUF171      1     0.30187     1
NUF171      1     0.22255     2
NUF171      2     0.1976      1
NUF171      2     0.04626     2
NUF171      3     0.1579      1
NUF171      3     0.0715      2
;
proc mixed data=norma1;
class exp cultivar;
model rpar=cultivar/outp=out_resid DDFM=SAT;
random exp;
lsmeans cultivar/pdiff adjust=tukey;
run;
proc gplot data=out_resid;
plot resid*pred;
plot pred*rpar;
run;
proc univariate data=out_resid normal plot;
var resid;
run;
```

* The same SAS code was used to analyze AUDPC and final severity for Brown patch and Large patch inoculations.

APPENDIX B
SAS PROC CONTRAST TO ANALYZE THE EFFECT OF PLOIDY AND MORPHOTYPES OVER DISEASE RESPONSE

```
title 'Experiment 1-0465';
data experiment1;
input cultivar $ rep AUDPC Ymax r;
cards;









;
proc glm;
class cultivar;

model audpc Ymax r = cultivar;

contrast'ploidy level 2 vs 3' cultivar 7 7 -13 -13 7 -13 7 7 7 7 7 7 7 -13
-13 7 7 -13 -13;

contrast 'morpho long vs short' cultivar 9 9 9 9 -11 9 9 -11 9 -11 -11 -11 -
11 9 9 9 -11 -11 9 -11;

means cultivar/LSD;


lsmeans cultivar/stderr tdiff;
run;
```

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# BIOGRAPHICAL SKETCH

Norma Cristina Flor, was born in Cali, Valle del Cauca, Colombia in 1975 to Carlos and Nelly. She only have one older sister, Marta Nelly, who is an economist and likes to work in the financial world. Norma is an Agronomist Engineer, like her father. She got her degree in December 1998 at Universidad Nacional de Colombia-Palmira. At International Center of Tropical Agriculture-CIAT-, she worked with the fungus *Pyricularia grisea* as her research topic along with Dr. Fernando Correa-Victoria, a rice pathologist. The research was twice awarded at national level. Then, she started to work to Dr. Daniel Debouck, a genetist, on cassava, *Manihot sculenta* Crantz., with four quarantine importance viruses. At CIAT she spend more than 8 years of her professional carreer.

On August 2005, she came to Gainesville to pursue her master's degree in plant pathology with Dr. Lawrence Datnoff, Dr. Philip Harmon, Dr. Richard Raid and Dr. Rusell Nagata. Her research was focused on characterizing *Rhizoctonia* isolates associated with warm season grasses at morphological and molecular level.

She arrived alone. Then, she got married with Juan Carlos, a Computer Engineer. Suddenly, she realized that she was going to enjoy the company of Isabella and Natalia, her two beautiful twins, who were born on July 2008. Now, Norma is planning to get a job related to her academic formation to continue working in her most favorite topic: plant pathology. Eventually, she would like to continue with her studies to pursue a Ph.D degree.