5-2016

Characterization and Control of Ophiosphaerella spp. Causing Spring Dead Spot of Bermudagrass in South Carolina, USA and Buenos Aires, Argentina

Alejandro L. Canegallo
Clemson University, acanega@g.clemson.edu

CHARACTERIZATION AND CONTROL OF *OPHIOSPHAERELLA* SPP. CAUSING SPRING DEAD SPOT OF BERMUDAGRASS IN SOUTH CAROLINA, USA AND BUENOS AIRES, ARGENTINA

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Plant and Environmental Sciences

---

by
Alejandro L. Canegallo
May 2016

Accepted by:
Dr. S. Bruce Martin, Committee Chair
Dr. Lambert B. McCarty
Dr. Paula Agudelo
Dr. Julia Kerrigan
Dr. Lane P. Tredway

ABSTRACT

Spring dead spot (SDS) disease is an important root disease of bermudagrass (*Cynodon* spp.) in transition zone habitats around the world. Causal agents have been identified in the U.S. as *Ophiosphaerella korrae* (OK), *O. herpotricha* (OH), and *O. narmari*, but they have not been identified in Argentina (ARG).

Pure cultures were obtained from symptomatic roots from six different bermudagrass cultivars from 24 different golf courses in SC (a total of 274 samples). In ARG, a total of 188 samples were collected from 17 different locations in the Buenos Aires province (BA), including golf courses, polo fields and a sod farm. 50 more samples were collected from three golf courses outside BA and one golf course in Uruguay. SDS pathogens were isolated from 78.5% of all soil cores collected in SC and 60.9% in ARG. The ITS region of genomic ribosomal DNA extracted from mycelia was amplified using the primers specific for OK: OKITS1/OKITS2, OH: OHITS1/OHITS2, and ON: ONITS1/ONITS2 to identify the causal agents of SDS of bermudagrass in SC and ARG. In SC, OKITS primers amplified a 454-bp fragment from 203 cultures out of 212. The amplification produced with OKITS primer set confirmed *O. korrae* as the causal agent of SDS in SC. 131 PCR products were successfully sequenced for phylogenetic analysis and 10 polymorphic sites were identified when they were compared with each other. Analysis of molecular variance (AMOVA) was used to compare variation by location or origin of isolates or by bermudagrass cultivar as the isolate source. Results of AMOVA showed that grouping populations by geographical regions better explained genetic diversity among *O. korrae* isolates: 43.27% of the genetic variation was due to

geographical regions, while percentage of genetic diversity due to bermudagrass cultivar isolate source was 21.33%.

In ARG, OHITS primers amplified a 454-bp fragment from all 145 cultures. This is the first report of *O. herpotricha* as the causal agent of SDS in ARG. 85 consensus sequences were obtained and 23 polymorphic sites identified. AMOVA analysis showed that 49.75% of the genetic variation was due to geographical regions. Grouped by cultivars, percentage of genetic variability due to bermudagrass cultivar isolate source was 24.17%. Pairwise F_{ST} showed no significant differences between BA and outside BA isolates but significant differences when compared them to isolates from the Midwest USA (Missouri and Oklahoma).

Using another approach to compare SDS fungi, amplified fragment length polymorphism (AFLP) was performed with 2 samples from each location where isolates where collected, both in SC and ARG. Isolates from Oklahoma, Missouri, North Carolina, and California were also included in this study. 53 genotypes corresponding to *O. korrae* and 59 to *O. herpotricha* were successfully amplified. A total of 256 fragments ranging in size from 50 to 500 bp were scored using two primer combination sets. Percentage of genetic similarity within *O. korrae* was higher than the percentage of genetic similarity of *O. herpotricha*. AMOVA for *O. korrae* accessions grouped by geographical regions showed that 62.32% of the genetic variability was among regions. F_{ST} pairwise comparisons showed significant genetic variation among the isolates collected in the three regions of SC. Genetic structure was observed with the program Structure and two genetically different clusters were observed, with some isolates

partially allocated to both groups, suggesting admixture between the two groups. AMOVA of accessions of *O. herpotricha* grouped by geographical regions showed that 73.45% of the genetic variability was due to different geographical regions. F_{ST} pairwise comparisons showed significant genetic variation between isolates from BA and outside BA, but also between ARG and US isolates. Genetic structure of *O. herpotricha* isolates analyzed with Structure resulted in three genetically different clusters, where all the isolates from ARG fall into the same cluster but some are partially allocated in any of the other groups. These partial allocations suggest some degree of admixture among different genetic groups.

Results of both molecular approaches support the conclusion that genetic variability of Ophiosphaerella spp. is due to geographical regions and it is not dependent on the host bermudagrass cultivar.

Some management practices influential in reducing SDS in the USA were examined in Argentina. A split-split plot randomized complete block design, 4 block experiment was conducted with nitrogen source as the whole plot, with aerification or no aerification (subplot), and no fungicide or fungicides as sub subplots. The experiment was placed on a 'Tifway' practice tee at Pilará Golf Club, Pilar, BA (Argentina) in 2012, 2013, and 2014. Disease severity (DS) and turf quality (TQ) were visually evaluated. Disease incidence (Din) and disease intensity (DIt) were evaluated using digital images assessed with the computer program Sigma Scan 80 Pro v. 5.0.

Ammonium sulfate reduced soil pH at root zone depths. There were no differences between ammonium sulfate vs. calcium nitrate on DS ant TQ in 2012 and 2014 while

calcium nitrate reduced DS and improved TQ in 2013. Aerification did not reduce DS or improve TQ. DS was best reduced by two fall applications of tebuconazole at 0.0875 gr/m^2. Two applications of fenarimol at 0.144 gr/m^2 also suppressed SDS in 2013. Both fungicides reduced SDS but did not differ in 2014. Ammonium sulfate best reduced Din in 2012, calcium nitrate was best in 2013 while no significant differences were found in 2014. Ammonium sulfate was the best source of nitrogen to reduce DIt both in 2013 and 2014. Hollow tine aerification reduced DIt in 2013 but no significant differences were found in 2014. DIn was reduced by the use of every fungicide applied when compared with the untreated control during the three years of the study, but no significant differences were found among fungicides. DIt was best reduced by two applications of tebuconazole at 0.175 gr/m^2 followed by two applications of fenarimol at 0.144 gr/m^2 and two applications of tebuconazole at 0.0875 gr/m^2. Based on this experiment, acceptable reduction of SDS requires fungicide treatment.

DEDICATION

This work is dedicated to my beloved wife Ivana and my loving daughter Justa. Without your unconditional love and support, this journey would have been far more difficult.

To my parents Maria Elena and Oscar, I would not be where I am today without their unwavering love and support throughout the years.

ACKNOWLEDGMENTS

I would first like to thank my major professor Dr. Bruce Martin, the most phenomenal source of inspiration I have ever had. Thank you so much for taking me in your graduate program, for being such a great mentor, teacher, advisor and friend.

I would like to express sincere gratitude to the members of my doctoral committee: Dr. Paula Agudelo, Dr. Lane Tredway, Dr. Bert McCarty, and Dr. Julia Kerrigan for their support and guidance throughout my time at Clemson.

To my friend Dr. Earl Elsner, who first encouraged me to come to Clemson. Thank you for all of your wonderful advice but much more for being always our family in the US.

I would like to give special thanks to Dr. Wonkeun John Park. Through countless hours of mentorship, he taught me how to deal with molecular biology. My friend, I would have never completed this work without your help and encouragement.

I want to thank Dr. Matias Vanotti who was always open to answer any question, and offered valuable advice whenever it was needed.

I would like to express gratitude to Dr. Sergio Marchant, for helping me to analyze PCR and AFLP results.

I also want to thank the people at Clemson University - Pee Dee Research and Education Center, Dr. Matt Smith, Dr. Bruce Fortnum, Dr. Francis Reay Jones, Dr. JC Chong, Ms. Sheila Goldwin, Mrs. Laurie Gregg McBride, Ms. Mandy Cribb Stephan, Ms. Pam Barnhill, Mr. Albert Lynn, Mr. Brandon Rodgers, Mr. Ryan Graham, Mr. Lanair Johnson, and Mr. Sam Goodman, for being a welcoming family.

My sincere gratitude to Mrs. Tammy Morton, for being always helpful and keep things running smoothly.

Thanks to my fellow graduate students Claudia Holguin, Brad Shaver, Saleh Ahmed, and Luke Dant, for his camaraderie, friendship, and advice.

Special thanks to Ing. Julio Alizon García, Ing. Guillermo Busso, and Ing. Santiago Zapiola, for their friendship, support and cooperation on taking great care of our business during my time in SC.

To Mr. Federico Zemborain, Mr. Diego Maidana, and Mr. Ruben Santa Maria, thank you so much for helping me make all of my applications, ratings, and pictures, and for all of your wonderful advice and friendship. Gratitude also to Pilará Golf Club and Nordelta Golf Club, for allowing me to place my experiments in their facilities.

I would like to thank the Universidad Católica Argentina for providing the lab space for microbiology and molecular work in Buenos Aires, and Dr. Jorge Galotta, Ing. Marcelo Schang, Ing. Gabriela Lalanne, Dr. Renata Cantoro, Dr. María Luz Zapiola, and Ing. Lucas Grajales for helping during all those years.

Thanks to John Mattheis, Scott and Rocio Brown, Fred and Ines Gillier, for making Florence and their homes our home.

To all my friends both in Argentina and the US, for their support and encouragement.

Finally, I want to thank all the golf clubs in Argentina and Uruguay where I serve as agronomist consultant, and their golf course superintendents who supported me through all these years.

TABLE OF CONTENTS

Table of Contents (Continued)

Page

LIST OF TABLES

List of Tables (Continued)

Table Page

LIST OF FIGURES

List of Figures (Continued)

Figure Page

List of Figures (Continued)

Figure Page

List of Figures (Continued)

Figure Page

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

The game of golf has been played in South Carolina since 1786 when Scottish immigrants, in a park known as Harleston's Green, founded the South Carolina Golf Club in Charleston. It is considered the first golf club established in the US (LynksMagazine, 2015). Today there are about 380 golf courses through the state (LynksMagazine, 2015). Located between the parallels 32°N and 35°N, South Carolina has a humid subtropical climate along the coast and coastal plain but a more temperate climate in the Upstate (northwestern region). In the summer, South Carolina is hot and humid, with daytime temperatures averaging between 30 and 34°C in most of the state and overnight lows averaging 19–24°C. Winter temperatures are much less uniform: coastal areas of the state have very mild winters, with high temperatures approaching an average of 16 °C and overnight low temperatures of 5–8°C. Inland, the average overnight low is around 0°C and well below freezing in the Upstate.

In South America, as in North America, the British people were responsible for the arrival of the game of golf. In 1890, San Martin Golf Club was established and its golf course was constructed in the Buenos Aires Province, Argentina (*Anonymous*, 2011). In 2015, there were 166 golf courses in the Buenos Aires Province registered in the Asociación Argentina de Golf. On the opposite side of the Americas, the Buenos Aires Province climate is temperate. The Northern part of the province, where most of the golf courses are located is between the parallels 33°S and 34°S. Summers are warm and humid with day temperatures between 28 and 32°C and night temperatures between 16

and 20°C. Winters are cool and dry, with highs between 13 and 18°C and nights between 2 and 5°C, with some waves of cold weather but temperature never falls below -4°C (Sala, 2015).

Bermudagrass and Use

Bermudagrass (*Cynodon spp*.), one of the most important warm season turfgrasses, (also known as couch grass in Australia and gramilla in Argentina), is the predominant species on golf courses both in South Carolina and the Buenos Aires Province. This perennial C4 grass originated in eastern Africa and now is widely distributed throughout most of the humid, tropical and subtropical regions of the world (Beard 1973). It grows between the latitudes of 45° N and 45° S (McCarty, 2005). Turf-type bermudagrasses include: 1) common bermudagrass (*C. dactylon* (L.) Pers. var. dactylon), a tetraploid with 36 chromosomes; 2) African bermudagrass (*C. transvaalensis* Burtt-Davy), a diploid of 18 chromosomes; and 3) the sterile triploid hybrid cultivars (*C. dactylon* (L.) Pers x *C. transvaalensis*), with 27 chromosomes, which are the most useful turfgrasses for golf course fairways and tees in Buenos Aires and in South Carolina. (McCarty, 2005). Hybrid bermudagrasses are widely used in polo fields as well.

Turf-type bermudagrasses are vigorous, dense, and aggressive, with leaf width ranging from medium to very fine texture. Color ranges from light green to very dark green and they are adapted to a wide range of soil conditions, a pH range from 5.5 to 7.5 (Beard, 1973) with excellent drought and salt tolerance (Beard, 1973; McCarty, 2005). Propagation is primarily vegetative by sprigs, sod or plugs, although common

bermudagrass is also sometimes propagated by seed. Establishment is fast, it spreads very rapidly by stolons and rhizomes, and recovery potential and wear tolerance are very good.

Bermudagrass requires full sunlight to perform best, having poor shade tolerance. As with all C-4 grasses, summer time is when bermudagrass grows best, with optimal temperatures for above-ground biomass production of 29 to 47 °C, and for root growth, 24 to 35 °C (McCarty, 2005). At the end of the summer, as days become shorter and temperatures decrease, its growth is slowed to a point where it becomes dormant at freezing temperatures in winter time. In the transitions zones, defined as the area or zone where both warm-season and cool-season turf species grow but sub-optimally (McCarty, 2005), bermudagrass remains dormant throughout the winter and resumes growing during the spring, as temperatures reach around 13-15 °C and the sunlight hours increase. Bermudagrass responds positively to the increase in temperatures and photoperiod and grows from the rhizomes and stolons, organs that store energy necessary for spring green-up.

Bermudagrass is susceptible to several nematodes, insect pests, and fungal disease problems. In transition zone areas, spring dead spot (SDS), a root disease caused by fungi in the genus *Ophiosphaerella*, is the most devastating disease of bermudagrass.

Spring Dead Spot of Bermudagrass

Spring dead spot (SDS) is caused by three different species of the genus *Ophiosphaerella:* 1) *O. korrae* (J. C. Walker and A. M. Smith) Shoemaker and Babcoock

(synonym: *Leptosphaeria korrae* Walker and Smith), 2) *O. herpotricha* (Fr.:Fr.) J. C. Walker, and 3) *O. narmari* (J. C. Walker and A. M. Smith) Wetzel, Hulbert and Tisserat (synonym: *L. narmari* Walker and Smith) (Wetzel et al., 1999a). The taxonomic classification of the genus *Ophiosphaerella* is as follows: Kingdom Eumycota, Phyllum Ascomycota, Class Dothidiomycetes, Subclass Pleosporomycete, Order Pleosporales, and Family Phaenosphaeriaceae (Schoch, 2006; Taylor et al., 2015).

SDS was first observed in common bermudagrass on a golf course in Oklahoma in 1936 but it was not officially named 'spring dead spot' until 1961 (Wadsworth and Young, 1961). However, in these earlier studies, the causal agent was thought to be a fungus in the genus *Helminthosporium*. The University of Georgia developed a three-year SDS research program in the mid 1960´s but again the symptoms were associated with species of *Pythium, Helinthosporium, Fusarium* and *Curvularia* (Kozelnicky, 1974).

In 1971, A. J. Smith first identified the causal agent of SDS of couch grass in Australia. It was a root colonizing fungus that produced dark-brown to black septate hyphae and with an ectotrophic growth visible microscopically along roots or stolons. It was first named *Ophiobulus herpotrichus* but its taxonomic status has changed over time: it was classified as *Leptosphaeria korrae* in a further taxonomic review in 1972 by Walker and Smith. It was later re-described by Shoemaker and Babcock in 1987 and reclassified as *Ophiosphaerella korrae* based on morphological characteristics of the ascoma cell walls and ascospores that matched the type description of *Ophiosphaerella*.

O. korrae is the prevalent causal organism of SDS of bermudagrass in the eastern United States where identifications have been conducted in North Carolina (Butler, 2004;

Iriarte et al., 2004) Mississippi (Perry and Tomaso-Peterson, 2006); Alabama, South Carolina, Virginia, West Virginia, and Kentucky (Iriarte et al., 2004). *O. korrae* was also identified as the causal agent of SDS in bermudagrass in California (Endo et al., 1985). It attacks other grasses as well. *O. korrae* was isolated from diseased roots of zoysiagrass (*Zoysia spp*.) (Tredway, 2011; Tredway and Butler, 2007). In cool season turfgrasses, *O. korrae* was identified as the organism causing necrotic ring spot of Kentucky bluegrass (*Poa pratensis* L) (Raffle and Hsiang, 1998; Smiley et al., 1986; Worf et al., 1986), annual bluegrass (*Poa annua* L) (Landschoot and June, 1996) and creeping red fescue (*Festuca rubra* L) (Dernoeden et al., 1995).

Leptosphaeria narmari, a second and similar root colonizing organism, was identified in 1972 as the most frequently encountered organism capable of producing SDS of couch grass in Australia (Walker and Smith, 1972). It was also identified in New Zealand (Walker and Smith, 1972) on couchgrass. In 1999 and based on molecular evidence, Wetzel et al. reported for the first time the presence of *L. narmari* in North America and proposed a change in its name to *Ophiosphaerella narmari* Wetzel, Hulbert & Tisserat based on the internal transcribed spacer (ITS) region similarities with *O. korrae* and *O. herpotricha* (Wetzel et al., 1999a).

In the US, *Ophiosphaerella herpotricha* has been reported as the causal organism of SDS of bermudagrass in Kansas and Oklahoma (Wetzel et al., 1999b), Kentucky, and less prevalent in North Carolina (Butler, 2004; Iriarte et al., 2004). DNA of *O. herpotricha* was similarly amplified from buffalograss (*Buchloe dactyloides* (Nutt)

Engelm.) and zoysiagrass (*Zoysia japonica* Steud.) roots collected from field-grown plants (Tisserat et al., 1994).

Symptoms and Signs of Spring Dead Spot of Bermudagrass

Symptoms are circular patches of bleached dead grass (Smith, 1971) and rings that are observed in the spring when bermudagrass resumes growth (Tredway et al., 2009b). Patches look sunken and more evident when temperatures increase in the early spring. In Australia and California patches are visible also during the fall and winter (Smiley et al., 2005). They reach from 10-20 cm. to 1 m. in diameter when patches coalesce. Roots of bermudagrass affected by *O. herpotricha* and *O. korrae* lose color and decrease root dry weight (Tisserat et al., 1989). Necrotic stolons, rhizomes and roots can be observed inside the dead patches. This produces a very slow recovery of the turfgrass, mainly thorough the healthy stolons that grow into the patches from its perimeter.

Ophiosphaerella Species Identification Based on Morphological Characteristics

The *Ophiosphaerella* species isolated from bermudagrass roots produce aerial mycelium when cultured in a specific media of potato dextrose agar (PDA) and on its host produces brown, septate, and branched hyphae 2.5-5 µm wide that can coalesce to form dark hyphal mats. It has ascocarps that form within stromal or are grouped in locules. The mature ascocarp, the pseudothecium, is partially or entirely immersed in the host tissue, and is not produced in culture unless there is host tissue present, and after a period of six to eight weeks incubation at 22 C (Landschoot, 1994). Another

characteristic of the genus is the presence of bitunicate asci, which possess a thin outer wall and a thick inner wall.

When cultured in a specific PDA medium, *O. korrae* produces a mycelium that is septate, aerial, hyaline, and white to light grey in color (Flores et al., 2012), and the cultures become light to dark grey from the center to the margins. Flask shaped pseudothecia may appear in nature, with dimensions of 300-550 µm tall x 300-500 µm wide, neck with thickened ridges at the base 50-150 µm length and 200 µm width, bitunicate asci, cylindrical to clavate, 150-185 µm in length and 10-13 µm width. Ascospores are filiform and slightly twisted in the ascus, 140-170 µm in length and 4-5 µm width, and contain up to 15 septa (Landschoot, 1994). In California, *L. korrae* (*O. korrae*) was isolated from diseased bermudagrass, cultured and first described morphologically compared to a culture produced from a single germinating ascospore previously described as *L. korrae* by J. Walker (Endo et al., 1985).

O. herpotricha produces cottony white mycelium turning tan to brown. Cultures grown on PDA become light to dark brown from the center to the margins. Pseudothecia are dark brown or black, spherical or flattened at the base, surrounded by dark brown thick-walled hairs, with body dimensions of 250-400 µm, neck 100-200 µm length and 80 µm width, with cylindrical to club-shaped asci, 150-185 µm in length and 10-13 µm wide. Asocospores are filiform and slightly twisted in the ascus, 150-190 µm in length and 7-9 µm width, that contain 8 to 20 septa (Landschoot, 1994). In 1989, Tisserat et al. described the morphological characteristics of *O. herpotricha* after isolation, culture, and inoculation of healthy plants in the greenhouse in Kansas.

O. narmari produce white to buff mycelium darkening as colony ages. On the host it forms 2.5-5.0 µm wide hyphae that is brown, septate and branched, and can form thicker strands 40-400 µm diameter-flattened sclerotia-like structures (Smith, 1971). Cultures on PDA produces white or buff, sparse aerial mycelium (Tredway et al., 2009b). *O. narmari* produce black, flask shaped pseudothecia 500-700 µm tall x 650 µm wide, neck lined with upwardly projecting periphysis with 2 thickened ridges at the base 100-300 µm length, and 300-450 µm width. Bitunicate asci are clavate with foot shaped base, 110-145 µm in length and 11-13 µm width, produce bi-seriate ascospores, pale brown, elliptical to fusiform. Ascospores measure 45-62 µm in length, 4-5 µm width and possess 3 to 5 septa (Landschoot, 1994).

Ophiosphaerella Species Identification Based on Molecular Techniques

Even though there are certain differences among species based on morphological characteristics (Landschoot, 1994), identification of the cause of SDS is complicated because the pathogens are morphologically similar in culture and do not produce ascocarps in artificial media (Tisserat et al., 1994). In this way, identification of the *Ophiosphaerella* species without using molecular techniques is time consuming and sometimes can be inconclusive.

Most phylogenetic analyses in fungi employ nuclear ribosomal DNA (rDNA), which contains tandem repeat of the rDNA genes, 28S, 5.8S and 18S, and non-genic or spacer regions, the ITS1 and ITS2 (Schoch et al., 2012). The rDNA generally exists in more than 100 copies per haploid genome (Tisserat et al., 1994), so it is easy to detect.

The internal transcribed spacer (ITS) region is a small fragment of the rDNA, and is highly variable, used in many phylogenetic studies because it's easy to amplify using PCR and shows differences among very closely related taxa. (O'Donnell, 1992). This region is flanked by the two more conserved coding genes 18S (encodes for the ribosomal small sub unit, SSU) and the 28S (encodes for the large sub unit LSU) that are commonly used for identification of higher taxa.

In 1994, Tisserat et al. developed species specific primers OKITS1, OKOTS2, OHITS1, and OHITS2 to amplify the internal transcribes spacer (ITS) regions of rDNA by polymerase chain reaction (PCR) for the identification of *O. korrae* and *O. herpotricha.* One bermudagrass sample from Georgia showing SDS symptoms, 3 samples from Maryland and a sample from Kentucky were cultured and identified as *O. korrae* while 49 samples from Kansas, 2 from Oklahoma, and 4 from Kentucky were identified as *O. herpotricha* (Tisserat et al., 1994).

In 1999, Wetzel et al. reported for the first time the presence of *O. narmari* in North America when they failed to amplify the ITS region of several isolations from Kansas, Oklahoma and California using *O. korrae* and *O. herpotricha* specific primers. An intense survey was conducted between 1994 and 1996 by Wetzel et al. over two golf courses in Oklahoma and one golf course in Kansas, where 390 samples were collected from fairways number 4, 11, and 16 at Shangri-La Resort, Afton OK; 190 samples were collected from fairways number 9, 14, and 16 at South Lakes G. C. Jenks, OK; and 140 samples were collected from fairways number 13 and 14 from Independence C. C., Independence, KS. Most isolates were positively identified with species-specific

polymerase chain reaction primers but some remained unidentified. As their cultural characteristics resembled Australian isolates of *O. narmari,* the entire ITS1-5.8S-ITS2 region and the NS rDNA region of those isolations were amplified, then sequenced and compared to all *Ophiosphaerella* species, showing high similarity to Australian isolates of *O. narmari*. Wetzel, et al. 1999 went on to develop oligonucleotide primers specific for *O. narmari*.

In another study conducted by Iriarte between 1998 and 2000 throughout the Southeast region, symptomatic roots from 204 bermudagrass samples were cultured and identified using species specific primers for *O. korrae, O. herpotricha* and *O. narmari* (Iriarte et al., 2004). In Alabama (one location), most of the samples collected were identified as *O. korrae* (18 out of 20); in Kentucky, 61 samples collected from 4 different locations resulted in 20 cultures identified as *O. herpotricha* and 35 as *O. korrae*; in Mississippi 20 samples collected from one location resulted in 16 *O. korrae* identifications; in North Carolina, 46 samples from eleven locations were collected and resulted in 10 *O. herpotricha* and 30 *O. korrae*; in South Carolina, all 11 cultures from 4 locations were identified as *O. korrae*. Consistent results were obtained in Tennessee, where 15 out of 16 samples collected in only one location were *O. korrae*; in Virginia, where 22 out of 27 samples collected in two locations were *O. korrae* and West Virginia where 3 samples collected from one location were identified as *O. korrae*.

Genetic Diversity among *Ophiosphaerella* Species

Raffle and Hsiang conducted a study in 1998 in Ontario, British Columbia and Quebec in Canada and Washington State in the USA, using randomized fragment length

polymorphism (RFLP) to examine genetic variation within a population of *O. korrae*. It was isolated from necrotic ring spot diseased Kentucky bluegrass roots in a sod farm in Ontario. All 21 fungal isolates collected corresponded to the same haplotype. This characteristic was also found in other locations. When isolations from 33 distinct locations from 19 different cities were compared, only six different haplotypes were detected. This finding indicates a low level of variation in *O. korrae* from Kentucky bluegrass in North America (Raffle and Hsiang, 1998).

In Oklahoma and Kansas, Wetzel et al. collected 517 turfgrass samples from three golf courses (Wetzel et al., 1999b) and identified 445 isolates as *O. herpotricha*, 45 as *O. korrae* and 27 as *O. narmari*. High genetic variability was found among the 445 *O. herpotricha* isolates, suggesting that a higher production of ascocarps by this species would produce more sexual recombination and be the cause of this high genetic variation (Wetzel et al., 1999b). The 45 isolates of *O. korrae* identified in the previous study showed low genetic variability but the 27 isolations of *O. narmari* collected, exhibited a high degree of genetic similarity and also were genetically similar to isolates from Australia and California (Wetzel et al., 1999b).

Iriarte et al. (2004) established regional differences in the distribution of SDS pathogens, after analyzing a total of 204 *O. korrae* isolates from eight different states in the SE region of the US. Based on amplified fragment length polymorphism AFLP analysis, genetic similarities among the many *O. korrae* isolates were found. This finding suggests that the low genetic variation is due to the small amount of genotypes that were introduced during the establishment of this pathogen in this region (Iriarte et al., 2004).

By contrast, a study conducted in Kansas showed that isolates of *O. korrae* collected in southern United States, exhibited a high degree of genetic similarity, but significant differences with isolates from western regions of the US which were very similar than isolates from Australia (Wetzel et al., 1999b).

To study the genetic variation of *Ophiosphaerella agrostis* that caused dead spot in creeping bentgrass, Kaminski et al. collected 77 samples from 21 different creeping bentgrass or hybrid bermudagrass putting greens in eleven States during a 5-year period (Kaminski et al., 2006). After PCR analysis using species specific oligonucleotide primers, high genetic variability was observed when AFLP was performed on 77 *O. agrostis* isolates.

Bermudagrass Infection and Colonization by *Ophiosphaerella* Species

Infection of all species of *Ophiosphaerella* in the field typically takes place during the autumn when daily temperatures are between 12 and 14°C and soil temperatures below 21°C (Crahay et al., 1988; Smiley et al., 2005; Walker et al., 2006). However, Walker et al. (2006) also demonstrated that new roots can be colonized and infected by *O. herpotricha* with cool soil temperatures during spring time in Oklahoma.

Infection of young and small branch roots caused by vascular wilt fungi occurs through the immature cortex of the apical region (Garret, 1970). However, a study with *O. herpotricha* established that the fungus colonizes the root surface, grows radially along the longitudinal axis with some branches growing transversally between the host cells (Caasi et al., 2010). It produces a dark network of ectotrophic hyphae which appears

to directly penetrate the epidermis, colonize it intercellularly and grow into the cortex, where some cells become necrotic. Vascular infection is produced only in advanced stages of disease development. Stolons are infected and colonized over the surface as well, and extensive colonization in the hollow center of the stolons was observed in Tifway and Midlawn cultivars (Caasi et al., 2010).

Factors Affecting Spring Dead Spot Development and Severity

Several studies demonstrated poor correlation between greenhouse and field experiments, suggesting that factors other than direct colonization and rotting of roots influence SDS occurrence and severity (Baird et al., 1998; Iriarte et al., 2005; Walker et al., 2006).

Temperature

The influence of cold temperatures on SDS development and severity is consistent with observations that SDS usually develops after winter dormancy: SDS weakens bermudagrass roots and predisposes it to winter injury. McCarty et al. (1991) demonstrated that diseased grass was more susceptible to freezing temperatures, and carbohydrate reserves in shoots were lower when the plant was exposed to freezing temperatures in the fall. A study in Maryland showed that the temperature at which inoculated plants with four isolations of *L. korrae* (*O. korrae*) did not survive (15°C), was significantly lower that the optimal temperature for in vitro growth of the isolates (25°C) at which most of the inoculated plants survived (Crahay et al., 1988). This suggests that

lower temperatures favor the pathogen in the field in its competition with other soil borne pathogens and antagonistic fungi to overcome bermudagrass resistance.

Optimal air temperatures for SDS development are between 12-14 °C for all species while optimal soil temperature for infection is between 10 °C and 21 °C (Crahay et al., 1988; Smiley et al., 2005; Tisserat et al., 1989; Walker et al., 2006). Long exposures of *O. korrae* and *O. herpotricha* infected grass to low temperatures can deplete carbohydrate reserves in crown and root tissues (Dunn et al., 1980) and may reduce spring green-up up to 70% when compared with healthy grass (McCarty et al., 1991). This conclusion was also supported by Nus and Shashikumar who found in a growth chamber study that plants infected with *O. korrae* and *O. herpotricha* have less tolerance to freezing temperatures (Nus and Shashikumar, 1993). In related experiments, Gatschet et al. demonstrated that a cold tolerant bermudagrass cultivar Midiron showed higher concentration of chitinases, antifungal proteins that function in plant defense, in the crowns when compared with the least cold tolerant bermudagrass cultivar Tifgreen.

Nitrogen source and soil pH

It is well known that nitrogen sources indirectly affect nutrient uptake and have direct effects on plant growth. For example, a study conducted in India showed that wheat plants supplied with nitrate sources of nitrogen produced significantly more root length and root surface than wheat plants supplied with ammonium sulfate (Kaur and Sadana, 2010). It was also demonstrated that nitrogen sources could have an impact (positive or negative) on patch diseases caused by *Ophiosphaerella spp.* (Cottrill and Miller, 2013; Dernoeden et al., 1991; Hill et al., 2003; Huber and Mccaybuis, 1993;

Kaminski and Dernoeden, 2005b; Lucas, 1980; Smiley and Cook, 1973; Thompson et al., 1995; Tredway et al., 2011). For example, a negative effect of N supply on SDS demonstrated that high applications of ammonium nitrate in late summer increased the severity of SDS (Lucas, 1980). By contrast, other studies have demonstrated a positive influence of ammonium sulfate on patch diseases: severity of take all disease of wheat (caused by *Ophiobolus graminis* = *Gaeummanomyces graminis* var. *tritici*) was progressively less as the pH dropped below 6.8 – 7 when ammonium sulfate or ammonium phosphate was used as nitrogen source (Smiley and Cook, 1973). These authors also reported that the hyphal growth was greatest at higher soil pH in a study where runner hyphae of *G.graminis* var. *tritici* were measured on sterile wheat straw inoculated with the pathogen and buried in sterile soil. Similar results were found on the living roots of wheat seedlings (the lower the soil pH, the less hyphal growth observed).

In a field study conducted by Dernoeden et al. in Maryland, the percent of turf area affected by SDS disease caused by *L. korrae*, was less on every plot fertilized with any source of nitrogen compared with the non-treated control but the best treatment was observed in the plots treated with ammonium sulfate. In addition, color, uniformity as well as recovery were significantly better. The authors also suggested that spring green-up was enhanced by the use of ammonium fertilizer source, suggesting that acidification of soil by ammonium may help to reduce the intensity of SDS. They also reported in a greenhouse study that survival of bermudagrass in pots inoculated with *korrae* were favored by fertilization with ammonium sulfate compared with other sources of nitrogen fertilizer (Dernoeden et al., 1991).

Similarly, it was reported in Kentucky bluegrass, that summer patch symptoms (caused by *Magnaporthe poae*) were more severe when nitrate sources of fertilizer were applied than ammonium sources (Thompson et al., 1995). Kaminski et al. (2005) also demonstrated that a reduction in pH using ammonium sulfate produce a faster recovery of bentgrass turf with dead spot disease. Cotrill et al. (2013) demonstrated in a field study in Missouri that pH manipulation may reduce Spring Dead Spot of bermudagrass severity caused by *O. herpotricha* and *O. korrae* but it was not correlated with *in vitro* studies. Vincelli, 2014 showed that soil pH in the range of 5.0 to 5.3 in the rhizosphere reduced significantly SDS severity; however fungal growth of *Ophiosphaerella* species was not reduced at this pH *in vitro*. The author speculated that a more favorable environment for microorganisms antagonistic to the pathogens also provided better conditions for the plant. Therefore, several beneficial rhizosphere microorganisms can inhibit the growth of the population or the metabolic activities of soilborne pathogens. Such disease-suppressive soils are especially well studied for take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici*. This is typically associated with an increase of *Pseudomonas* spp. that produce the antifungal compound 2,4-diacetylphloroglucinol (Berendsen et al., 2012). It was found also that three different species of bacteria (*Comamonas acidovorans, Pseudomonas* spp, and *Enterobacter* spp.) act as biological control agents and helped reduce the occurrence of summer patch, caused by the fungus, *Magnaporthe poae* (Thompson et al., 1998). Biological control agents compete with fungi for space at the root surface and for nutrients, especially for iron that are essential for growth and activity of the fungi (pathogen). This competition is particularly

significant where soil pH is high that leads to low iron solubility (Raaijmakers et al., 2009).

Using ammonium based fertilizers like ammonium sulfate typically takes several years of application for pH reduction. During three years of study on previously SDS inoculated research plots, Tredway demonstrated that ammonium sulfate fertilization significantly reduced the SDS index (which combines disease severity and diameter of the patch) when *O. herpotricha* was the causal organism. But calcium nitrate fertilization produced the same positive effect reducing the SDS index when *O. korrae* was the causal agent (Tredway et al., 2009a). They also established that there was no positive or negative effect of fall applications of any amendments which reduce or raise the soil pH, suggesting there is not only a pH effect on SDS but also a nitrogen source effect.

The change in soil pH of the rhizosphere has a remarkable influence on the availability of micronutrients. The concentration of available Fe, Mn, Zn and Cu in the rhizosphere increased when pH decreased by the use of ammonium as source of nitrogen fertilizer and vice versa using nitrates as the source of nitrogen fertilizer (Chen et al., 2002). In addition, the concentration of these micronutrients in the leaves was positively correlated with the level of available nutrients in the rhizosphere. Manganese (Mn) is required in higher plants in much higher concentration than by fungi and bacteria and that appears to be an opportunity for the pathogen to exploit this difference in requirement (Marschner in (Dordas 2009)). Mn is involved in respiration, photosynthesis, and plant hormone metabolism, but also controls lignin and suberin biosynthesis through the activation of several enzymes (both important biochemical barriers), and the synthesis of

secondary metabolites related with the defense of plants against plant pathogens (Hill, Heckman et al. 1999, Heckman, Clarke et al. 2003, Dordas 2009). Mn deficiency in plant tissue was associated with the area where take-all disease of wheat was most severe (Huber and McCaybuis 1993).

It has been demonstrated that positive effects result with acidic soil rhizosphere pH by reducing severity of patch diseases of wheat, bentgrass and bermudagrass; it appears to be not the direct effect of soil pH on the pathogen (since *in vitro* experiments for both *O. korrae* and *O. herpotricha* did not show a correlation). These benefits have been demonstrated in field studies by Cottrill et al. (2013) and are presumed to be due to the influence of low rhizosphere pH on the availability of other nutrients, mainly manganese, and on the population of microorganisms present in the rhizosphere that can act as biological control agents. Also, it was demonstrated that potassium acidifying fertilizer potassium sulfate (K_2SO_4) did not help to reduce summer patch severity, meaning there may be a relationship between strongly acidifying nitrogen fertilizers and disease reduction (Hill et al., 2003).

Disease Management

Current research on disease management agrees with the importance of an integrated management program over consecutive years in order to reduce SDS incidence and severity, since individual practices have been inconsistent and often ineffective. This integrated management program needs to be focused on achieving healthier plants, to

tolerate the environmental conditions that favor SDS pathogen colonization and infection of roots, rhizomes, and stolons.

The use of resistant bermudagrass cultivars may be an effective strategy to reduce severity of SDS. It has been demonstrated that there are differences among cultivars in response to *Ophiosphaerella* spp. infection. Selection of the proper cultivar is one of the most important and fundamental decisions to be made when establishing a turfgrass since it must be capable of meeting the expectations for the purpose it was selected. Increased resistance to SDS has been noted in cultivars that exhibited increased freezing resistance (Iriarte et al., 2005; Martin et al., 2001). Cultivars with improved cold hardiness like Midiron, Guymon, Midfield, Yukon, and Mirage, exhibit more tolerance to spring dead spot and it was demonstrated that chitinases were observed in higher concentration in the crowns of a cold tolerant cultivar Midiron when compared with the least cold tolerant Tifgreen (Gatschet et al., 1996). Chitinases are thought to be antifungal proteins functioning in disease resistance by attacking cell walls of invading fungal pathogens and signaling plant defense response (Graham and Striclen, 1994). In winter rye (*Secale cereale*), chitinases accumulate in the plant during cold acclimation, when temperatures range from -2 °C to -5 °C (Yeh et al., 2000). During this period of acclimation, ice crystals are formed in intercellular spaces and xylem vessels of the leaves. When temperatures go further -5°C, the intercellular ice creates a vapor pressure differential that results in the progressive loss of water from the cell (Burke et al., 2000). Antifreeze proteins inhibit the growth and recrystallization of ice crystals that are formed during this cold acclimation by adsorbing onto the surface of ice crystals (Yeh et al., 2000).

Chitinase involvement in disease and cold resistance suggest the correlation between cold hardiness and SDS resistance (Baird et al., 1998).

Low mowing height, thatch accumulation and soil compaction are generally associated with increased SDS (Martin et al., 2001). Studies indicate that severe disturbance of the rootzone of bermudagrass can reduce SDS damage caused by *O. herpotricha.* Thereby, colonized roots, rhizomes and stolons are removed resulting in the production of new roots, that will be re-colonized with the inoculum present in the soil, but it would take a relatively long period of time to disrupt normal root function. It was demonstrated that yearly removal of infected soil is the best SDS cultural control, but impractical for any golf course, so a combination of intense late summer core aerification and verticutting may promote the generation of new roots (Tisserat and Fry, 1997). Raising mowing height results in higher levels of carbohydrates reserves of crowns and roots, increasing cold hardiness (McCarty et al., 1992). The use of dinitroaniline herbicides such as prodiamine and pendimethalin as pre-emergent control of spring weeds should be avoided because they may cause abnormal inhibition of bermudagrass roots tips resulting in much slower spring green-up (Beck et al., 2013).

As discussed above, studies show different results concerning the source of nitrogen. In North Carolina, a negative effect on disease severity was observed from heavy late applications of ammonium nitrate (Lucas, 1980). Tredway *et al* (2011) demonstrated the positive effect of ammonium sulfate and calcium nitrate when *O. herpotricha* and *O. korrae* respectively were the causal agents.

According to Dernoeden (1991), fertilization with potassium enhances cold hardiness and increased the survival of Tufcote bermudagrass inoculated with *L. korrae* when compared with unfertilized inoculated plants. On the other hand, Cotrill and Miller (2012) found no statistical differences on disease severity among plots receiving urea, ammonium sulfate, and calcium nitrate, however two applications of tebuconazole showed the best result when was combined with ammonium sulfate. However, McCarty (McCarty et al., 1992) found that fall applications of potassium sulfate increased SDS incidence. For turf recovery, increased nitrogen fertilization needs to be applied during the spring to fill in the patches.

Concerning chemical control, scientists have been testing fungicides for spring dead spot control since 1960 with variable results (Dernoeden, 1993). Reasons for the erratic outcomes are many, since epidemiology of spring dead spot and plant-fungus interaction below ground and inside the roots are not well understood. There has not been an established exact soil temperature that triggers infection, and there is a general lack of specific recommendations regarding product effectiveness, application timing or application methods. Also aggressiveness of the three different causal organisms in different regions may explain inconsistencies in fungicide performance. Tredway et al. 2008 established that systemic fungicides must be sprayed before dormancy therefore the active ingredient can be absorbed and translocated by an active growing plant. It is also important to deliver the fungicide to the infected plant organs through irrigation prior to drying. Morning applications when dew is present and immediate activation of the irrigation system may increase the effectiveness of the fungicide treatment (Walker,

2010). In the Mid-Western US, attempts for SDS control were less successful than in the Eastern US (Tredway et al., 2009b). When disease pressure is high, two fall applications of fungicides were not sufficient for SDS control (Walker, 2010). Suppression of SDS caused by *O. herpotricha* have been erratic in Kansas trials when fenarimol, propiconazole, myclobutanyl and azoxystrobin were tested to reduce disease severity (Tisserat and Kennelly, 2008). Similar results were obtained in Missouri in 2011-2012 and 2012-2013 at no significant differences occurred in disease severity among fungicide plots treated with fenarimol, tebuconazole, thiophanate methyl and untreated control (Earlywine and Miller, 2013; Earlywine and Miller, 2014). However Walker (2005) found significant reduction on SDS incidence with propiconazole, fenarimol and tebuconazole in Oklahoma. Research in the southern US has shown positive results. In 1980 Lucas established in North Carolina that the best timing for preventive SDS control with fungicides was before bermudagrass goes dormant (Lucas, 1980). He reduced spring dead spot incidence and severity applying high rates of benomyl (two to five times more than its normal rate for most turf diseases) on a golf course fairway that showed severe symptoms the previous year. In North Carolina, it was also found a wide window exists in the fall to apply preventive fungicide when soil temperatures ranged from 15°C to 27°C, since similar results on SDS control were obtained when treatments were applied in late summer and in late fall (Butler and Tredway, 2006). Wetzel (2001) tested fungicides for preventive SDS control in Tifway bermudagrass and found only fenarimol provided significant disease incidence reduction. Similar results were obtained by Tredway and Butler (2011) with two fall applications of fenarimol on Tifway

bermudagrass. In South Carolina, the greatest reduction in spring dead spot incidence was observed with 2 or 3 preventive fall applications of fenarimol, while late winter applications provided some control but resulted in unacceptable turf quality (Luc and Martin, 2006). In Argentina, two consecutive experiments were conducted on TifEagle bermudagrass, where preventive applications of fenarimol showed the highest disease reduction followed by two applications of myclobutanil (Luc et al., 2005).

At this time, it appears that best cultural management practices will not provide an acceptable level of disease control but are necessary as a part of an integrated long term management of spring dead spot together with the application of recommended fungicides, which have also demonstrated various levels of inconsistencies (Walker, 2009).

Objectives of a Two-Hemisphere Study

During the last decade, SDS has been a tremendous problem for golf courses and polo fields in the Buenos Aires Province, where bermudagrasses are the main turfgrass species. Since the early 2000s, several golf courses and polo fields in Argentina were planted with several cultivars of certified bermudagrass from sources in the Southeastern US, and those were the most affected by SDS. Besides the reduced playability, safety, and aesthetic quality, the economic impact on facility budgets, either because of reduced revenue or the cost of pesticide application has been enormous. Therefore, a study was conducted in both hemispheres to determine the variability between the pathogen affecting bermudagrass in South Carolina and the Buenos Ares Province, the potential

correlations with cultivars and locations, and to determine if best practices for SDS control developed in the northern hemisphere are applicable to Argentina

The isolation and identification through a combination of classic and molecular techniques of the pathogen causing SDS were the first two objectives of this research. Samples were collected and isolations of the pathogen were realized both in South Carolina and Buenos Aires. Isolates were analyzed first according to traditional description of the cultures, identification of the organisms, geographic location, photographs, etc. This was also complemented and supported by the molecular analysis of the DNA of the pathogen, to determine genetic variability across the regions under study, and define if the inoculum causing SDS in Argentina were transported with the plant material imported from North America. At the same time, knowing the population of the causal organism of SDS in Argentina, it would be possible to determine if management practices developed in the northern hemisphere can be translated to Argentina.

Finally, improved disease control measures are what the industry needs. Thereby, a field experiment was carried out in the Buenos Aires Province to evaluate the combined effect of three different nitrogen sources, two aerification treatments, and four fungicide treatments, to develop best practices for management of SDS in Argentina.

Literature Cited

Anonymous. (2011) Los primeros pasos, Asociacion Argentina de Golf http://www.aag.com.ar/aag2011-dev/articulo051120110002225.asp.

Baird J.H., Martin D.L., Taliaferro C.M., Payton M.E., Tisserat N.A. (1998) Bermudagrass resistance to spring dead spot caused by *Ophiosphaerella herpotricha*. Plant Disease 82:771-774.

Beard J.B. (1973) Turfgrass Science and Culture Prentice Hall Inc. Englewood Cliffs, NJ 07632.

Beck L.L., Cooper T., Hephner A., Straw C., Henry G. (2013) Effect of pre-emergence herbicides on bermudagrass recovery from SDS, Golf Course Management. pp. 80-85.

Berendsen R., Pieterse C.M.J., Bakker P.A.H.M. (2012) The rhizosphere microbiome and plant health. Trends in Plant Science 17:478-486.

Burke M.J., Anderson J.A., Buchanan D.W., Rajashekar C.B., George M.F. (2000) Evidence for large negative pressure potentials in plant cells during freezing.

Butler E.L. (2004) Development of novel strategies for control of spring sead spot of bermudagrass, Department of Plant Pathology, North Carolina State Unversity, Raleigh, NC.

Butler E.L., Tredway L.P. (2006) Method and timing of fungicide application for control of spring dead spot in hybrid bermudagrass, Plant Health Progress, doi: 10.1094/PHP-2006-0901-01-RS.

Caasi O.C., Walker N.R., Marek S.M., Enis J.N., Mitchell T.K. (2010) Infection and colonization of turf-type bermudagrass by *Ophiosphaerella herpotricha* expressing green or red fluorescent proteins. Phytopathology 100:415-423.

Cottrill D.J., Miller G.L. (2013) Impact of nitrogen source and pH on mycelial growth of the spring dead spot pathogens, *Ophiosphaerella herpotricha* and *O. korrae*, 2013 APS Meeting.

Crahay J.H., Dernoeden P.H., O'Neill N.R. (1988) Growth and pathogenicity of *Leptosphaeria korrae* in bermudagrass. Plant Disease 72:945-949.

Chen, Han, Zhou. (2002) The rhizosphere pH change of *Pinus koraiensis* seedlings as affected by N sources of different levels and its effect on the availability and uptake of Fe, Mn, Cu, and Zn. Journal of Forrestry Research 13:37-40.

Dernoeden P.H. (1993) Integrating strategies for the management of patch diseases caused by ectotrophic fungi, Turfgrass Patch Diseases, APS Press The American Phytopathological Society - St Paul, Minnesota. pp. 123-161.

Dernoeden P.H., Crahay J.N., Davis D.B. (1991) Spring Dead Spot and Bermudagrass Quality as Influenced by Nitrogen-Source and Potassium. Crop Science 31:1674-1680.

Dernoeden P.H., Zhang M., Wetzel H.C. (1995) First report of necrotic ring spot (*Leptosphaeria korrae*) in creeping red fescue in Maryland. Plant Disease 79:966.

Dunn J.H., Nelson C.J., Sebaugh J.L. (1980) Characterization of thatch, rhizomes, carbohydrates, and spring dead spot in twenty cultivars of bermudagrass (Abstract). Journal of the American Society for Horticultural Science 105:653-657.

Earlywine, Miller. (2013) Evaluation of fungicides for spring dead spot control on bermudagrass, Division of Plant Science University of Missouri Columbia, MO 65201.

Earlywine D., Miller G. (2014) Evaluation of Torque and 3336f for spring dead spot control on bermudagrass, 2012-2013, Plant Disease Management Reports 8:T021.

Endo R.M., Ohr H.D., Krausman E.M. (1985) *Leptosphaeria korrae*, a cause of the spring dead spot disease of bermudgrass in California. Plant Disease 69:235-237.

Garret S.D. (1970) Pathogenic root-infecting fungi. Cambridge University Press.

Gatschet M.J., Taliaferro C.M., Porter D.R., Anderson M.P., Anderson J.A., Jackson K.W. (1996) A cold-regulated protein from bermudagrass crowns is a chitinase. Crop Science 36:712-718.

Graham L.S., Striclen M.B. (1994) Plant chitinases (abstract). Canadian Journal of Botany 72:1057-1083.

Hill W.J., Heckman J.R., Clarke B.B., Murphy J.A. (2003) Summer patch disease severity on Kentucky bluegrass in response to fertilizer source. Journal of Plant Nutrition 26:1499-1512. DOI: Doi 10.1081/Pln-120021057.

Huber D.M., Mccaybuis T.S. (1993) A Multiple Component Analysis of the Take-All Disease of Cereals. Plant Disease 77:437-447.

Iriarte F.B., Fry J.D., Martin D.L., Todd T.C., Tisserat N.A. (2005) Effect of cold acclimation and freezing on spring dead spot severity in bermudagrass. Hortscience 40:421-423.

Iriarte F.B., Wetzel H.C., III, Fry J.D., Martin D.L., Tisserat N.A. (2004) Genetic diversity and aggressiveness of *Ophiosphaerella korrae*, a cause of spring dead spot of bermudagrass. Plant Disease 88:1341-1346.

Kaminski J., Dernoeden P.H. (2005) Nitrogen source impact on dead spot (*Ophiosphaerella agrostis*) recovery in creeping bentgrass. International Turfgrass Society Research Journal 10.

Kaminski J.E., Dernoeden P.H., Mischke S., O'Neill N.R. (2006) Genetic diversity among *Ophiosphaerella agrostis* strains causing dead spot in creeping bentgrass. Plant Disease 90:146-154.

Kaur A.J., Sadana U.S. (2010) Nitrogen source and manganese application effects on manganese dynamics in the rhizosphere of wheat cultivars grown on manganese-deficient soils. Journal of Plant Nutrition 33:831-845.

Kozelnicky G.M. (1974) Updating 20 years of research: Spring dead spot on bermudagrass, USGA Green Section Record, USGA. pp. 12-15.

Landschoot P.J. (1994) Taxonomy and biology of ectotrophic root-infecting fungi associated with patch diseases of turfgrasses, in: B. B. Clarke and A. B. Gould (Eds.), Turfgrass Patch Diseases: Caused by Ectotrophic Root-Infecting Fungi, The American Phytopathological Society., St. Paul, Minnesota. pp. 41-71.

Landschoot P.J., June P.D. (1996) First report of necrotic ring spot on *Poa annua* putting greens in Pennsylvania. Plant Disease 80:712.

Luc J.E., Canegallo A., Martin S.B. (2005) Spring dead spot in bermudagrass greens in Argentina and South Carolina, Golf Course Management. pp. 92-95.

Luc J.E., Martin S.B. (2006) Control of spring dead spot in bermudagrass ptting greens in South Carolina, F&N Tests Vol 61:T010.

Lucas L.T. (1980) Control of spring dead spot of bermudagrass with fungicides in North Carolina. Plant Disease 64:868-870.

LynksMagazine. (2015) History of golf in South Carolina, Lynks Magazine http://www.linksmagazine.com/special_sections/history-of-golf-in-south-carolina.

Martin D.L., Bell G.E., Baird J.H., Taliaferro C.M., Tisserat N.A., Kuzmic R.M., Dobson D.D., Anderson J.A. (2001) Spring dead spot resistance and quality of seeded bermudagrasses under different mowing heights. Crop Science 41:451-456.

McCarty L.B. (2005) Best golf course management practices Pearson Prentice Hall - Upper Saddle RIver, NJ 07458.

McCarty L.B., DiPaola J.M., Lucas L.T. (1991) Regrowth of bermudagrass infected with spring dead spot following low temperature exposure. Crop Science 31:182-184.

McCarty L.B., Lucas L.T., Dipaola J.M. (1992) Spring dead spot occurrence in bermudagrass following fungicide and nutrient applications. Hortscience 27:1092-1093.

Nus J.L., Shashikumar K. (1993) Fungi associated with Spring Dead Spot reduces freezing resistance in bermudagrass. HortScience 28:306-307.

O'Donnell K. (1992) Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). Current Genetics 22:213-220.

Perry H., Tomaso-Peterson M. (2006) Frequency of occurrence of *Ophiosphaerella korrae* on Tifway bermudagrass roots in Mississippi. Phytopathology 96:S92.

Raaijmakers J.M., Paulitz T.C., Steinberg C., Alabouvette C., Moenne-Loccoz Y. (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant and Soil 321:341-361. DOI: Doi 10.1007/S11104-008-9568-6.

Raffle V.L., Hsiang T. (1998) Low level of DNA polymorphisms in isolates of *Leptosphaeria korrae* pathogenic on *Poa pratensis.* Can. J. Plant Path 20:48-54.

Schoch C.L. (2006) A multigene phylogeny of the Dothideomycetes using four nucrear loci. Mycologia 98:1041-1052.

Schoch C.L., Seifert K.A., Huhndorf S., Robert V., Spouge J.L., Levesque C., Chen W. (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences, USA 109:6241-6246.

Smiley R.V., Cook R.J. (1973) relationship between take-all of wheat and rhizosphere pH in soils fertilized with ammonium vs nitrate-nitrogen. Phytopathology 63:882-890.

Smiley R.W., Dernoeden P.H., Clarke B.B. (2005) Compendium of turfgrass diseases APS Press The American Phytopathological Society, St. Paul, Minnesota.

Smiley R.W., Fowler M.C., Hummel N.W., Petrovic A.M., Pidduck H.M., Strang C. (1986) Necrotic ring spot and summer patch of inoculated Kentucky bluegrasses 1:58.

Smith A.M. (1971) Spring dead spot of couch grass turf in New South Wales. The Journal of the Sports Turf Research Institute 47:54-59.

Taylor J., Spatafora J., Barbee M., Schoch C. (2015) Tree of life web project, tolweb.org/Ascomycota/20521.

Thompson D.C., Clarke B.B., Heckman J.R. (1995) Nitrogen form and rate of nitrogen and chloride application for the control of summer patch in Kentucky bluegrass. Plant Disease 79:51-56.

Thompson D.C., Kobayashi D.Y., Clarke B.B. (1998) Suppression of summer patch by rhizosphere competent bacteria and their establishment on Kentucky bluegrass. Soil Biology & Biochemistry 30:257-263. DOI: Doi 10.1016/S0038-0717(97)00114-4.

Tisserat N., Fry J. (1997) Cultural practices to reduce spring dead spot *(Ophiosphaerella herpotricha)* severity in Cynodon dactylon. Int. Turfgrass Soc. Res. J. 8:931-936.

Tisserat N., Kennelly. (2008) Spring dead spot, K-State Turfgrass Research, www.plantpath.ksu.edu/doc985.ashx.

Tisserat N.A., Hulbert S.H., Sauer K.M. (1994) Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiosphaerella korrae* and *O. herpotricha*. Phytopathology 84:478-482.

Tisserat N.A., Pair J.C., Nus A. (1989) *Ophiosphaerella herpotricha* , a cause of spring dead spot of bermudagrass in Kansas. Plant Disease 73:933-937.

Tredway, Soika, Butler. (2011) Impact of fertilization programs and preventive fungicide applications on spring dead spot in hybrid bermudagrass. , http://www.slideshare.net/turfpathology/impact-of-fertilization-programs-and-preventive-fungicides-on-spring-dead-spot.

Tredway L. (2011) Spring dead spot of zoysiagrass, NCSU, Turf Diseases: Golf Course Disease Updates by University Professors. pp. 1.

Tredway L.P., Butler E.L. (2007) First report of spring dead spot of zoysiagrass caused by *Ophiosphaerella korrae* in the United States. Plant Disease 91:1684.

Tredway L.P., Soika M.D., Butler E.L. (2009a) Response of spring dead spot caused by *Ophiosphaerella korrae* and *O. herpotricha* to fertilization programs and preventive fungicide applications, Phytopathology. pp. S129.

Tredway L.P., Tomaso-Peterson M., Perry H., Walker N.R. (2009b) Spring dead spot of bermudagrass: A challenge for researchers and turfgrass managers, APSnet, Plant Health Progress. pp. 1-18.

Vincelli P. (2005) Managing spring dead spot of bermudagrass. University of Kentuky. Cooperative Extension Service, http://www2.ca.uky.edu/agc/pubs/id/id130/id130.htm.

Wadsworth D.F., Young H.C. (1961) Spring dead spot of bermudagrass. USGA Journal and Turf Management, Reprint from Plant DIsease Reporter 44 (7) July 15, 1960.

Walker J., Smith A.M. (1972) *Leptosphaeria narmari* and *L. korrae*, two long-spored pathogens of grasses in Australia. British Mycological Society 58:459-466.

Walker N. (2010) Timing fungicide applications for spring dead spot, Golf Course Management. pp. 84-92.

Walker N.R. (2009) Influence of fungicide application timings on the management of bermudagrass spring dead spot caused by *Ophiosphaerella herpotricha*. Plant Disease 93:1341-1345.

Walker N.R., Mitchell T.K., Marek S.M. (2006) Influence of temperature and time of year on colonization of bermudagrass roots by *Ophiosphaerella herpotricha*. Plant Disease 90:1326-1330.

Wetzel H.C. (2001) Evaluation of fungicides for control of spring dead spot of bermudagrass, 2000, F&N Tests 56:T3.

Wetzel H.C., III, Hulbert S.H., Tisserat N.A. (1999a) Molecular evidence for the presence of *Ophiosphaerella narmari* n. comb., a cause of spring dead spot of bermudagrass, in North America. Mycological Research 103:981-989.

Wetzel H.C., III, Skinner D.Z., Tisserat N.A. (1999b) Geographic distribution and genetic diversity of three *Ophiosphaerella* species that cause spring dead spot of bermudagrass. Plant Disease 83:1160-1166.

Worf G.L., Stewart J.S., Avenius R.C. (1986) Necrotic ring spot disease of turfgrass in Wisconsin. Plant Disease 70:453-458.

Yeh S., Moffatt B.A., Griffith M., Xiong F., Yang D.S.C., Wiseman S.B., Sarhan F., Danyluk J., Xue Y.Q., Hew C.L., Doherty-Kirby A., Lajoie G. (2000) Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. Plant Physiology 124:1275-1284.

CHAPTER TWO

GENETIC VARIABILITY OF *OPHIOSPARELLA* SPP. CAUSING SPRING DEAD SPOT OF BERMUDAGRASS IN SOUTH CAROLINA, USA, AND BUENOS AIRES, ARGENTINA, BASED ON NUCLEAR RIBOSOMAL DNA SEQUENCES

Introduction

Spring dead spot (SDS) is the most devastating disease of bermudagrass (*Cynodon* spp.) in the southern United States, Australia, and Argentina, where common bermudagrass [*C. dactylon* (L.) Pers. var. *dactylon*] and the interspecific hybrids *C. dactylon* (L) × *C. transvaalensis* Burtt-Davy are the prevalent turfgrass species in golf courses and polo fields (Canegallo et al., 2015; Luc et al., 2005; Lucas, 1980; Smiley et al., 2005; Smith, 1971).

SDS can be caused by any of three ectotrophic root infecting fungi of the genus *Ophiosphaerella*: 1) *O. korrae* (J. C. Walker and A. M. Smith) Shoemaker and Babcock (synonym: *Leptosphaeria korrae* Walker and Smith), 2) *O. herpotricha* (Fr.:Fr.) J. C. Walker, and 3) *O. narmari* (J. C. Walker and A. M. Smith) Wetzel, Hulbert and Tisserat (synonym*: L. narmari* Walker and Smith) (Wetzel, Hulbert, and Tisserat 1999). Symptoms are circular patches of bleached dead grass (Smith, 1971) and rings that are observed in the spring when bermudagrass resumes growth (Tredway et al., 2009b). Patches reach from 0.1-0.2 m to 1 m. in diameter when patches coalesce. Patches look sunken and more evident when temperatures increase in the early spring. The influence of cold temperatures on SDS development and severity is consistent with observations that SDS usually develops after winter dormancy: SDS weakens bermudagrass roots and

predisposes it to winter injury. McCarty et al. 1991 demonstrated that diseased turfgrass was more susceptible to freezing temperatures which resulted in lower carbohydrate reserves in shoots, when the plant was exposed to freezing temperatures in the fall (McCarty et al., 1991). Long exposures of *O. korrae* and *O. herpotricha* infected grass to low temperatures can deplete carbohydrate reserves in crown and root tissues (Dunn et al., 1980). Spring green-up may be reduced up to 70% in diseased bermudagrass when compared with healthy grass (McCarty et al., 1991), what is produced mainly thorough the healthy stolons that grow into the patches from its perimeter. In Australia and California, patches are visible also during the fall and winter (Smiley et al., 2005). In the Buenos Aires Province, Argentina, severe epidemics occur every year although temperatures rarely reach the freezing point. Mean temperature for March, April, and May (fall time in the Southern hemisphere) is 18.4 °C with a minimum mean temperature of 7.7 °C for the same period, while the mean temperature for winter months (June, July and August) is 11.2 °C and the mean minimum temperature for the same period is 1.8 °C (Sala, 2015).

SDS was first observed in common bermudagrass in a golf course in Oklahoma in 1936 (Wadsworth and Young, 1961), but the causal agent was misidentified. A. J. Smith first identified the causal agent of SDS of couch grass (bermudagrass) in Australia. It was identified as a root colonizing fungus that produced dark-brown to black septate hyphae, with an ectotrophic growth visible microscopically along roots or stolons. It was first named *Ophiobolus herpotrichus* but later was classified as *Leptosphaeria korrae* in 1972 by Walker and Smith. It was later re-described by Shoemaker and Babcock in 1987 and

reclassified as *Ophiosphaerella korrae* based on morphological characteristics of the ascoma cell walls and ascospores. Also in 1972, *Leptosphaeria narmari*, a similar root colonizing fungus, was also identified and was the most frequently isolated organism producing SDS of couch grass in Australia and New Zealand (Walker and Smith, 1972). *Ophiosphaerella* species were initially identified based on morphological characteristics, where the production and size of ascospore, shape of hyphopodia, color and shape of the colony in pure culture, and the size and shape of pseudothecia, asci, and ascospores were taken into account for species identification (Landschoot, 1994). However, in 1994 in Kansas, species specific primers for the identification of *O. korrae* and *O. herpotricha* were developed (Tisserat et al., 1994) for more accurate species identification. As a result, a 454-bp fragment of the internal transcribed spacer (ITS) region of rDNA was amplified from DNA of *O. korrae* when the primers OKITS1/OKITS2 were used. In the same way, primers OHITS1/OHITS2 amplified a 454-bp fragment of the ITS region of rDNA when DNA of *O. herpotricha*. In 1999, species specific primers were developed for the identification of *O. narmari*, which was isolated in the US by Wetzel. (Wetzel et al., 1999a).

Molecular identification of the causal agents of SDS has been conducted in North Carolina (Butler, 2004; Crahay et al., 1988; Iriarte et al., 2004); Mississippi (Perry and Tomaso-Peterson, 2006); Alabama, South Carolina, Virginia, West Virginia, and Kentucky (Iriarte et al., 2004) , California (Endo et al., 1985), where *O. korrae* was found to be the prevalent species causing SDS of bermudagrass. It has also been identified as the causal agent of SDS of bermudagrass in Italy (Gullino et al., 2007). It attacks some

other warm and cool season turfgrasses grasses as well: *O. korrae* was isolated from diseased roots of zoysiagrass (*Zoysia* spp.) with spring dead spot symptoms (Tredway, 2011; Tredway and Butler, 2007). On cool season grasses it has been identified as the causal agent of necrotic ringspot in Kentucky bluegrass (*Poa pratensis* L.) (Raffle and Hsiang, 1998; Smiley et al., 1986; Worf et al., 1986), annual bluegrass (*Poa annua* L.) (Landschoot and June, 1996) and creeping red fescue (*Festuca rubra* L.) (Dernoeden et al., 1995). Similarly, *O. herpotricha* has been reported as a causal organism of SDS of bermudagrass in Kansas, Oklahoma, Kentucky (H. C. Wetzel et al., 1999; Iriarte et al., 2004; Sauer et al., 1993; Tisserat et al., 1994; Walker, 2007), and less prevalent in North Carolina (Iriarte et al., 2004). DNA of *O. herpotricha* was similarly amplified from isolates from roots of buffalograss (*Buchloe dactyloides* (Nutt) Engelm.) and zoysiagrass (*Zoysia japonica* Steud.) (Tisserat et al., 1994).

Ribosomal ITS region sequencing has been used to compare isolates of *O. korrae* and *O. herpotricha* (Tisserat et al., 1994). Furthermore, Wetzel et al. compared 159 isolates of *O. korrae*, 225 of *O. herpotricha* and 49 of *O. narmari* from Kansas, Oklahoma, California, Wisconsin, and Michigan (USA) and Australia. They demonstrated, based again on the comparison of sequences of the ITS regions of rDNA that the two species clustered into separate monophyletic groups (Wetzel et al., 1999a).

Epidemics of SDS on bermudagrass have been reported every year both in South Carolina and Buenos Aires, therefore the objectives of this study were to determine 1) prevalent species of *Ophiosphaerella* present in SC and BA, 2) genetic variation of *Ophiosphaerella spp.* between regions and among populations within each region 3)

genetic variability of *Ophiosphaerella spp* among host cultivars and among populations within cultivars, and 4) phylogenetic relationships among isolates collected in SC and ARG, and other *Ophiosphaerella* isolates collected in the US.

Materials and Methods

Sample collection

Collection of samples from bermudagrass with SDS symptoms was carried out in South Carolina, US (SC) and in Argentina, mainly in the Buenos Aires Province (BA) in 2012 and 2013. In SC 36 golf courses were surveyed but only in 22 of these were symptoms of SDS observed. Samples were collected in the following cities: Myrtle Beach, Charleston, Florence, Pageland, Cheraw, Santee, Columbia, Lexington, Aiken, North Augusta, Greenville, Clemson, and Sunset. In BA, 41 golf courses, polo fields and a sod farm were surveyed. Samples were collected from 17 locations exhibiting SDS symptoms from the following locations: Pilar, Matheu, Lujan, Tigre, General Rodriguez, Moreno, Hurlingham, Escobar, Lobos, and Pinamar. Collection of samples was completed with additional cities outside BA: Cordoba, Cordoba Province; Rosario, Santa Fe Province; and Cafayate, Salta Province (Table 1).

In order to assess genetic diversity of SDS pathogens on a local level, a first survey was conducted in two locations in SC and another two locations in Argentina. The sampling pattern was modified from a previous study in Kansas and Oklahoma. (H. C. Wetzel et al., 1999). In our survey, a 3-m-diameter circle was delineated and a turfgrass core 60.32 mm diameter, 63.5 mm in depth was removed from the edge of every

symptomatic SDS patch inside the circle. Additionally, 10 cores were randomly taken from 10 SDS patches outside but close to the circle (Figure 2.1). In SC this sampling pattern was conducted in the practice area at Myrtle Beach National and in the fairway # 10 at Columbia Country Club. In Argentina this sampling pattern was replicated at Pilará Golf Club at the fairway # 9, and at Jockey Club de Rosario over its fairway # 18.

A second sampling pattern was used to determine genetic variability of *Ophiosphaerella* spp. pathogens causing SDS of bermudagrass on a State/Province level. A total of 10 samples were randomly taken from 3 different selected sites exhibiting SDS symptoms per location surveyed both in SC and ARG. A total of 274 samples were collected in SC and 238 in ARG.

Fungal culture and DNA Isolation

Diseased turf cores (60.32 mm diameter, 63.5 mm in depth) were taken from the margins of symptomatic patches during the month of May 2012 in SC and October and November 2012 in Argentina and kept in a cooler until processed for fungal isolations. In South Carolina, at the Clemson University Pee Dee Research and Education Center (PDREC) in Florence, SC, cores were gently washed and the soil was completely removed under tap water. Plants were observed under a 10× magnification dissecting microscope and 4 to 5 pieces of 1.5 to 2 cm of symptomatic roots per sample were selected. In a laminar flow hood, roots were surface sterilized in a solution of 20 % sodium hypochlorite for 5 minutes, and blotted on sterilized paper to dry. Tissue was immediately placed into petri dishes previously prepared with a SDS semi-selective media. Media was prepared by autoclaving for 20 minutes a solution of 500 ml of

deionized water, 5 g of potato dextrose agar (PDA), 5.5 g of agar (Bacto Agar), and 50 mg of chloramphenicol. Streptomycin sulfate, 50 mg, was added to cooled liquid media (50 °C) before plates were prepared. Cultures were incubated in the dark at room temperature for 4 to 5 days and actively growing mycelium was transferred to new media prepared as described. Petri dishes were incubated in the dark at room temperature for 14 days and the morphology of the cultures were described and photographed. 216 cultures resembling previously identified *Ophiosphaerella* species were successfully isolated from 274 samples collected. A new batch of cultures was prepared prior to DNA extraction, where mycelium were transferred to new media, but at this time, three pieces of 0.5 cm × 2 cm of autoclaved filter paper were placed into each petri dish to let the mycelium grow over the paper. Once papers were colonized by the mycelium, they were harvested to store in an ultralow freezer at – 80 °C.

Cultures were grown on potato dextrose broth for DNA extraction. A solution of 40 mg l^{-1} of Difco potato dextrose broth (PDB) was prepared and 50-ml vials were filled with 40 ml of PDB solution with no antibiotics. Media were autoclaved for 20 minutes. Four to five pieces of approximately 3 mm × 10 mm agar plugs of each isolate were placed into the autoclaved vials containing PDB. Vials were placed in a rack on a shaker table with gentle agitation and incubated in the dark at room temperature for 7 days. A small ball of mycelium (0.5 to 1 cm in diameter) was extracted from each vial using a 1 ml pipette, drained into the vial, placed immediately into a 1.5-ml microcentrifuge tube and stored at – 80 °C. Genomic DNA was extracted using the Easy DNA Kit (Invitrogen Corp, Pittsburgh, PA 15275) following the protocol provided by Dr. L. Tredway

(Appendix 1). To produce the lysis of the cells, two scoops of glass beads (unwashed, 425-600 µm, Sigma Aldrich) and 175 µl of the Solution A of the Easy DNA Kit were added to each tube. Tubes were vortexed at maximum speed for 15 seconds, then placed into a bead beater (SoniBeast™ Mini-Beadbeater 24, BioSpec Products Inc.) for 3 minutes and finally vortexed for two additional minutes. After incubation of 15 minutes at 65 °C, 75 µl of the Solution B of the Easy DNA Kit were added to each tube and immediately mixed by vortexing. To remove proteins, carbohydrates, and lipids, 250 µl of chloroform was added, vortexed until a homogeneous mix was obtained, and tubes were centrifuged for 15 minutes. The upper phase was transferred to new 1.5-ml microcentrifuge tubes placed on ice containing 1 ml of solution of 95% ethanol and 2.5 µl of mussel glycogen. Tubes were placed at – 20 °C for 30 minutes and then centrifuged at maximum speed for 15 minutes for nucleic acid precipitation. Mussel glycogen is an additive that helps nucleic acids to precipitate. The 95% ethanol was decanted, a pellet was visualized at the bottom of the tubes, and 500 µl of cold 80% ethanol was added and centrifuged for 5 minutes for cleaning the rest of the contaminants while DNA and RNA are at the bottom of the tube. Ethanol was decanted and tubes were placed over a sterilized filter paper with the lids open until they were dry. Once they were completely dry, 50 µl of TE (Tris EDTA) buffer was added to dissolve the pellet and tubes were refrigerated overnight. Next day, 2.5 µl of RNAse were added to each tube and mixed thoroughly before incubation in a water bath at 37 °C for 45 minutes to remove RNA from the solution. Tubes were labeled and stored at - 20 °C.

In Argentina, the same protocol for isolations was carried out at Universidad Católica Argentina, Facultad de Ciencias Agrarias (UCA) in Buenos Aires. 145 cultures resembling *Ophiosphaerella* species were successfully isolated from 238 samples collected. DNA extraction was completed in the same lab at UCA. The process was the same as it was in SC, but the mechanical work for cell lysis was performed manually with micro pestles because a bead better was not available. Genomic DNA was stored at -20 °C. For transportation to SC for molecular analysis, 10 µl of genomic DNA of each isolate were dropped on autoclaved filter paper discs (Owens and Szalanski, 2005), where a circle was drawn with a pencil to identify the place where DNA was dropped. Papers were left until dry for 4 hours at the lab hanging from a pin, saved in plastics bags and transported to SC for analysis.

In addition to the 207 isolates from SC and the 145 from Argentina, two isolates of *O. herpotricha* from North Carolina provided by Dr. Lane Tredway were included as positive controls, as well as 17 isolates from Oklahoma provided by Dr. Nathan Walker, and 25 isolates from Missouri provided by Dr. Lee Miller. Also two isolates of *O. korrae* from a golf course in California and two isolates of *O. korrae* from a golf course in North Carolina that were sent to Dr. Martin's diagnostic lab were included as positive controls.

Amplification of ITS Region Using Polymerase Chain Reaction

Genomic DNA from SC isolates, the positive controls for *O. korrae* and *O. herpotricha*, and the isolates from NC and CA golf courses averaged a concentration of 2500 n gr μl^{-1}. Therefore all the isolates were diluted to 1:10 volume in pure water to reach a concentration of the diluted template of 250 n gr μl^{-1}. DNA isolates from

Argentina were re-suspended from filter paper. One of the two circles drawn on each filter paper disc were cut with a sterilized scissors and placed into a 1.5-ml microcentrifuge tube, where 300 µl of TE buffer was added. After 1 hour at room temperature and overnight at 4°C, DNA concentration was measured on every template, averaging 50 n gr μl^{-1}. Isolates from Oklahoma and Missouri were purified in new media as described. The same protocol was performed for DNA extraction, but DNA concentration was lower, ranging 10 to 25 n gr μl^{-1}.

For each fungal isolate, PCR was carried out with the species specific primers developed by Tisserat et al. (Tisserat et al., 1994) OKITS and OKITS2 for *Ophiosphaerella korrae*, and OHITS1 and OHITS2 for *O. herpotricha*. Amplification of the internal transcribed spacer (ITS) region was performed in a programmable thermal cycler (Bio-Rad Laboratories Inc. Hercules, CA), containing 2 µl of 10× PCR buffer, 0.4 µl of 10 nM Primer OKITS1 (5-CCAAGTGCAGCACAAACTGCATG), 0.4 µl of 10 nM primer OKITS2 (5-CCAAGTGTAGCACAAACTAGCG), 0.4 µl of dNTP, 0.1 µl of 5 u μl^{-1} Taq DNA polymerase, 1 µl of the diluted template from SC, and distilled water to complete a 20 µl reaction. For the Argentine PCR, 5 µl of the re-suspended template were used per reaction mixture. To run the isolates from Oklahoma and Missouri, 5 µl of concentrated DNA of each sample was used as template. An *O. korrae* template extracted from a previously known isolate provided by Dr. Lane Tredway was run as positive control. A similar PCR was performed using *O. herpotricha* primers from Tisserat, et al 1994. These were OHITS1 (5-AAGAGGCTTAATGGGTGCCCAC-3) and OHITS2 (5-AAAAGGCTTATTGGGTGCCTAT-3), again with a known template of *O. herpotricha*

as positive control provided by Dr. Tredway. PCR cycling parameters were, for *O. korrae*, 94 °C for 5 min, followed by 30 cycles (94 °C for 1.5 min, 65 °C for 45 s, and 72 °C for 1.5 min) followed by the elongation time of 7 min at 72 °C. For *O. herpotricha* cycling parameters were modified as follows: 94 °C for 5 min, followed by 35 cycles (94 °C for 1.5 min, 58 °C for 45 s, and 72 °C for 1.5 min) followed by 72 °C for 7 min. Following amplification, PCR products were separated by electrophoresis at 140 V for 30 min in 1.5 % agarose gels in 0.5× TBE (tris-borate-EDTA) buffer with gel-red at 2 µl 100 ml^{-1}, and viewed with transmitted UV light and photographed.

DNA Cloning

The presence of double bands was detected when PCR products from Oklahoma were viewed in gels. Therefore it was possible that within one isolate of *Ophiosphaerella* spp. there could be more than one amplified ITS region. Selected samples from every geographical region were chosen at random and cloned. Four samples from SC, three samples from Argentina, three samples from Oklahoma and two samples from Missouri were selected. A PCR reaction using species specific primers pairs OKITS and OHITS was performed as described before on the selected samples (starting from genomic DNA), and amplification (presence of bands) was checked in the electrophoresis chamber. PCR products were used as the templates for the cloning reactions.

The process of cloning started with the ligation of the PCR product to the vector. It was performed using the NEB Cloning Kit (New England BioLabs, Ipswich, MA). Ligation was performed by mixing the following: 0.2 µl of linearized p mini vector (25 ng/µl), 2 µl of cloning master mix, 0.5 µl of template, and 1.3 µl of ultrapure water for a

total volume reaction of 4 µl. Mix was incubated at room temperature (25 °C for 10 minutes, then incubated on ice for 2 minutes. The second step was bacteria transformation, implemented adding 2 µl of the complete ligation reaction to 50 µl of competent cells [NEB 10-beta Competent *E. coli* (Cloning Efficiency)]. Mix was incubated on ice for 20 minutes, then heat shocked at 42 °C for 30 s, and incubated on ice again for 5 more minutes. 950 µl of SOC (medium to obtain maximum transformation efficiency of *E. coli*) was added and placed at 37 °C for 60 minutes with shaking at 250 rpm. in a benchtop orbital shaker MaxQ 4450 (ThermoFisher Scientific, Waltham MA). 50 µl of the outgrowth were spread on warm (37 °C) *petri* dishes containing agar media amended with 100 ppm of ampicillin. Plates were incubated inverted overnight at 37 °C for 24 hours in a benchtop orbital shaker MaxQ 4450 (ThermoFisher Scientific, Waltham MA) for bacterial colony development.

Twenty four hours later, five bacterial colonies were selected from each plate and tested for transformation by a PCR reaction. With a pipette tip, each colony was punched two times, the tip was submerged in the PCR mix previously prepared in a 96-well plate, and discarded. PCR mix was as follows: 1 µl of 10× PCR buffer, 0.2 µl of 10 nM Primer NEB F1 (5-ACCTGCCAACCAAAGCGAGAAC), 0.2 µl of 10 nM primer NEB R1 (5-TCAGGGTTATTGTCTCATGAGCG), 0.2 µl of dNTP, 0.05 µl of 5 u μl^{-1} Taq DNA polymerase, and 8.35 µl of distilled water to complete a 10 µl reaction. PCR cycling parameters were, 94 °C for 5 m, followed by 32 cycles (94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 m) followed by elongation of 2 min at 72 °C.

Samples with positive amplification were transferred from the colonies to 1.5 ml microcentrifuge tubes with 600 µl of Luria Broth using pipette tips as described before for bacterial colony multiplication. Microcentrifuge tubes were left overnight at 37 °C in a benchtop orbital shaker MaxQ 4450 (ThermoFisher Scientific, Waltham MA). Products were purified using *Zyppy ™ Plasmid Miniprep Kit* (Zymo Research Corp, Irvine CA) according to manufacturer instructions to separate the plasmid DNA from *E. coli* for sequencing.

PCR Product Purification and DNA Sequence Alignment

Each PCR product was purified with the *DNA Clean & Concentrator* kit (Zymo Research Corp., Irvine CA). According to manufacturer's instructions, PCR product was diluted with DNA binding buffer 1 to 5 (v/v) in 1.5 ml centrifuge tubes. Each mix was then transferred to a Zymo-Spin™ column in a collection tube and it was centrifuged for 30 s and the flow through was discarded. 200 µl of DNA wash buffer was added to the column and centrifuged for 30 s. This washing step was repeated. 10 µl of DNA elution buffer was added directly to the column and incubated at room temperature for one minute. The column was then transferred to a 1.5 ml microcentrifuge tube and centrifuged for 30 s to elute the DNA. Tubes with ultra-pure DNA from PCR were stored at –20°C. DNA concentration were measured with a NanoDrop 2000 (Thermo Scientific) and prepared in 96-well plates for sequencing: 10ng of each sample was combined with 0.5ul of 2uM of the forward primer. Separately another 10 ng of each sample were combined with 0.5ul of 2uM of the reverse primer. Samples were dried down in a laminar flow hood for 5 hours and sent to Clemson University Genomics Computational

Laboratory (105 Collings St, Clemson, SC 29634). For *O. korrae* positive isolates, samples were combined with OKITS1/OKITS2. For *O. herpotricha* positive isolates, samples were combined with OHITS1/OHITS2 primers.

Raw sequences from both strands resulting from Clemson University Genomics Computational Laboratory analysis were assembled to produce a consensus sequence with FinchTV software with manual adjustment. The identities of the sequences were determined with BLAST analyses from the National Center for Biotechnology Information (NCBI).

Phylogenetic Analysis and Analysis of Molecular Variance

Sequences were aligned with Clustal W2 software and saved in Phyllip format. The multiple sequence alignment was carried out with the software package MEGA 6 (Tamura, Stecher, Peterson, and Kumar, 2013) using the Kimura's Two Parameters Distance Model and Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The typology of the resulting tree was tested by bootstrapping with 1000 resampling of the data.

Genetic structure refers to the amount and distribution of genetic variation within and among populations (MacDonald, 1997). In 1992, Excoffier et al. and Michalakis and Excoffier in 1996, extended the analysis of variance (ANOVA) approach so that it can be applied to other kinds of population genetic data, including DNA sequences (Halliburton, 2004). The method was called analysis of molecular variance (AMOVA) (Halliburton, 2004).

AMOVA was performed using Arlequin ver. 3.5.2.1 software [(c) Laurent Excoffier, 1998-2015] separately on each of the two species identified, *O. korrae* and *O. herpotricha*. To determine which source of variation better explained genetic variability, two different approaches were performed to run AMOVA, grouping the populations in two different ways. The first grouping approach tested genetic variability based on the regions where the fungi were isolated. In this way, isolates identified as *O. korrae* were separated into 6 groups: 1) Coastal South Carolina, 2) Inland SC, 3) Upstate SC, 4) North Carolina, 5) California, and 6) Oklahoma. The populations within each region are the locations or golf courses from where samples were collected. Isolates identified as *O. herpotricha* were grouped as follows 1) Buenos Aires, Argentina, 2) Inland, ARG., 3) Missouri, 4) North Carolina, and 5) Oklahoma. Again populations within each region correspond to the golf courses or polo fields where the samples were collected. The second grouping approach tested the genetic variability based on the bermudagrass cultivars from the causal organism was isolated. *O. korrae* isolates were grouped as follows: 1) 'Tifway', 2) 'Ultradwarf bermudagrass', and 3) 'Unknown bermudagrass'. Populations within each region are the golf courses where samples were collected. *O. herpotricha* isolates were grouped as follows: 1) 'Tifway UGA', 2) 'Tifway EDIN', 3) 'Old Tifway', 4) 'Dwarf bermudagrass', 5) 'Tifsport', 6) 'Common seeded', 7) 'Unknown NC', 8) 'Unknown OK', 9) 'Common seeded MO', and 10) 'Patriot'. Again, populations within each region are the golf courses and polo fields where samples were collected.

Pairwise genetic distance (F_{ST}) and their significance by performing 10100 permutations among the individuals between populations were also calculated using Arlequin ver. 3.5.2.1 to compare genetic differences between populations.

Results

Fungal Isolation

SDS pathogens were isolated from 78.5% of all soil cores collected from golf courses in South Carolina in 2012 and 2013. In Argentina, the percentage of SDS pathogen isolated from soil cores were 60.9% of the samples collected during the same period of time. Colony morphology of 216 SC isolates grown on quarter strength PDA media in Petri dishes, exhibited a white color at the beginning, with centers become grayish/black after 10 to 14 days (Figure 2.1), and the whole culture developing to greyish black after 4 to 6 weeks. In Argentina, all the 145 cultures on quarter strength PDA developed a whitish growing during the first 7 to 10 days and turned brown when cultures became mature after 20 to 30 days (Figure 2.2). Identification based on pseudothecia, asci and ascospores size and shape was not performed. Color of aerial mycelium in pure culture of *O. korrae* was the same as it was described before (Tredway et al., 2009b), and as it was reported in Australia (Smith, 1971), California (Endo et al., 1985), and Italy (Gullino et al., 2007). The color of Argentinian cultures matched with the description of cultures of *O. herpotricha* (Landschoot, 1994; Tisserat et al., 1989).

Species Identification Based on PCR Amplification

In total, 207 of the 216 isolations tentatively identified by culture color were identified with the molecular method described above (Tisserat et al, 1994). According to this method, 203 isolates from SC, 2 from NC, and 2 from CA produced amplification with PCR primer par OKITS1/OKITS2. Amplicons were visualized as bands in 1.2 % agarose gel under UV light. The amplification produced with this species specific primer set confirmed *O. korrae* as the causal agent of SDS in the regions where those samples were collected (Figure, 2.4). It was the only SDS pathogen isolated in South Carolina. Only 9 isolates remained unidentified.

All the 145 cultures from Argentina were identified using the same method but amplification was positive with the OHITS1/OHITS2 species specific primer set. Amplicons were visualized as bands in 1.2 % agarose gel under UV light. *O. herpotricha* was the only SDS pathogen isolated in Argentina (Figure 2.5).

Sequence Analysis and Phylogeny of *Ophiosphaerella* spp. in South Carolina and Argentina

PCR amplification of the ITS region with primers OKITS1/OKITS2 and OHITS1/OHITS2 gave a single product of 454 bp for each isolate. This fragment includes part of the ITS1 region, the 5.8s rDNA, and part of the ITS2 region.

PCR purified products amplified by the pair of primers OKITS of 131 from SC. A total of 10 polymorphic sites out of the 454 bp (2.2%) were identified when the 131 consensus sequences from SC were compared with each other. From the 145 PCR products from Argentina, amplified by primers OHITS, 85 isolates were successfully sequenced. A total of 23 polymorphic sites out of the 454 bp (5.18%) were identified when the 85 consensus sequences from Argentina were compared with each other.

The ITS sequences of the 14 strains collected at a single site 'Reserve Myrtle Beach' for the study of variability within a location were compared with each other. They were nearly identical, only two samples with a nucleotide difference in two sites, #25 and #398. The same study performed at a second site in SC 'Columbia Country Club' with 8 samples showed only one isolate with a difference at only site #165. Similar results were obtained in Argentina. At the single site 'Pilará GC', only one nucleotide varied at site # 424 from 14 samples collected. At the single site 'Jockey Club de Rosario', no difference on any nucleotide was found on the 6 samples evaluated.

Phylogenetic analysis generated with Bayesian inference based on the ITS sequence shows a phylogenetic tree presented in Figure 2.6. The 234 accessions fell into two main clusters based on *Ophiosphaerella* species: isolates previously identified as *O. korrae* are separated from the isolates previously identified as *O. herpotricha* (Figure 2.6). Within the *O. korrae* group, cluster I includes most of the Coastal SC accessions and the ones isolated in Oklahoma. Cluster II includes all the inland SC accessions that fall together with most of the Upstate SC accessions and the isolates from California and North Carolina. Within the *O. herpotricha* isolates, two main clusters can be differentiated. Cluster III includes the accessions from Midwest USA (Oklahoma and Missouri isolates), while all the Argentinian isolates (Buenos Aires ARG and Inland ARG) fall in cluster IV (Figure 2.6).

Analysis of Molecular Variance (AMOVA) - Diversity and Population Structure of *O. korrae*

Comparison of the two approaches to measure genetic variability through analysis of molecular variance showed that grouping the populations by geographical regions

better explains the diversity among *O. korrae* isolates than cultivars from they were isolated.

AMOVA showed that the percentage of genetic variability found among regions from where the pathogen was isolated was 43.27% (Table 2.2 A), while percentage of variation among populations (defined as golf courses or locations) but within regions was 8.38% (Table 2.2 A). By contrast, based on the AMOVA performed grouping populations by cultivars, the percentage of genetic variation among cultivars was 21.33%, with a F statistics of 0.21329 and a P-*value* of 0.0847, over the significance level (Table 2.2 B), resulting no significant differences among sequences because of cultivars from which they were isolated.

When pairwise F_{ST} was performed, significant differences ($P < 0.05$) were found for the following pair of populations (Table 2.3): Coastal SC/Inland SC, Coastal SC/Upstate SC, Coastal SC/California, Coastal SC/Oklahoma, Inland SC/Oklahoma, Upstate SC/Oklahoma. No significant differences ($P > 0.05$) were found between the following pairs of populations: Inland SC – Upstate SC, North Carolina with every population from SC, California and Oklahoma, isolates from California when compared with Inland SC, Upstate SC and Oklahoma.

Analysis of Molecular Variance (AMOVA) - Diversity and Population Structure of *O. herpotricha*

Grouping the populations by geographical regions best explained genetic variability among *O. herpotricha* isolates, similar to results with *O. korrae*. The AMOVA analysis showed that the percentage of genetic variability found among regions was 49.75% (Table 2.4 A), with a P-value < 0.05. Percentage of variation among populations

but within regions was 32.58% (Table 2.4 A). By contrast, AMOVA when grouped by cultivars, the percentage of genetic variation among cultivars was 24.17%, (Table 2.2 B), with a F statistics of 0.24175 and a P-*value* of 0.11901, over the significance level (Table 2.2 B), meaning no significant differences among cultivars. Also the percentage of variation among populations but within cultivars was higher (52.08%, Table 2.4 B).

When pairwise F_{ST} was performed, no significant differences ($P > 0.05$) were found for the isolates collected in Argentina, the isolates collected at the Midwest USA (Missouri and Oklahoma), but significant differences were found when both populations from Argentina (Buenos Aires and Inland) were compared with both populations isolated at the Midwest USA (Oklahoma and Missouri). No significant differences were found when North Carolina isolates were compared with any of the other populations in this study (Table 2.5).

Discussion

This study represents the first attempt to identify and compare the species that produce spring dead spot disease of bermudagrass in South Carolina, Northern hemisphere, and Argentina in the Southern hemisphere. The 212 South Carolinian isolates were representative of all areas where bermudagrass is grown in golf courses in South Carolina as well as the isolates from Argentina, which were more intensively collected from the area where the concentration of golf courses and polo fields is the highest, but also included some other more distant locations.

The current results show that, based on the ITS region, the isolates from South Carolina differ from Argentinian isolates. We report for the first time the presence of *O. herpotricha* causing spring dead spot in Argentina, where 145 isolates collected during 2012 and 2013 were positively identified with oligonucleotide specific primers for *O. herpotricha*. At the same time, this current study confirmed previous studies (Iriarte et al., 2004) that *O. korrae* is the most prevalent cause of SDS of bermudagrass in South Carolina, since 203 of 212 isolates from 22 different locations along the entire state, were positively amplified with PCR using *O. korrae* oligonucleotide specific primers.

The distribution of SDS pathogens both in SC and Argentina seems to be less complex than the distribution reported by Wetzel et al. (1999), when all the three SDS pathogens were identified in Kansas and Oklahoma. Although with less samples analyzed, *O. korrae* was found as the only cause of SDS in Georgia and Maryland (Tisserat et al., 1994) as well as in a survey in SC (only four locations), Alabama, Virginia, West Virginia, and Tennessee, (Iriarte et al., 2004). Similar results were obtained in Mississippi (Perry, 2006) where only *O. korrae* was associated with SDS. However, in other studies in North Carolina (Butler, 2004; Iriarte et al., 2004), and Kentucky (Iriarte et al., 2004; Tisserat et al., 1994), both *O. korrae* and *O. herpotricha* were identified; with *O. korrae* predominating in occurrence. By contrast *O. herpotricha* is the most prevalent SDS pathogen isolated in Oklahoma and Kansas, the primary cause of SDS in the Southern Great Plains (Tisserat et al., 2004; Wetzel, 1999).

The differentiation of pathogen populations within a species may be inferred from the analysis of the sequences of the ITS region from different geographical regions.

The phylogenetic tree generated with the ITS sequences obtained from SC, and Argentina, plus accessions extracted from GenBank, Oklahoma, North Carolina, California and Missouri, separated the ITS sequences into two distinct groups, where all the *O. korrae* isolates fall in one group and all the *O. herpotricha* cluster together, similar to Wetzel, 1999, who generated a phylogenetic tree with three groups, one per each species of *Ophiosphaerella* identified. In this study, a large set of isolates of *O. korrae* from South Carolina were compared with isolates of the same species from California, North Carolina, and Oklahoma. In the same way, isolates of *O. herpotricha* from Argentina were compared with isolates from Oklahoma, Missouri, and North Carolina.

When ITS region sequences were compared with each other within both species, we found that the percentage of polymorphic sites for *O. korrae* was 2.2 %, and the same measure for *O. herpotricha* was 5.18 %. This is coincident with previous studies, where *O. korrae* showed higher genetic variability than *O. herpotricha* (Raffle and Hsiang, 1998; Wetzel et al., 1999b).

AMOVA to measure genetic variability within each of the *Ophiosphaerella* species identified, based on ITS sequences, has not been reported before the present study. Our results based on AMOVA indicate geographic regional differences within each species. In this way, although in a short sequence of 454 bp, we found genetic differences between two populations inside SC (isolations from Coastal SC significantly differ from population Inland SC). However, in previous studies, Iriarte et al. (2004) found no regional genetic difference among populations of *O. korrae* collected in Southern US, although genetic variability was calculated using AFLP. We also found

significant differences between the populations from SC when they are compared with the population of Oklahoma.

On the other hand, based on the nuclear ribosomal DNA sequences, the genetic diversity of *Ophiosphaerella* populations isolated from symptomatic bermudagrass turf is not dependent on the host bermudagrass cultivar. The lack of structure supports the idea that genetic variation is due to regional location. Since the ITS region is a small fragment, further analysis with different techniques are necessary to support the idea of geographic genetic differences among populations within each of the *Ophiosphaerella* species causing spring dead spot of bermudagrass.

Table 2.1: Geographic origin of the isolates of *Ophiosphaerella spp.* collected from symptomatic hybrid and common bermudagrass in golf courses in South Carolina and in golf courses and polo fields in Argentina. Hosts with the same letter have the same origin.

Location			Host	Isolates	Species Identified	
Myrtle Beach	Reserve Myrtle Beach	Fairways	'Tifway'	33	33	*Ophiosphaerella korrae*
Myrtle Beach	Myrtle Beach National	Fairways	'Tifway'	8	5	*O. korrae*
Myrtle Beach	Blackmoore	Fairways	'Tifway'	10	10	*O. korrae*
Charleston	Daniel Island GC	Greens	'Tifway'	8	8	*O. korrae*
Florence	Pee Dee REC	Collar	'Tifway'	8	8	*O. korrae*
Pageland	White Plains	Fairways	'Tifway'	7	6	*O. korrae*
Cheraw	Cheraw State Park	Fairways	'Tifway'	7	7	*O. korrae*
Santee	Santee Cooper	Fairways	'Tifway'	9	9	*O. korrae*
Santee	Santee Lake Marion	Greens	'Miniverde'	9	9	*O. korrae*
Columbia	Fort Jackson	Fairways	'Tifway'	8	7	*O. korrae*
Columbia	Woodlands	Greens	'Champion'	8	8	*O. korrae*
Columbia	Woodlands	Fairways	'Tifway'	8	8	*O. korrae*
Columbia	Columbia CC	Fairways	'Tifway'	28	27	*O. korrae*
Columbia	Forrest Lake Club	Fairways	'Tifway'	9	9	*O. korrae*
Lexington	Lexington CC	Fairways	'Tifway'	8	8	*O. korrae*
North Augusta	River Club	Fairways	'Tifway'	9	8	*O. korrae*
Aiken	Houndslake	Fairways	'Tifway'	2	2	*O. korrae*
Greenville	Furman University	Fairways	'Tifway'	9	8	*O. korrae*
Greenville	Greenville CC	Fairways	'Tifway'	8	8	*O. korrae*
Greenville	Thornblade	Fairways	'Tifway II'	7	7	*O. korrae*
Clemson	The Walker Course	Fairways	'Tifway'	7	7	*O. korrae*
Sunset	Reserve Lake Keowee	Fairways	'Tifway'	5	5	*O. korrae*
Pilar, BA	Pilara GC	Fairways	'Tifway'[a]	22	22	*Ohiosphaerella herpotricha*
Pilar, BA	Martindale CC	Fairways	'Tifway' [b]	4	4	*O. herpotricha*
Pilar, BA	Martindale CC	Greens	'Tifdwarf'	3	3	*O. herpotricha*
Matheu, BA	Campo Chico CC	Fairways	'Tifway'	6	6	*O. herpotricha*
Lujan, BA	La Colina CC	Fairways	'Tifway'[c]	8	8	*O. herpotricha*
Lujan, BA	Everlinks CC	Fairways	'Tifway'[c]	4	4	*O. herpotricha*
Tigre, BA	Nordelta GC	Fairways	'TifSport'	8	8	*O. herpotricha*
Hurlingham, BA	Hurlingham Club	Fairways	'Tifway' [b]	5	5	*O. herpotricha*
Moreno, BA	San Diego CC	Greens	'TifEagle'	8	8	*O. herpotricha*
General Rodriguez, BA	El Relincho	Polo Field	'Tifway'[a]	6	6	*O. herpotricha*
General Rodriguez, BA	EDIN	Sod Farm	'Tifway'[c]	6	6	*O. herpotricha*
General Rodriguez, BA	La Herradura	Polo Field	'Tifway'[a]	3	3	*O. herpotricha*
Escobar, BA	El Canton CC	Fairways	'Tifway'[a]	7	7	*O. herpotricha*
Lobos, BA	San Eugenio	Polo Field	'Tifway'[a]	4	4	*O. herpotricha*
Lobos, BA	San Francisco	Polo Field	'Tifway'[c]	4	4	*O. herpotricha*
Lobos, BA	Velay	Polo Field	Common cv 'Sundevil'	10	10	*O. herpotricha*
Pinamar, BA	Costa Esmeralda CC	Fairways	'Tifway'[a]	7	7	*O. herpotricha*
Rosario, Santa Fe	Jockey Club de Rosario	Fairways	'Tifway'[c]	16	16	*O. herpotricha*
Cordoba, Cordoba	Jockey Club de Cordoba	Fairways	'Tifway'[a]	2	2	*O. herpotricha*
Cafayate, Salta	Cafayate GC	Fairways	'Tifway'[a]	7	7	*O. herpotricha*
Colonia, Uruguay	Sheraton Colonia Hotel	Fairways	'Tifway'[a]	5	5	*O. herpotricha*

Table 2.2: Summary of the analysis of molecular variance table (AMOVA) for the ITS region sequences of *Ophiosphaerella korrae* from South Carolina, North Carolina, California, and Oklahoma, grouped by geographical location.

Source of Variation	Geographical Location Variability					
	df	**Sum of Squares**	**Variance Components**	**Percentage of Variation**	**Fixation Indices**	***p-value ****
Among Regions	5	33.188	0.3728 Va	43.27	F_{CT}: 0.4327	0.0027 + - 0.0005
Among Populations (Locations) Within Regions	18	14.734	0.0722 Vb	8.38	F_{SC}: 0.1477	0.0174 + - 0.0013
Within Populations (Locations)	110	45.818	0.4165 Vc	48.35	F_{ST}: 0.5165	0.0000 + - 0.0000
Total	133	93.740	0.8615			

*Significant at $p < 0.05$ after 10100 permutations.
Fixation indices F_{CT}, F_{SC}, and F_{ST} are *F*-statistics

Table 2.3: Summary of the analysis of molecular variance table (AMOVA) for the ITS region sequences of *Ophiosphaerella korrae* from South Carolina, North Carolina, California, and Oklahoma, grouped by host cultivar.

Source of Variation	Host Cultivar Variability					
	df	**Sum of Squares**	**Variance Components**	**Percentage of Variation**	**Fixation Indices**	***p-value* ***
Among Cultivars	2	11.259	0.1788 Va	21.33	F_{CT}: 0.2132	0.085 + - 0.00274
Among Populations (Locations) Within Cultivars	21	36.664	0.2433 Vb	29.01	F_{SC}: 0.3687	0.0000 + - 0.0000
Within Populations (Locations)	110	45.818	0.4165 Vc	49.67	F_{ST}: 0.5033	0.0000 + - 0.0000
Total	133	93.740	0.8386			

*Significant at $p < 0.05$ after 10100 permutations.
Fixation indices F_{CT}, F_{SC}, and F_{ST} are *F*-statistics

Table 2.4: Pairwise F_{ST} table among geographical regions of *Ophiosphaerella korrae* isolates.

	Coastal SC	Inland SC	Upstate SC	North Carolina	California	Oklahoma
Coastal SC	*					
Inland SC	0.00000+-0.00000	*				
Upstate SC	0.00000+-0.00000	0.11613+-0.0033	*			
North Carolina	0.99990+-0.00000	0.99990+-0.00000	0.99990+-0.00000	*		
California	0.02544+-0.0015	0.07772+-0.0021	0.18127+-0.0036	0.99990+-0.00000	*	
Oklahoma	0.00277+-0.0005	0.00000+-0.00000	0.00000+-0.00000	0.29858+-0.0042	0.07455+-0.0025	*

Data in red color are significantly different.
Number of permutations: 10100
Significant at $p < 0.003$
Bonferroni corrections were performed because of multiple comparisons (15 pairs)

Table 2.5: Summary of the analysis of molecular variance table (AMOVA) for the ITS region sequences of Ophiosphaerella korrae from Argentina, North Carolina, Missouri, and Oklahoma, grouped by geographical location.

Source of Variation	Geographical Region Variability					
	df	**Sum of Squares**	**Variance**	**Percentage of Variation**	**Fixation Indices**	***p-value* ***
Among Regions	5	52.899	0.9995	49.75	F_{CT}: 0.4327	0.0027 + - 0.0005
Among Populations (Locations) Within Regions	19	55.020	0.6544	32.58	F_{SC}: 0.6869	0.0174 + - 0.0013
Within Populations (Locations)	73	25.925	0.3551	17.68	F_{ST}: 0.7626	0.0000 + - 0.0000
Total	97	133.844	2.00905			

*Significant at $p < 0.05$ after 10100 permutations.
Fixation indices F_{CT}, F_{SC}, and F_{ST} are *F*-statistics

Table 2.6: Summary of the analysis of molecular variance table (AMOVA) for the ITS region sequences of *Ophiosphaerella korrae* from Argentina, North Carolina, Missouri, and Oklahoma, grouped by host cultivar.

Source of Variation	Host Cultivar Variability					
	df	**Sum of Squares**	**Variance**	**Percentage of Variation**	**Fixation Indices**	***p-value* ***
Among Cultivars	9	58.587	0.3616 Va	24.17	F_{CT}: 0.2132	0.1198 + - 0.0034
Among Populations (Locations) Within Cultivars	15	48.332	0.7790 Vb	52.08	F_{SC}: 0.3687	0.0000 + - 0.0000
Within Populations (Locations)	73	25.925	0.3551 Vc	23.74	F_{ST}: 0.5033	0.0000 + - 0.0000
Total	97	133.844	14.957			

*Significant at $p < 0.05$ after 10100 permutations.
Fixation indices F_{CT}, F_{SC}, and F_{ST} are *F*-statistics

Table 2.7: Pairwise F_{ST} table among geographical regions of *Ophiosphaerella herpotricha* isolates.

	BA ARG	Inland ARG	Missouri	Oklahoma	North Carolina
BA ARG	*				
Inland ARG	0.09237+-0.0029	*			
Missouri	0.99990+-0.0000	0.99990+-0.0000	*		
Oklahoma	0.00000+-0.00000	0.00030+-0.0002	0.99990+-0.0000	*	
North Carolina	0.00238+-0.0005	0.00020+-0.0001	0.99990+-0.0000	0.99990+-0.0000	*

Data in red color are significantly different.
Number of permutations: 10100
Significant at $p < 0.005$
Bonferroni corrections were performed because of multiple comparisons (10 pairs)

Figure 2.1: Sampling method to determine genetic variation within a location. A 3-m-diameter circle was delineated and a turfgrass cores were removed from the edge of every symptomatic spring dead spot (SDS) patch inside the circle. Additional 10 cores were randomly taken from 10 SDS patches outside the circle

Figure 2.2: Colony morphology of a four weeks old *Ophiosphaerella korrae* isolate from Columbia Country Club, Columbia, South Carolina (A) and six weeks old isolate from Oklahoma (B) grown on quarter strength potato dextrose agar.

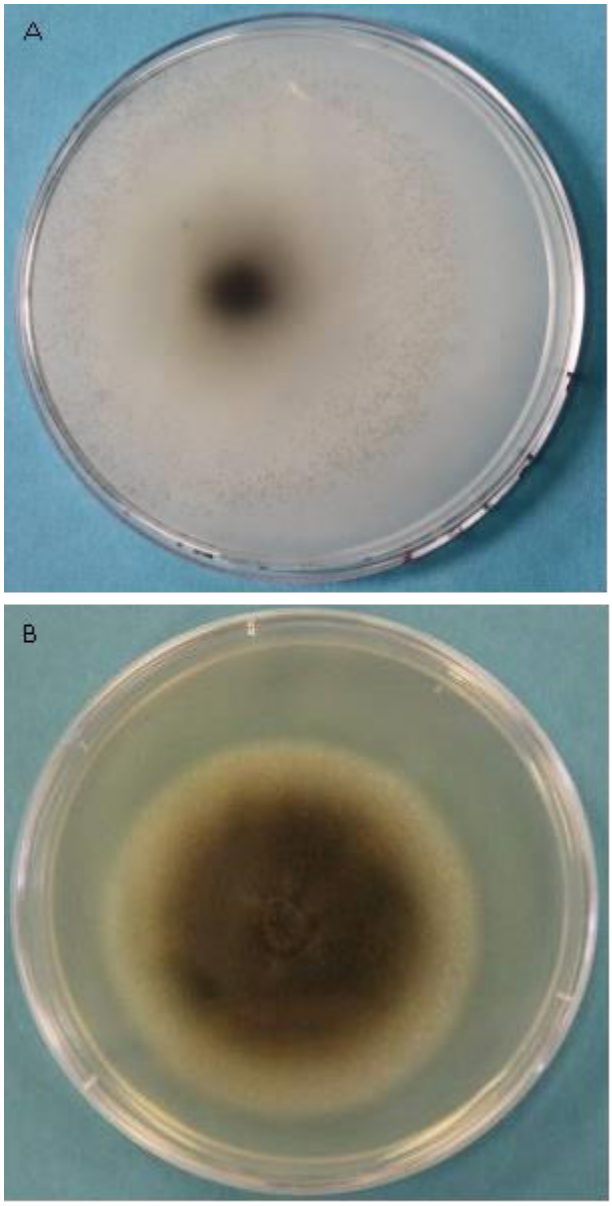


Figure 2.3: Colony morphology of a four weeks old *Ophiosphaerella herpotricha* isolate from Costa Esmeralda Golf Club, Pinamar, Argentina (A) and a six weeks old isolate from Missouri (B) grown on quarter strength potato dextrose agar.

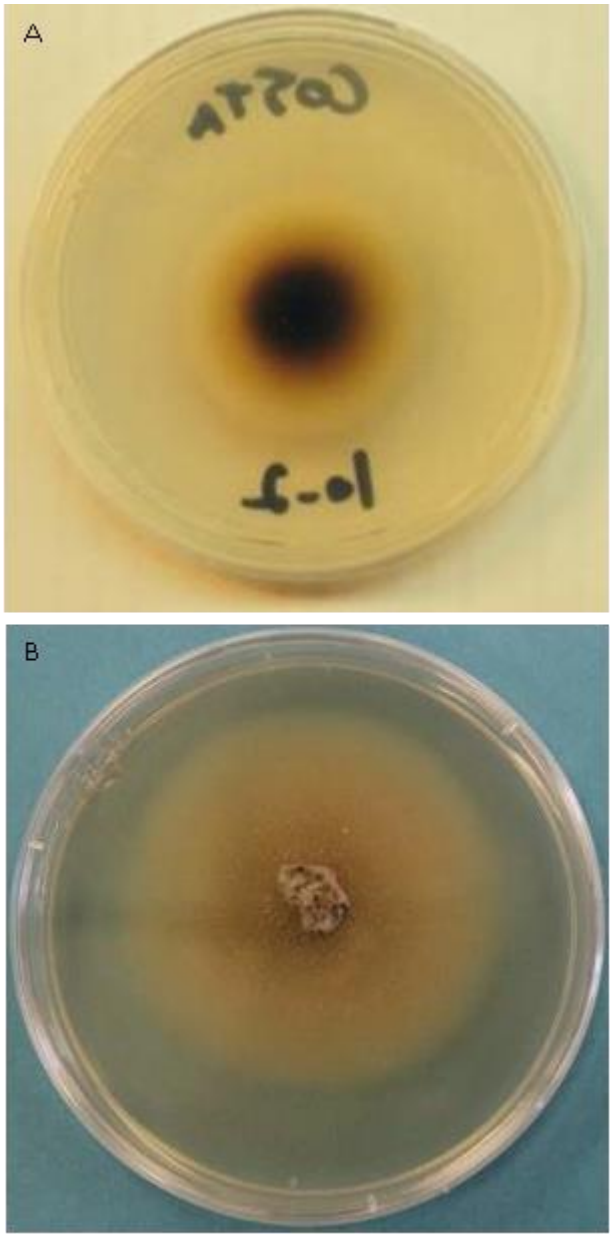


Figure 2.4: Polymerase chain reaction (PCR) products amplified from the internal transcribed spacer region obtained using species specific primers designed by Tisserat et al. (1994) of genomic DNA of putative *Ophiosphaerella korrae* from South Carolina.

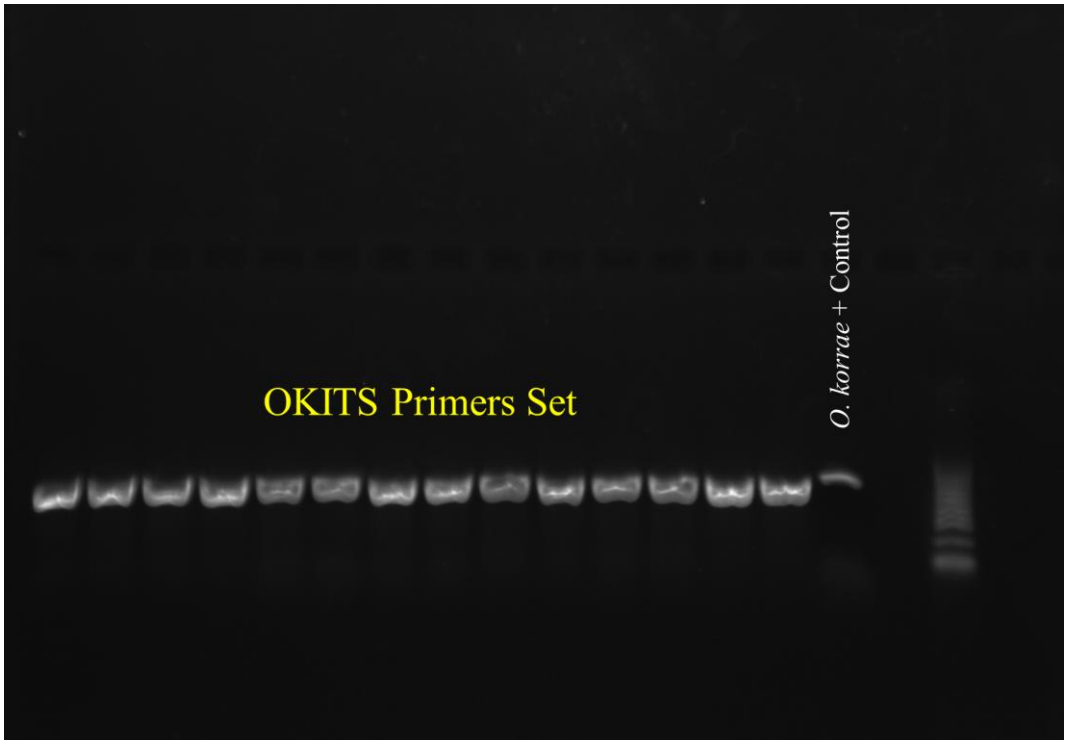


Figure 2.5: Polymerase chain reaction (PCR) products amplified from the internal transcribed spacer region obtained using species specific primers designed by Tisserat et al. (1994) of genomic DNA of putative *Ophiosphaerella herpotricha* from Argentina

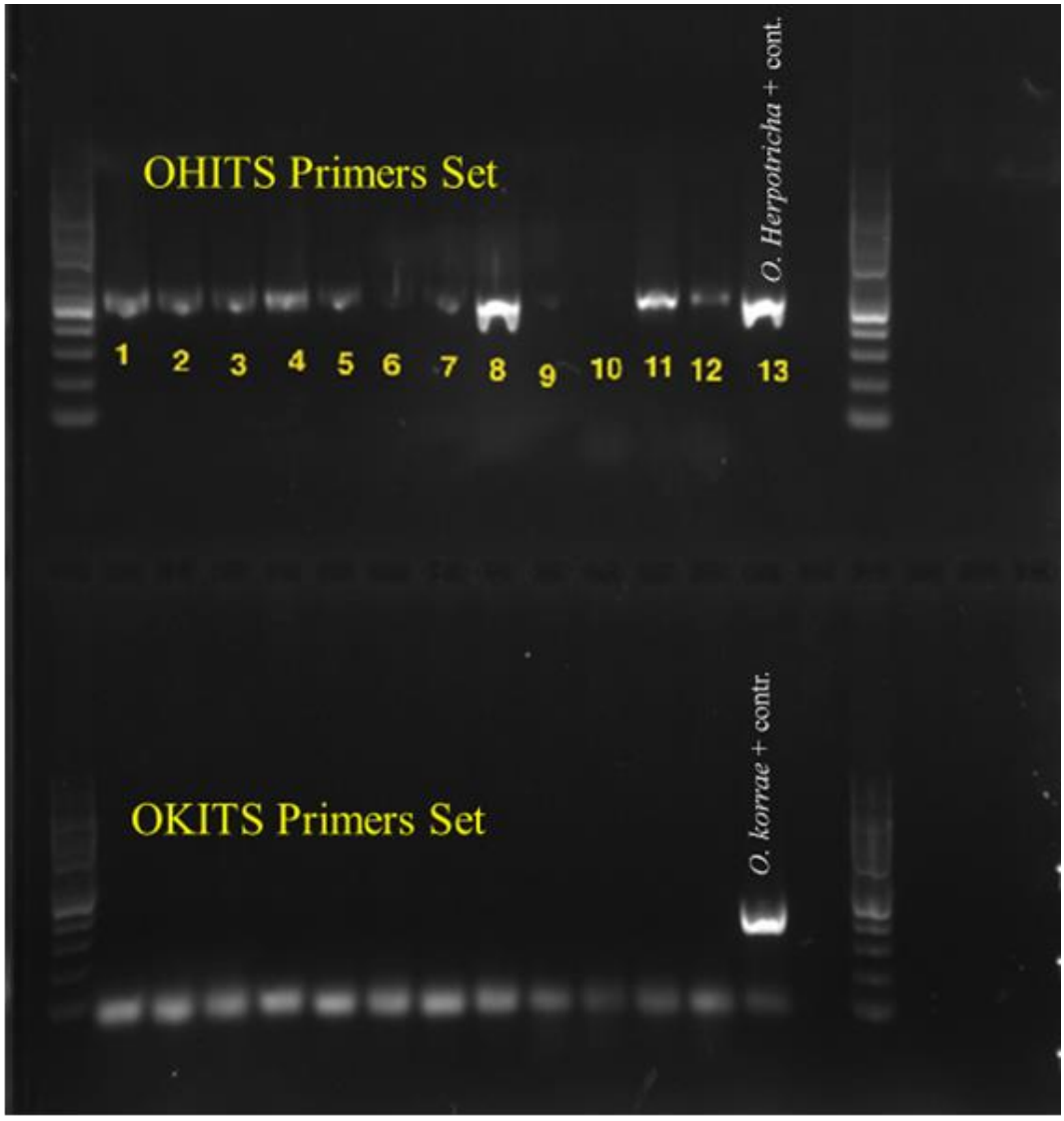


Figure 2.6: Phylogenetic tree generated from Bayesian inference of a data set of ITS sequences of 234 isolates of *Ophiosphaerella* spp. collected in South Carolina, North Carolina, California, Oklahoma, Missouri, and Argentina.

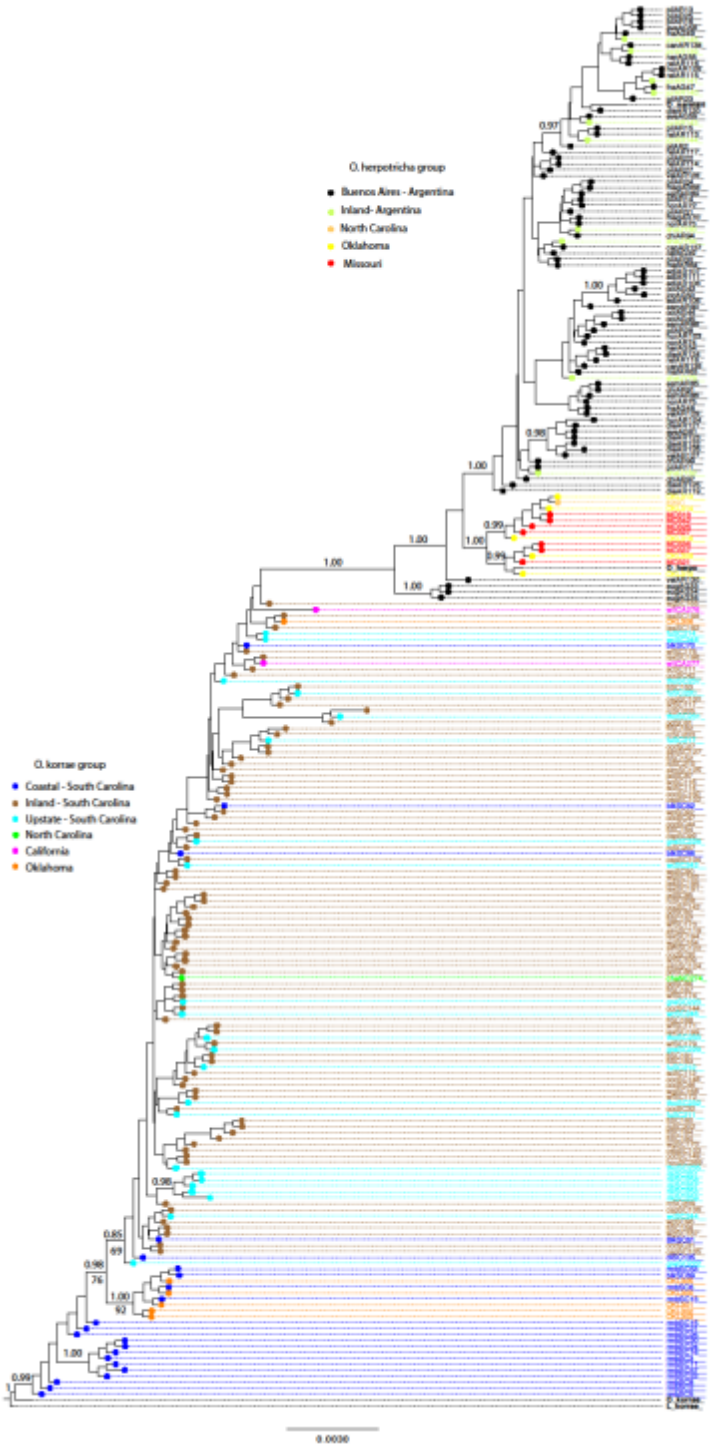
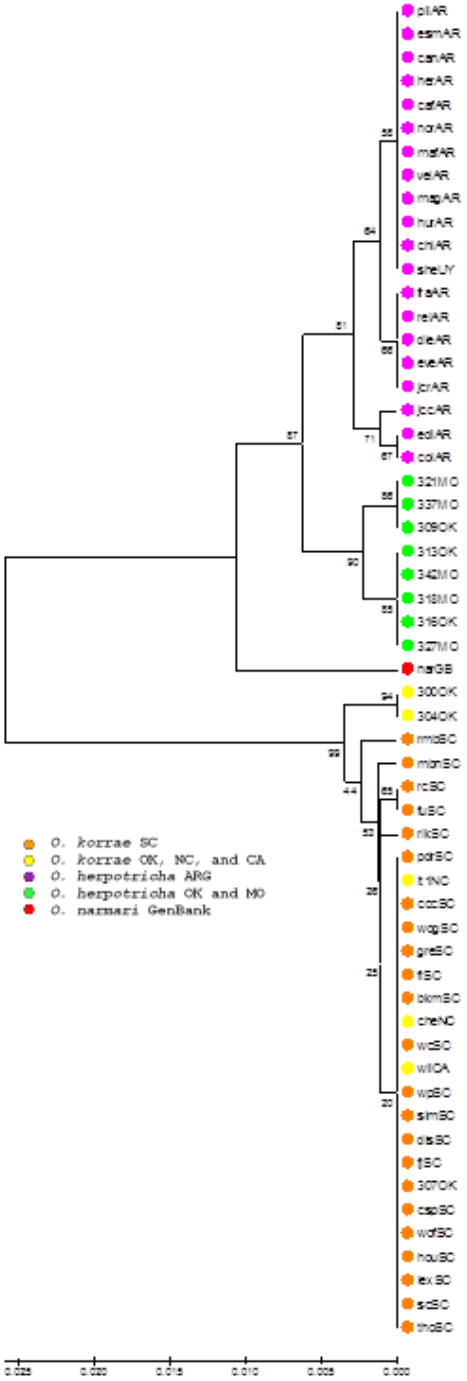


Figure 2.7: Phylogenetic tree of *Ophiosphaerella* spp. based on ITS region. Dendrogram generated with UPGMA of isolates collected in South Carolina, North Carolina, California, Oklahoma, Missouri, and Argentina. Data was subjected to 1000 bootstrap replicates.



Literature Cited

Butler E.L. (2004) Development of novel strategies for control of spring sead spot of bermudagrass, Department of Plant Pathology, North Carolina State Unversity, Raleigh, NC.

Canegallo A., Martin S.B., Park W., Agudelo P., McCarty L., Kerrigan J., Tredway L., Cantoro R. (2015) Characterization of *Ophiosphaerella* species causing spring dead spot disease of bermudagrass in South Carolina and Buenos Aires, Argentina., in: T. A. P. Society (Ed.), APS Annual Meeting, Pasadena, CA.

Crahay J.H., Dernoeden P.H., O'Neill N.R. (1988) Growth and pathogenicity of *Leptosphaeria korrae i*n bermudagrass. Plant Disease 72:945-949.

Dernoeden P.H., Zhang M., Wetzel H.C. (1995) First report of necrotic ring spot (*Leptosphaeria korrae*) in creeping red fescue in Maryland. Plant Disease 79:966.

Dunn J.H., Nelson C.J., Sebaugh J.L. (1980) Characterization of thatch, rhizomes, carbohydrates, and spring dead spot in twenty cultivars of bermudagrass (Abstract). Journal of the American Society for Horticultural Science 105:653-657.

Endo R.M., Ohr H.D., Krausman E.M. (1985) *Leptosphaeria korrae*, a cause of the spring dead spot disease of bermudgrass in California. Plant Disease 69:235-237.

Gullino M., Mocioni, M., Tittone P. (2007) First report of spring dead spot of bermudagrass in Italy 91:1200.

Iriarte F.B., Wetzel H.C., III, Fry J.D., Martin D.L., Tisserat N.A. (2004) Genetic diversity and aggressiveness of *Ophiosphaerella korrae*, a cause of spring dead spot of bermudagrass. Plant Disease 88:1341-1346.

Landschoot P.J. (1994) Taxonomy and biology of ectotrophic root-infecting fungi associated with patch diseases of turfgrasses, in: B. B. Clarke and A. B. Gould (Eds.), Turfgrass Patch Diseases: Caused by Ectotrophic Root-Infecting Fungi, The American Phytopathological Society., St. Paul, Minnesota. pp. 41-71.

Landschoot P.J., June P.D. (1996) First report of necrotic ring spot on *Poa annua* putting greens in Pennsylvania. Plant Disease 80:712.

Luc J.E., Canegallo A., Martin S.B. (2005) Spring dead spot in bermudagrass greens in Argentina and South Carolina, Golf Course Management. pp. 92-95.

Lucas L.T. (1980) Control of spring dead spot of bermudagrass with fungicides in North Carolina. Plant Disease 64:868-870.

MacDonald B. (1997) The population genetics of fungi: tools and techiques. Phytopathology P1997:448-453.

McCarty L.B., DiPaola J.M., Lucas L.T. (1991) Regrowth of bermudagrass infected with spring dead spot following low temperature exposure. Crop Science 31:182-184.

Owens C.B., Szalanski A.L. (2005) Filter paper for preserving insect DNA samples.

Perry H., Tomaso-Peterson M. (2006) Frequency of occurrence of Ophiosphaerella korrae on Tifway bermudagrass roots in Mississippi. Phytopathology 96:S92.

Raffle V.L., Hsiang T. (1998) Low level of DNA polymorphisms in isolates of *Leptosphaeria korrae* pathogenic on Poa pratensis. Can. J. Plant Path 20:48-54.

Sala D. (2015) Datos historicos. clima sur GBA, http://www.climasurgba.com.ar/historia.php.

Sauer K.M., Hulbert S.H., Tisserat N.A. (1993) Identification of *Ophiosphaerella herpotricha* by cloned DNA probes. Phytopathology 83:97-102.

Smiley R.W., Dernoeden P.H., Clarke B.B. (2005) Compendium of turfgrass diseases APS Press The American Phytopathological Society, St. Paul, Minnesota.

Smiley R.W., Fowler M.C., Hummel N.W., Petrovic A.M., Pidduck H.M., Strang C. (1986) Necrotic ring spot and summer patch of inoculated Kentucky bluegrasses 1:58.

Smith A.M. (1971) Spring dead spot of couch grass turf in New South Wales. The Journal of the Sports Turf Research Institute 47:54-59.

Tisserat N., Iriarte F.B., Wetzel III H.C., Fry J., Martin D.L. (2004) Identification, distribution, and aggresiveness of spring dead spot pathogens of bermudagrass, USGA Green Section Record, USGA, USGA. pp. 15-18.

Tisserat N.A., Hulbert S.H., Sauer K.M. (1994) Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiosphaerella korrae* and *O. herpotricha*. Phytopathology 84:478-482.

Tisserat N.A., Pair J.C., Nus A. (1989) *Ophiosphaerella herpotricha* , a cause of spring dead spot of bermudagrass in Kansas. Plant Disease 73:933-937.

Tredway L. (2011) Spring dead spot of zoysiagrass, NCSU, Turf Diseases: Golf Course Disease Updates by University Professors. pp. 1.

Tredway L.P., Butler E.L. (2007) First report of spring dead spot of zoysiagrass caused by *Ophiosphaerella korrae* in the United States. Plant Disease 91:1684.

Tredway L.P., Tomaso-Peterson M., Perry H., Walker N.R. (2009) Spring dead spot of bermudagrass: A challenge for researchers and turfgrass managers, APSnet, Plant Health Progress. pp. 1-18.

Wadsworth D.F., Young H.C. (1961) Spring dead spot of bermudagrass. USGA Journal and Turf Management, Reprint from Plant DIsease Reporter 44 (7) July 15, 1960.

Walker J., Smith A.M. (1972) *Leptosphaeria narmari* and *L. korrae* spp.nov., two long-spored pathogens of grasses in Australia. British Mycological Society 58:459-466.

Walker N. (2007) Root colonization of several species of native grasses by *Ophiosphaerella herpotricha*, 46th Annual Meeting of the Society of Nematologists and Annual Meeting of the American Phytopathological Society (SON/APS 2007).

Wetzel H.C. (1999) Geographic distribution and genetic diversity of three *Ophiosphaerella* species that cause spring dead spot of bermudagrass, Department of Plant Pathology, Kansas State University, Manhattan, Kansas.

Wetzel H.C., III, Hulbert S.H., Tisserat N.A. (1999a) Molecular evidence for the presence of *Ophiosphaerella narmari* n. comb., a cause of spring dead spot of bermuda grass, in North America. Mycological Research 103:981-989.

Wetzel H.C., III, Skinner D.Z., Tisserat N.A. (1999b) Geographic distribution and genetic diversity of three *Ophiosphaerella* species that cause spring dead spot of bermudagrass. Plant Disease 83:1160-1166.

Worf G.L., Stewart J.S., Avenius R.C. (1986) Necrotic ring spot disease of turfgrass in Wisconsin. Plant Disease 70:453-458.

CHAPTER THREE

GENETIC DIVERSITY OF *OPHIOSPHARELLA* SPP. ISOLATES BASED ON ANALYSES OF AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS (AFLP) IN SOUTH CAROLINA, USA, AND BUENOS AIRES, ARGENTINA.

Introduction

Fungi in the genus *Ophiosphaerella* are pathogens that cause diseases on several cool season and warm season turfgrasses (Raffle and Hsiang, 1998; Smiley et al., 2005; Tredway and Butler, 2007; Tredway et al., 2009b). In bermudagrass turf (*Cynodon* spp.), three species of *Ophiosphaerella* have been proved to cause spring dead spot disease (SDS) in the United States. *O. korrae* (J. C. Walker and A. M. Smith) Shoemaker and Babcock (synonym: *Leptosphaeria korrae* Walker and Smith) has been documented as the most prevalent causal agent in California (Endo et al., 1985), Mississippi (Perry et al., 2010), North Carolina (Butler, 2004) and South Carolina (Canegallo et al., 2015), whereas *O. herpotricha* (Fr.:Fr.) J. C. Walker is the main cause of SDS in Kansas (Tisserat et al., 1989), and Oklahoma (Wetzel et al., 1999b). *O. narmari* (J. C. Walker and A. M. Smith) Wetzel, Hulbert and Tisserat (synonym*: L. narmari* Walker and Smith) (Wetzel et al., 1999a) is the primary cause of SDS in Australia and New Zealand (Walker and Smith, 1972), and occasionally has been isolated in North America (Wetzel et al., 1999a; Wetzel et al., 1999b). In Argentina, only *O. herpotricha* have been recently documented as the cause of SDS of bermudagrass (Canegallo et al., 2015).

These species were initially distinguished based on morphological characteristics of the pseudothecia, asci, and ascospores (Landschoot, 1994). However identification of

the cause of SDS is complicated because the pathogens are morphologically similar in culture and do not produce ascocarps in artificial media (Tisserat et al., 1994). Therefore, an alternative approach such as amplified fragment length polymorphism (AFLP) has been considered better way for study of population diversity of *Ophiosphaerella* species.

Most phylogenetic analyses in fungi employ nuclear ribosomal DNA (rDNA). It generally rDNA exists in more than 100 copies per haploid genome (Tisserat et al., 1994), so it is easy to detect. The internal transcribed spacer (ITS) region is a small fragment of the rDNA, and is highly variable, used in many phylogenetic studies because it's easy to amplify using PCR and shows differences among very closely related taxa (O'Donnell, 1992). In 1994, Tisserat et al. developed species specific primers OKITS1, OKOTS2, OHITS1, and OHITS2 to amplify the internal transcribed spacer (ITS) regions of rDNA by polymerase chain reaction (PCR) for the identification of *O. korrae* and *O. herpotricha*. Furthermore, species specific primers to detect *O. narmari* were later developed (Wetzel et al., 1999a). However, although useful for species identification, species-specific primers targeting ITS regions can be limiting in its ability to describe population structure and genetic diversity (Douhan et al., 2009). According to Mallet and Willmott (2003) it is best not to rely on a single sequence to identify a species, but almost always need several genomic regions to distinguish closely related species.

AFLP-PCR is a DNA fingerprinting technique used to visualize DNA polymorphisms between samples, and is used as a tool to distinguish closely related organisms (Vos et al., 1995). It is based on the digestion of genomic DNA and the generation of a set of selective fragments amplified using PCR. AFLP-PCR is a powerful

technique used for the analysis of genetic variation below species level, particularly in investigations of population structure and differentiation, based on measures of genetic distance between and across the populations (Hou and Lou, 2011; Milla-Lewis et al., 2013). It has been previously used in *Ophiosphaerella* spp. to establish phylogenetic relationships based on genetic distances (Camara, 1999; Iriarte et al., 2004; Kaminski et al., 2006; Wetzel, 1999).

Materials and Methods

Sample Collection and Genomic DNA Extraction

Fungi were collected from SDS symptomatic bermudagrass during May 2012 in SC. Following the isolation of purported causal organisms, genomic DNA was extracted for analysis using a protocol provided as described in Chapter Two (Appendix A). A total of 46 samples from SC, previously identified as *O. korrae*, were analyzed, two per each golf course where the causal agent was isolated. Two *O. korrae* isolates from North Carolina, two from California, and seven isolates from Oklahoma were included in this study. Isolates from Argentina, previously identified as *O. herpotricha*, were collected in October and November 2012 and DNA was extracted using the same protocol (Appendix A). A total of 40 samples (two per location, from either golf course, polo field or sod farm) were included in the analysis. Two *O. herpotricha* isolates from North Carolina, seven isolates from Oklahoma and fourteen from Missouri were included in the study. AFLP-PCR was performed to examine the genetic variation of each species among geographical regions and locations within the regions under study.

Amplified Fragment Length Polymorphism (AFLP) Reaction

AFLP reactions started with the digestion of the genomic DNA of the samples. Restricted endonuclease digestion reaction was prepared in 200 µl PCR tubes, where 300 ng of DNA template of each sample was digested with 1µl *Eco*RI/*Mse*I enzyme mix (AFLP Core Reagent Kit, Invitrogen Corp, Pittsburgh, PA 15275), 2.5 µl of 5× reaction buffer (AFLP Core Reagent Kit, Invitrogen), and water to complete a 12.5-µl final reaction volume. Tubes were incubated for 2 hours at 37 °C followed by 15 min at 70 °C to inactivate the enzymes in a programmable thermal cycler (Bio-Rad Laboratories Inc. Hercules, CA).

After digestion, ligation of adapters was performed. Tubes were placed on ice and 12.5 µl of Adaptor/Ligation solution (AFLP Core Reagent Kit, Invitrogen), and 0.5 µl of T4 DNA ligase (AFLP Core Reagent Kit, Invitrogen), were added to the digested DNA fragments, gently mixed, and after a brief centrifugation they were incubated at 16 °C overnight in the programmable thermal cycler.

Pre-amplification reaction was initiated when ligated products were diluted 1:10 in volume with TE buffer (10 mM Tris·Cl pH 8.0, 0.1 mM EDTA). Nonselective amplification reaction was performed with 2.5 µl of the diluted ligation product, 0.4 µl of 10 µM *Eco*RI Pre-amp primer 5'- CTC GTA GAC TGC GTA CCA ATT C -3', 0.4 µl of 10 µM *Mse*I Pre-amp primer 5'- GAC GAT GAG TCC TGA GTA A -3', 0.4 µl of dNTP, 2.5 µl of 10× PCR buffer plus Mg, 0.1 µl of 5 u μl^{-1} *Taq* DNA Polymerase (Quiagen), and 18.7 µl of distilled PCR water for a final reaction of 25 µl. Reactions were performed in the programmable thermal cycler programmed as follows: 2 min at 72 °C; 20 cycles of 30

s at 94°C, 30 s at 56°C, 1 min at 72°C; and final extension of 7 min at 72°C and cooling to 4°C. Amplicons were checked on 1.2 % agarose gel and visualized with gel-red dye under UV illumination. Pre-amplification products were diluted 1:50 in volume with TE buffer (10 mM Tris·Cl pH 8.0, 0.1 mM EDTA) for selective amplification.

Selective AFLP amplification was performed with two different set of primers. Reaction 1 was prepared by mixing 2.5 µl of diluted pre-amplified product, 2.0 µl of 10× PCR buffer, 0.4 µl of dNTPs, 0.4 µl of E-AG FAM primer (10 µM) 5'- ACT GCG TAC CAA TTC AG -3', 2 µl of *Mse*I-CG primer 5'- GAT GAG TCC TGA GTA ACG -3' (10 µM), 0.1 µl of *Taq* DNA Polymerase, and 12.6 µl of water for a final reaction volume of 20 µl. Reaction 2 was performed the same but the primer set was 0.4 µl of E-AG FAM primer (10 µM) 5'- ACT GCG TAC CAA TTC AG -3', 2 µl of *Mse*I-CT primer 5'- GAT GAG TCC TGA GTA ACT -3' (10 µM). Reactions were performed under the following conditions: 2 min at 94°C; 12 cycles of 30 s at 94°C, 30 s at 65°C, and 60 s at 72°C (annealing temperature was lowered by 0.7°C during each cycle), followed by 23 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C and a final extension of 7 min at 72°C and cooling to 4°C. Products were checked as before in a 1.2% agarose gel with gel-red and a DNA ladder of 100 bp. Products were diluted 1:8 in water and sent frozen in dry ice to Clemson University Genomic Institutes (105 Collings St, Clemson, SC 29634) for analysis.

AFLP Products Analysis

AFLP products were analyzed by Clemson University Genomics Computational Laboratory with a 3730 ABI sequencer using the standard Liz600 orange size marker.

Raw traces were delivered in .fsa format and analyzed using GeneMarker version 2.6.4 software (Softgenetics LLC, State College, PA 16803). Once the raw data files were uploaded to the software, a synthetic gel image displays the unprocessed data in a traditional gel format (Figures 3.1 to 3.4). Red, green, and yellow dye colors were disabled, for the program shows the fluorescent line traces for only blue dye, (color of FAM, the dye associated with the primer used in selective amplification). The signal intensities, recorded in Relative Fluorescent Units (RFUs), are plotted along a frame scale in the raw data analysis window with fragment mobility from right to left. The smallest size fragments are on the far left of the trace (Figure 3.5).

AFLP raw data were processed using GenMarker software (Softgenetics, 100 Oakwood Avenue, Suite 350, State College, PA 16803). Size standard color Liz600 (orange) was selected. An allelic panel, that was created using all the samples generated from the two set of primers, was selected to compare the samples with a known panel. On the allele call box, size of fragments were selected from 50 to 500 bp, peak detector threshold was set from 2500 RFU to 45000 RFU, to avoid the program call peaks below and above those values. The stutter peak filter was selected with 5% to the right and 5% to the left. The rest of the settings were left as default. Every sample was revised and corrected manually to add or remove miscalled peaks. Results were shown in a bin table and exported as an Excel file. Percentages of genetic similarity within each species based on genetic distances were calculated with GenMarker.

Analysis of Molecular Variance

Analysis of molecular variance (AMOVA) was performed with the binary data generated with AFLP for phylogenetic analysis. AMOVA was performed using Arlequin ver. 3.5.2.1 software [(c) Laurent Excoffier, 1998-2015] separately on each of the two species identified, *O. korrae* and *O. herpotricha*. Populations were grouped based on the regions where the causal organism was isolated to test geographic variability of the causal organism of SDS. In this way, isolates identified as *O. korrae* were separated into 6 groups: 1) Coastal South Carolina, 2) Inland SC, 3) Upstate SC, 4) North Carolina, 5) California, and 6) Oklahoma. The populations within each region are the locations or golf courses where samples were collected. Isolates identified as *O. herpotricha* were grouped as follows 1) BA, ARG + Inland, ARG., 2) Missouri + Oklahoma + North Carolina. Populations within each region are the golf courses or polo fields where the samples were collected. Genetic variability analysis based on the bermudagrass host cultivars was not performed.

Pairwise genetic distance (F_{ST}) and their significance by performing 10100 permutations among the individuals between populations were also calculated by Arlequin ver. 3.5.2.1. Data were also analyzed with Structure program, a program that can perform estimation of membership coefficients (Rosenberg, 2004). To visualize the Structure results, Clump and Distruct software were used. A graphic was performed for each species where membership coefficients generated by Structure software represents subgroups in different colors.

Results

A total of 256 fragments ranging in size from 50 to 500 bp were scored using two primer combinations sets. The number of scored fragments amplified per sample ranged from 4 to 48 (Figures 3.1, 3.2, 3.3, and 3.4). Due to some failures during the process, 109 genotypes were analyzed (53 genotypes corresponding to *O. korrae* and 59 to *O. herpotricha*) out of the initial 120 samples. Data from both primer sets were combined for further analysis. Percentage of genetic similarity was estimated using simple matching coefficient with GeneMarker software, based on the presence/absence of each band. Percentage of genetic similarity for isolates of *O. korrae* ranged from 79.3 to 98 %, while for *O. herpotricha* they ranged from 69.9 to 99.2%.

Diversity and Population Structure for *O. korrae*

Analysis of molecular variance (AMOVA) for *O. korrae* indicated that the majority of genetic variation occurred among regions (62.32%), while the variation among populations but within regions was 37.68% (Table 3.3). F_{ST} pairwise comparisons showed significant genetic variation among the isolates collected in the three regions of SC (Table 3.5). Due to the multiple pairwise simultaneous tests, Bonferroni corrections were implemented to define p-value (Rice, 1989). No significant differences were found between Californian isolates and each of the three regions of SC. North Carolina isolates are genetically similar to Upstate and Coastal SC, however, significant differences were found when compared with Inland SC. Isolates of *O. korrae* from all three SC regions are genetically different from Oklahoma isolates (Table 3.5).

A phylogenetic tree using maximum parsimony created with PAUP software shows no structure related with geographical regions (Figure 3.8).

Genetic structure of *O. korrae* is better observed with the model implemented in the program Structure, resulting in two genetically different clusters represented with two different colors (Figure 3.6). Inland SC, the majority of Upstate SC, and half of Coastal SC isolates fall in the same cluster with the green color, meaning they are genetically similar. The red group includes all the individuals from Oklahoma and California, which showed no genetic differences between them. The other half of the isolates from Coastal SC and a few from Upstate SC also are located in this green cluster. Some isolates are partially allocated to both green and red groups, suggesting admixture between the two groups.

Diversity and Population Structure for *O. herpotricha*

Analysis of molecular variance (AMOVA) for *O. herpotricha* resulted again in much higher regional differences (73.45% of the genetic variation) than genetic variation among locations but within regions (26.55%) (Table 3.4). Pairwise F_{ST} comparison showed that isolates from the two Argentinian regions are significantly different. Isolates from BA-ARG are also genetically different compared to all the other isolates used in this study, NC, Oklahoma and Missouri isolates (Table 3.6). Inland-ARG isolates are different from Oklahoma and Missouri isolates but no genetic variation were found when compared with NC isolates. Significant genetic variation was found between Oklahoma and Missouri but no differences were found when those two were compared with NC isolates.

The phylogenetic tree using maximum parsimony created with PAUP software shows no structure related with geographical regions (Figure 3.9).

However, analysis of *O. herpotricha* with Structure resulted in three genetically different clusters represented with three different colors (Figure 3.7). Isolates from Buenos Aires, ARG and Inland, ARG fall in the same cluster with the green color, meaning there are no significant differences between them. However, some of the isolates are partially allocated to both green and red groups. Isolates from Missouri fall into the purple group but some are partially allocated to the red and green groups. Isolates from Oklahoma fall also into the purple group but some isolates are also partially allocated to the red group and one isolate is partially allocated to the green group. North Carolina isolates fall in the purple color group but partially mixed with green group (Figure 3.7). These partial allocations suggest some degree of admixture among different genetic groups.

Discussion

AFLP results support the idea of genetic variability of *Ophiosphaerella* spp. due to geographical regions, as was suggested in Chapter Two, where ITS rDNA region was analyzed. Genetic variability within each of the *Ophiosphaerella* species identified measured with AMOVA, pairwise F_{ST} comparison based on AFLP bands, and Structure analysis has not been reported before the present study.

In this study using AFLP, the percentage of genetic similarity within *O. korrae* (79.3 to 98%) was higher than the percentage of genetic similarity of *O. herpotricha* (69.9 to 99.2%) as reported by Wetzel et al. (1999). Similarly, the study of ITS rDNA region in Chapter Two, variation within *O. korrae* isolates (2.2%) was less than one half of the variability within the isolates of *O. herpotricha* (5.18% different). Genetic similarity of

these isolates of *O. korrae* (analyzed from AFLP data) collected in SC, NC, Oklahoma and CA were different from the range of 62 to 100% reported by Iriarte et al. (2004), and similar to Wetzel et al. (1999) with an average of 88% similarity. Similarly, a low level of genetic variation was found in a study with *O. korrae* isolated from Kentucky bluegrass (*Poa pratensis*) (Raffle and Hsiang, 1998). On the other hand, in our study, the percentage of genetic similarity of *O. herpotricha* (69.9 to 99.2%) with isolates from geographically more remote places, was higher than similarities reported by Wetzel et al. (1999) who found a similarity range of 60 to 98% among isolates from Kansas, Kentucky, North Carolina, Oklahoma, and Texas.

AMOVA for both *O. korrae* and *O. herpotricha* clearly indicated that variation among regions is higher than variation among populations within regions. The same results were obtained with the AMOVA performed with sequences of ITS rDNA regions. Pairwise comparison performed with data from AFLP showed significant genetic diversity among Coastal SC, Inland SC, and Upstate SC. Similar results were obtained with comparisons of ITS rDNA sequences, where Coastal SC isolates are significantly different from Upstate SC and Inland SC, although no significant differences were found between Inland SC and Upstate SC. In previous studies using AFLP, Iriarte et al. (2004) found no regional genetic differences among populations of *O. korrae* collected in the southern US, as well as Wetzel et al. (1999) who found a high degree of genetic similarity among isolates from Arkansas, Georgia, Mississippi and Tennessee.

Results obtained from both molecular techniques (AFLP and sequencing analysis of ITS rDNA region) agree that isolates from Coastal SC, Inland SC, and Upstate SC are

genetically different from Oklahoma isolates. In the same way, Iriarte et al. (2004) found significant differences between southern US isolates and western isolates from Colorado and California as well as Wetzel et al (1999) who found significant differences among isolates from Georgia, Mississippi, Arkansas and Tennessee with isolates from western regions of North America and Australia.

Although phylogenetic trees built for both *O. korrae* and *O. herpotricha* show no resolution concerning genetic structure, a clear pattern of geographical differences can be seen with Structure analysis. Isolates of *O. korrae* and *O. herpotricha* partially allocated to more than one group in the Structure analysis suggest the presence of gene flow (admixture). It could be explained by the transmission of SDS pathogens with the plant material of bermudagrass when it is transported from place to place for bermudagrass establishment (Pair et al., 1986; Wetzel et al., 1999b). This could partially explain the gene flow among populations of *O. korrae* and among populations of *O. herpotricha* within the US, what is most likely to occur between neighboring populations (Hou and Lou, 2011), since plant material for planting new stands of bermudagrass are usually produced in local sod farms. However, it is less likely to explain the occurrence of admixture between Argentina and US isolates of *O. herpotricha*. There are no records of plant stock imported from the Midwest to Argentina and vice versa, therefore there were probably no transmission of SDS pathogens on plant material in this way. In addition, the absence of *O. korrae* isolates in the survey in Argentina contrast with the idea of immigration of the pathogen with the plant material, since existing hybrid bermudagrass in Argentina have origin in the southeastern US (mainly the state of Georgia), where *O.*

korrae has been reported as the main cause of SDS (Tredway et al., 2009b; Wetzel et al., 1999b).

In conclusion, genetic variability due to geographic location is supported by AFLP fingerprinting analysis of *Ophiosphaerella korrae* and *O. herpotricha* isolates, as it was established in the previous chapter with the study of the sequences of the ITS rDNA. The lack of *O. korrae* isolates in Argentina and the different pattern of AFLP bands from Argentinian isolates when compared with US southeastern isolates suggests that immigration of genotypes of *Ophiosphaerella spp*. with plant material is unlikely. More research needs to be done to determine the mechanisms of admixture between regions distantly located.

Table 3.1 Geographic origin of the isolates of *Ophiosphaerella* spp. collected from symptomatic hybrid and common bermudagrass in golf courses in South Carolina and in golf courses and polo fields in Argentina.

Species	Region	Sample ID	Location	
Ophiosphaerella korrae	Coastal SC	rmb10SC	Myrtle Beach	Reserve Myrtle Beach
O. korrae		rmb15SC	Myrtle Beach	Reserve Myrtle Beach
O. korrae		rmb52SC	Myrtle Beach	Reserve Myrtle Beach
O. korrae		rmb53SC	Myrtle Beach	Reserve Myrtle Beach
O. korrae		blk63SC	Myrtle Beach	Blackmoore
O. korrae		blk67SC	Myrtle Beach	Blackmoore
O. korrae		mbn71SC	Myrtle Beach	Myrtle Beach National
O. korrae		mbn72SC	Myrtle Beach	Myrtle Beach National
O. korrae		di139SC	Charleston	Daniel Island GC
O. korrae	Inland SC	ccc34SC	Columbia	Columbia CC
O. korrae		ccc37SC	Columbia	Columbia CC
O. korrae		ccc141SC	Columbia	Columbia CC
O. korrae		ccc143SC	Columbia	Columbia CC
O. korrae		wog168SC	Columbia	Woodlands (greens)
O. korrae		wog170SC	Columbia	Woodlands (greens)
O. korrae		wof174SC	Columbia	Woodlands (fairways)
O. korrae		wof179SC	Columbia	Woodlands (fairways)
O. korrae		fj58SC	Columbia	Fort Jackson GC
O. korrae		fl187SC	Columbia	Forrest Lake Club
O. korrae		fl188SC	Columbia	Forrest Lake Club
O. korrae		lex192SC	Lexington	Lexington CC
O. korrae		lex194SC	Lexington	Lexington CC
O. korrae		slm119	Santee	Santee Lake Marion
O. korrae		slm121	Santee	Santee Lake Marion
O. korrae		sc109SC	Santee	Santee Cooper
O. korrae		sc118SC	Santee	Santee Cooper
O. korrae		rc201SC	North Augusta	River Club
O. korrae		rc203SC	North Augusta	River Club
O. korrae		csp103SC	Cheraw	Cheraw State Park
O. korrae		csp104SC	Cheraw	Cheraw State Park
O. korrae		pdr85SC	Florence	Pee Dee REC
O. korrae		wp92SC	Pageland	White Plains
O. korrae		wp94SC	Pageland	White Plains
O. korrae	Upstate SC	gre225SC	Greenville	Greenville CC
O. korrae		gre227SC	Greenville	Greenville CC
O. korrae		tho235SC	Greenville	Thornblade
O. korrae		tho237SC	Greenville	Thornblade
O. korrae		fu216Sc	Greenville	Furman University
O. korrae		fu218SC	Greenville	Furman University
O. korrae		keo253SC	Sunset	Reserve Lake Keowee
O. korrae		keo257SC	Sunset	Reserve Lake Keowee
O. korrae		wc242SC	Clemson	The Walker Course
O. korrae		wc245Sc	Clemson	The Walker Course
O. korrae	North Carolina	che273NC	Charlotte	Cherokee CC
O. korrae		che274NC	Charlotte	Cherokee CC
O. korrae	California	wil277CA		
O. korrae		wil278CA		
O. korrae	Oklahoma	300OK		
O. korrae		301OK		
O. korrae		302OK		
O. korrae		303OK		
O. korrae		304OK		
O. korrae		305OK		

Table 3.2 Geographic origin of the isolates of *Ophiosphaerella* spp. collected from symptomatic hybrid and common bermudagrass in golf courses in South Carolina and in golf courses and polo fields in Argentina.

Species	Region	Sample ID	Location	
Ophiosphaerella herpotricha	Buenos Aires, ARG	pil21AR	Pilara GC	Pilar
O. herpotricha		pil27AR	Pilara GC	Pilar
O. herpotricha		mag68AR	Martindale CC	Pilar
O. herpotricha		mag69AR	Martindale CC	Pilar
O. herpotricha		maf3AR	Martindale CC	Pilar
O. herpotricha		maf65AR	Martindale CC	Pilar
O. herpotricha		chi97AR	Campo Chico CC	Matheu
O. herpotricha		chi99AR	Campo Chico CC	Matheu
O. herpotricha		nor7AR	Nordelta GC	Tigre
O. herpotricha		nor72AR	Nordelta GC	Tigre
O. herpotricha		can137AR	El Canton CC	Escobar
O. herpotricha		her54AR	La Herradura Polo	Grl. Rodriguez
O. herpotricha		edi106AR	EDIN	Grl. Rodriguez
O. herpotricha		edi107Ar	EDIN	Grl. Rodriguez
O. herpotricha		rel112AR	El Relincho Polo	Grl. Rodriguez
O. herpotricha		rel113AR	El Relincho Polo	Grl. Rodriguez
O. herpotricha		die120AR	San Diego CC	Moreno
O. herpotricha		die121AR	San Diego CC	Moreno
O. herpotricha		eve59AR	Everlinks CC	Lujan
O. herpotricha		eve60Sc	Everlinks CC	Lujan
O. herpotricha		col40AR	La Colina CC	Lujan
O. herpotricha		col43AR	La Colina CC	Lujan
O. herpotricha		hur103AR	Hurlingham Club	Hurlingham
O. herpotricha		hur104AR	Hurlingham Club	Hurlingham
O. herpotricha		fra8AR	San Francisco Polo	Lobos
O. herpotricha		fra49AR	San Francisco Polo	Lobos
O. herpotricha		vel128AR	Velay Polo	Lobos
O. herpotricha		vel129AR	Velay Polo	Lobos
O. herpotricha		eug33AR	San Eugenio Polo	Lobos
O. herpotricha	Inland ARG	esm86AR	Pinamar, BA	Costa Esmeralda CC
O. herpotricha		esm88AR	Pinamar, BA	Costa Esmeralda CC
O. herpotricha		jcr1AR	Rosario, Santa Fe	Jockey Club de Rosario
O. herpotricha		jcr140AR	Rosario, Santa Fe	Jockey Club de Rosario
O. herpotricha		caf36AR	Cafayate, Salta	Cafayate GC
O. herpotricha		caf39AR	Cafayate, Salta	Cafayate GC
O. herpotricha		she79UY	Colonia, Uruguay	Sheraton Colonia Hotel
O. herpotricha		she84UY	Colonia, Uruguay	Sheraton Colonia Hotel
O. herpotricha	North Carolina	lt2NC		
O. herpotricha		lt3NC		
O. herpotricha	Oklahoma	307OK		
O. herpotricha		308OK		
O. herpotricha		309OK		
O. herpotricha		310OK		
O. herpotricha		311OK		
O. herpotricha		313OK		
O. herpotricha		314OK		
O. herpotricha	Missouri	321MO		
O. herpotricha		325MO		
O. herpotricha		326MO		
O. herpotricha		327MO		
O. herpotricha		328MO		
O. herpotricha		331MO		
O. herpotricha		335MO		
O. herpotricha		337MO		
O. herpotricha		341MO		
O. herpotricha		342MO		

Table 3.3: Summary of the analysis of molecular variance table (AMOVA) for *Ophiosphaerella korrae* isolates from South Carolina, North Carolina, California, and Oklahoma, analyzed by comparison of AFLP, grouped by geographical location.

Source of Variation	Host Cultivar Variability					
	df	**Sum of Squares**	**Variance Components**	**Percentage of Variation**	**Fixation Indices**	***p-value* ***
Among Cultivars	4	540.698	9.098	62.32	F_{CT}: 0.62324	0.13089 + - 0.00338
Among Populations (Locations) Within Cultivars	1	11.000	5.500	37.68	F_{SC}: 1.0000	0.33515 + - 0.00467
Within Populations (Locations)	47	0.000	0.000	0.000	F_{ST}: 1.0000	0.0000 + - 0.0000
Total	52	551.698	14.598			

Table 3.4: Summary of the analysis of molecular variance table (AMOVA) for *Ophiosphaerella herpotricha* isolates from Argentina, North Carolina, Missouri, and Oklahoma, analyzed by comparison of AFLP, grouped by geographical location

Source of Variation	Geographical RegionVariability					
	df	Sum of Squares	Variance	Percentage of Variation	Fixation Indices	p-value *
Among Regions	1	441591	12.0108	73.45	F_{CT}: 0.73447	0.32921+-0.00455
Among Populations (Locations) Within Regions	2	65307	4.3422	26.55	F_{SC}: 1.0000	0.0000 + - 0.0000
Within Populations (Locations)	55	0.000	0.000	0.000	F_{ST}: 1.0000	0.0000 + - 0.0000
Total	58	478.898	16.3531			

Table 3.5: Summary of F_{ST} pairwise comparisons among isolates of *Ophiosphaerella korrae* isolates from Coastal SC, Inland SC, Upstate SC, North Carolina, California, and Oklahoma, based on AFLP results.

	Coastal SC	**Inland SC**	**Upstate SC**	**North Carolina**	**California**	**Oklahoma**
Coastal SC	*					
Inland SC	0.00000+-0.00000	*				
Upstate SC	0.00000+-0.00000	0.00000+-0.00000	*			
North Carolina	0.01832+-0.0014	0.00396+-0.0006	0.01455+-0.0013	*		
California	0.01653+-0.0013	0.00158+-0.0004	0.01980+-0.0016	0.33700+-0.0052	*	
Oklahoma	0.00030+-0.0002	0.00000+-0.00000	0.00000+-0.00000	0.03356+-0.0019	0.03277+-0.0015	*

Data in red color are significantly different.
Significant at $p < 0.003$
Bonferroni corrections were performed because of multiple comparisons (15 pairs)

Table 3.6: Summary of F_{ST} pairwise comparisons among isolates of *Ophiosphaerella herpotricha* isolates from Buenos Aires AR, Inland AR, Missouri, Oklahoma, and North Carolina, based on AFLP results.

	BA ARG	**Inland ARG**	**Missouri**	**Oklahoma**	**North Carolina**
BA ARG	*				
Inland ARG	0.00000+-0.00000	*			
Missouri	0.00000+-0.00000	0.00000+-0.00000	*		
Oklahoma	0.00000+-0.00000	0.00079+-0.0003	0.00000+-0.00000	*	
North Carolina	0.00238+-0.0005	0.003336+-0.0018	0.01277+-0.0011	0.02921+-0.0017	*

Data in red color are significantly different.
Significant at $p < 0.005$
Bonferroni corrections were performed because of multiple comparisons (10 pairs)

Figure 3.1: Synthetic gel showing polymorphic bands generated with primer set E-AG FAM/MseI-CG with DNA from *Ophiosphaerella korrae* isolates from South Carolina (SC), North Carolina (NC), Oklahoma (OK), and California (CA)

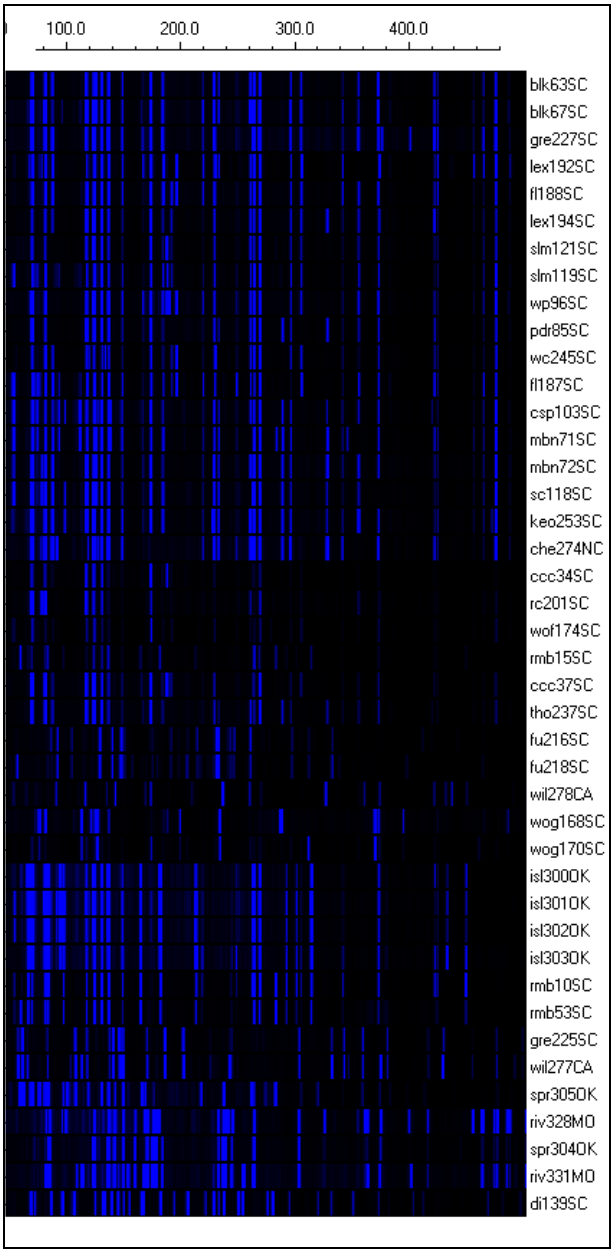


Figure 3.2: Synthetic gel showing polymorphic bands generated with primer set E-AG FAM/MseI-CT with DNA from *Ophiosphaerella korrae* isolates from South Carolina (SC), North Carolina (NC), Oklahoma (OK), and California (CA)

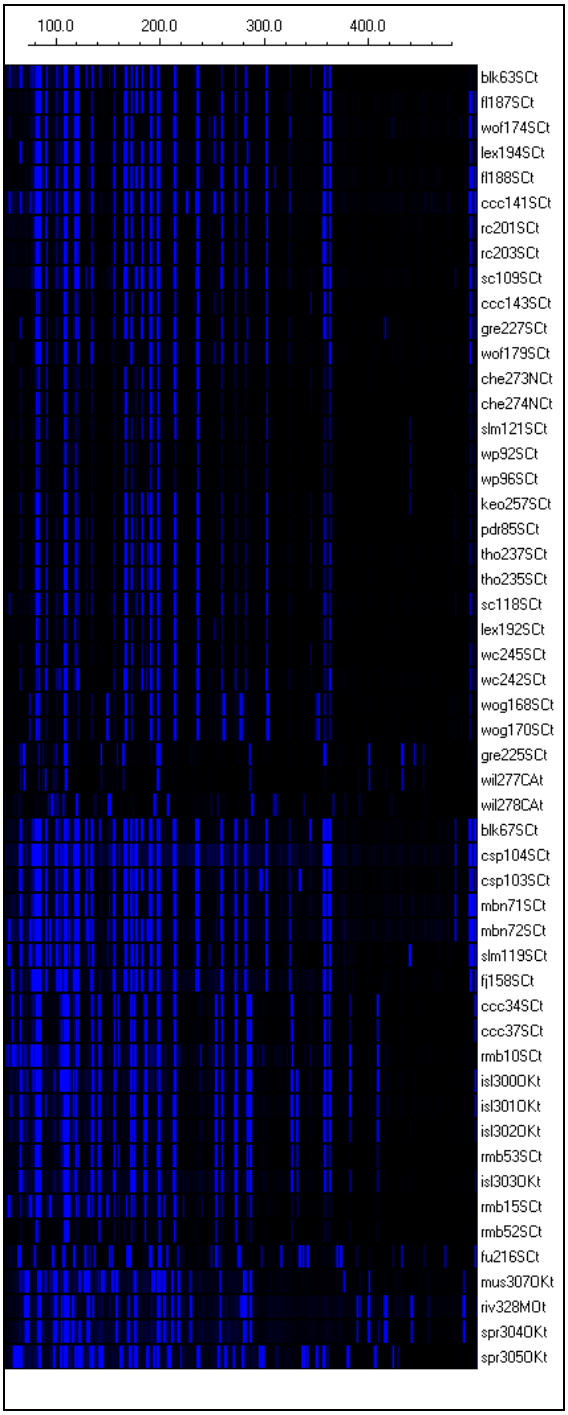


Figure 3.3: Synthetic gel showing polymorphic bands generated with primer set E-AG FAM/MseI-CG with DNA from *Ophiosphaerella herpotricha* isolates from North Carolina (NC), Oklahoma (OK), Missouri (MO), and Argentina (AR)

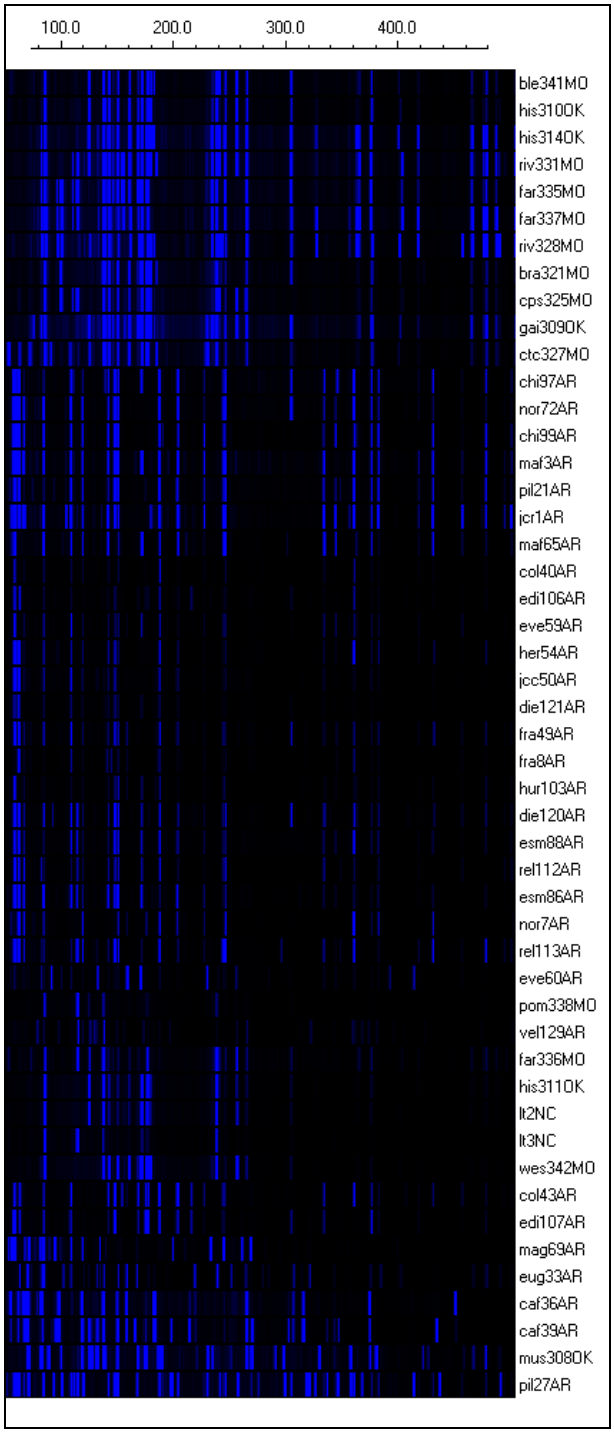


Figure 3.4: Synthetic gel showing polymorphic bands generated with primer set E-AG FAM/MseI-CT with DNA from *Ophiosphaerella herpotricha* isolates from North Carolina (NC), Oklahoma (OK), Missouri (MO), and Argentina (AR)

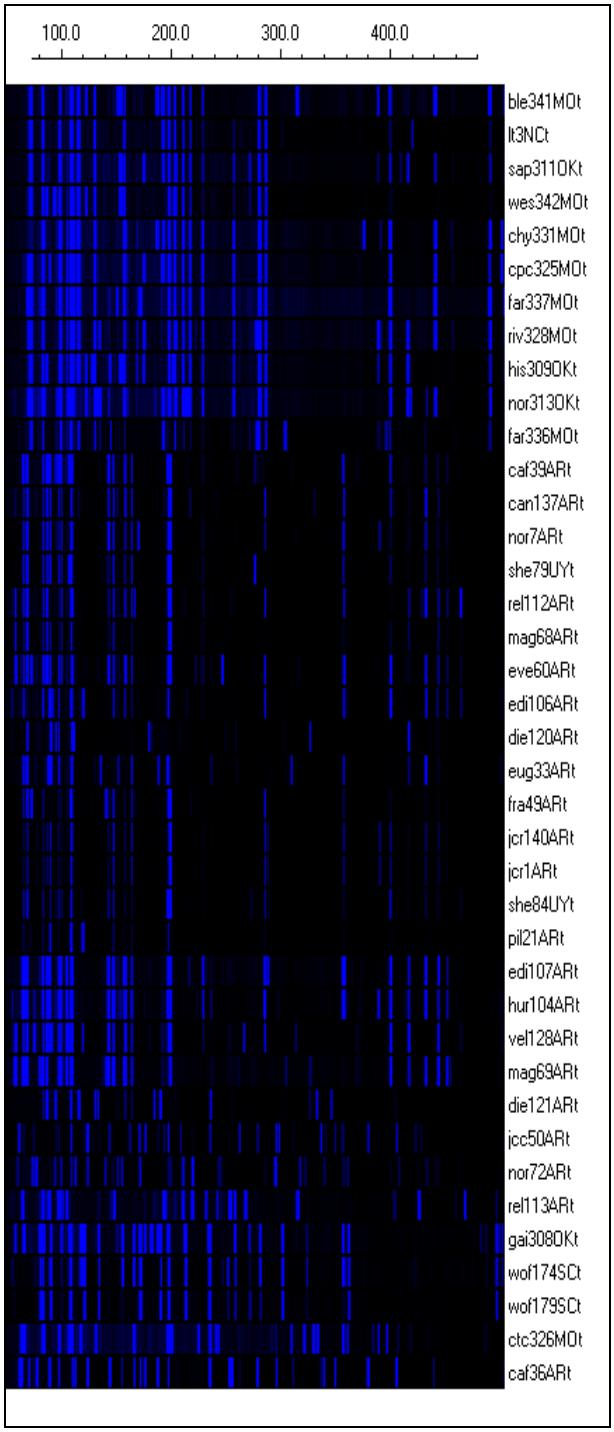
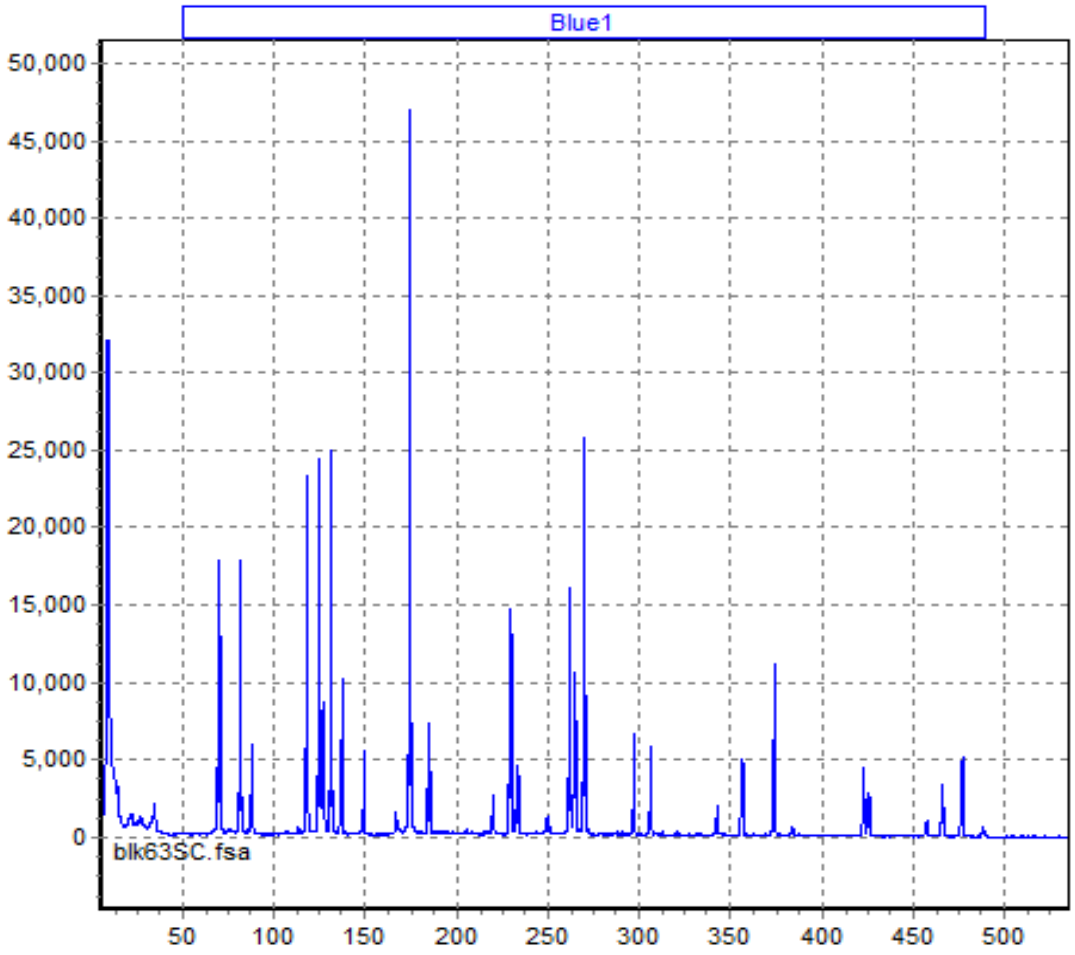
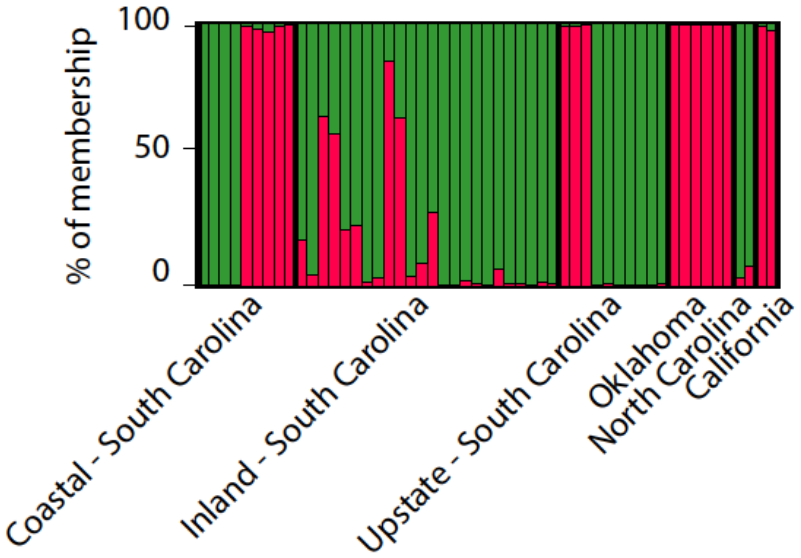


Figure 3.5: Example of a raw data of AFLP processed with GeneMarker software. Only traces for fluorescent blue dye are shown (color of FAM, the dye associated with the primer used in selective amplification).



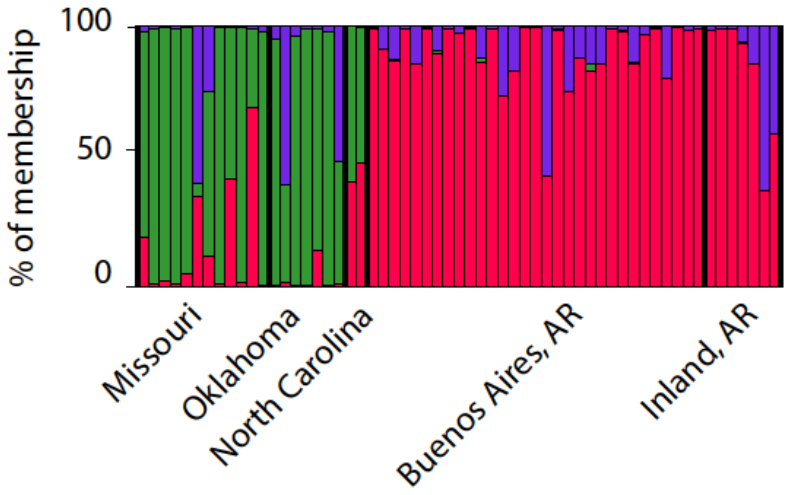
Plot shows fragments recorded in Relative Fluorescent Units (0 to 50,000 RFUs) on the y axis (heights of the peaks denote the intensity of the signal in RFUs). Fragment sizes (50 to 500 bp) are plotted in the x axis.

Figure 3.6: Graphical representation of *Ophiosphaerella korrae* genotypes of populations from South Carolina, North Carolina, Oklahoma, and California.



Genotypes were obtained using AFLP with two different set of primers pairs. Images were produced from the program DISTRUCT and are based on population grouping assignments determined by the program STRUCTURE V 2.3.
Each column on the X-axis represents the genotype of one isolate. Y-axis is percentage based and represents the homogeneity of the genotypes.
Different colors show different groups (red and green)

Figure 3.7: Graphical representation of *Ophiosphaerella herpotricha* genotypes of populations from Missouri, North Carolina, Oklahoma, and Argentina generated with AFLP



Genotypes were obtained using AFLP with two different set of primers pairs. Images were produced from the program DISTRUCT and are based on population grouping assignments determined by the program STRUCTURE V 2.3.
Each column on the X-axis represents the genotype of one isolate. Y-axis is percentage based and represents the homogeneity of the genotypes.
Different colors show different groups (red, green, and purple)

Figure 3.8: Maximum parsimony phylogenetic tree (created using PAUP software) of *O. korrae* isolates based on AFLP analysis.

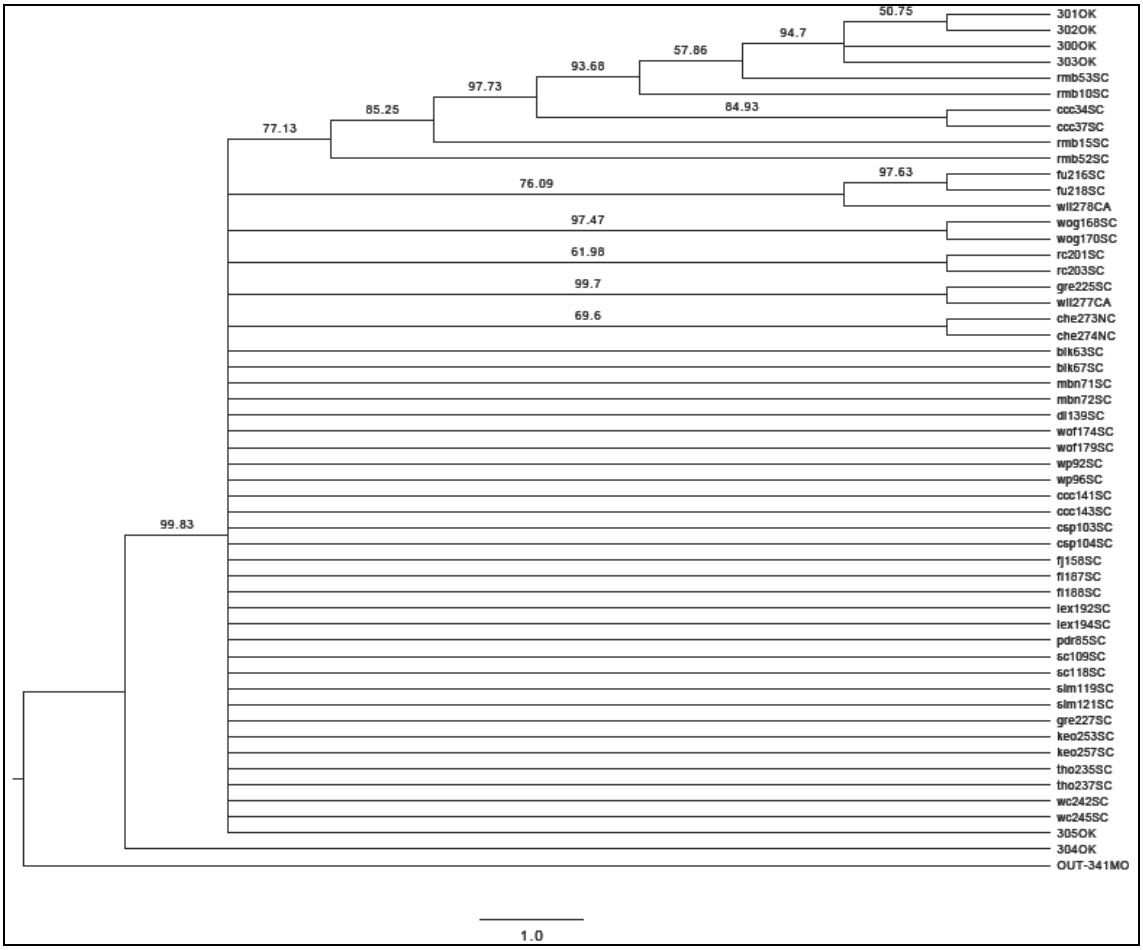
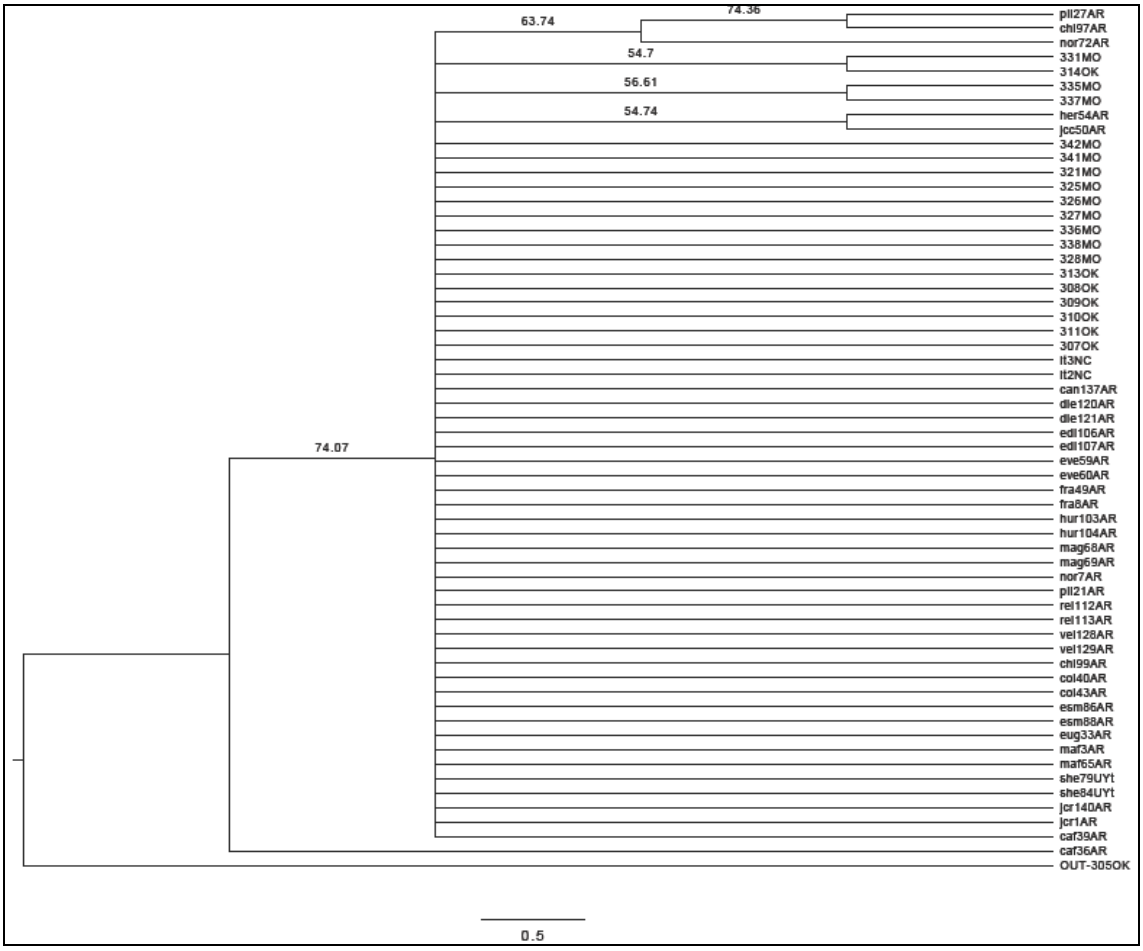


Figure 3.9: Maximum parsimony phylogenetic tree (created using PAUP software) of *O. herpotricha* isolates based on AFLP analysis. It shows no genetic structure associated with geographical regions.



Literature Cited

Butler E.L. (2004) Development of novel strategies for control of spring sead spot of bermudagrass, Department of Plant Pathology, North Carolina State Unversity, Raleigh, NC.

Camara M.P.S. (1999) Systematics of *Ophiosphaerella, Paraphaeosphaeria and Phaeosphaeria*: a molecular and morphological approach, Plant Pathology, The Pennsylvania State University.

Canegallo A., Martin S.B., Park W., Agudelo P., McCarty L., Kerrigan J., Tredway L., Cantoro R. (2015) Characterization of *Ophiosphaerella* species causing spring dead spot disease of bermudagrass in South Carolina and Buenos Aires, Argentina., in: T. A. P. Society (Ed.), APS Annual Meeting, Pasadena, CA.

Douhan G.W., Olsen M.W., Herrell A., Winder C., Wong F., Entwistle K. (2009) Genetic diversity of *Labyrinthula terrestris,* a newly emergent plant pathogen, and the discovery of new Labyrinthulid organisms. Mycological Research 113:1192-1199.

Endo R.M., Ohr H.D., Krausman E.M. (1985) *Leptosphaeria korrae*, a cause of the spring dead spot disease of bermudgrass in California. Plant Disease 69:235-237.

Hou Y., Lou A. (2011) Population genetic diversity and structure of a naturally isolated plant species, *Rhodiola dumulosa* (crassulaceae). PLoS ONE 6.

Iriarte F.B., Wetzel H.C., III, Fry J.D., Martin D.L., Tisserat N.A. (2004) Genetic diversity and aggressiveness of *Ophiosphaerella korrae*, a cause of spring dead spot of bermudagrass. Plant Disease 88:1341-1346.

Kaminski J.E., Dernoeden P.H., Mischke S., O'Neill N.R. (2006) Genetic diversity among *Ophiosphaerella agrostis* strains causing dead spot in creeping bentgrass. Plant Disease 90:146-154.

Landschoot P.J. (1994) Taxonomy and biology of ectotrophic root-infecting fungi associated with patch diseases of turfgrasses, in: B. B. Clarke and A. B. Gould (Eds.), Turfgrass Patch Diseases: Caused by Ectotrophic Root-Infecting Fungi, The American Phytopathological Society., St. Paul, Minnesota. pp. 41-71.

Mallet J., Willmott K. (2003) Taxonomy: Ranaissance or tower of babel?, Trends in Encology and Evolution. pp. 57.

Milla-Lewis S.R., Zuleta M.C., van Esbroeck G.A., Quesenberry K.H., Kenworthy K.E. (2013) Cytological and molecular characterization of genetic diversity in *Stenotaphrum*. Crop Science 53:296-308.

O'Donnell K. (1992) Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). Current Genetics 22:213-220.

Pair J.C., Crowe F.J., Willis W.G. (1986) Transmission of spring dead spot disease of bermudagrass by turf/soil cores. Plant Disease 70:877-878.

Perry D.H., Tomaso-Peterson M., Baird R. (2010) Seasonal variation in frequency of isolation of *Ophiosphaerella korrae* from bermudagrass roots in Mississippi and pathogenicity and optimal growth of selected isolates. Mycopathologia 169:395-402.

Raffle V.L., Hsiang T. (1998) Low level of DNA polymorphisms in isolates of *Leptosphaeria korrae* pathogenic on Poa pratensis. Can. J. Plant Path 20:48-54.

Rice W.R. (1989) Analyzing tables of statistical tests. Evolution 43:223-225.

Rosenberg N.A. (2004) Distruct: a program for the graphical display of population structure. Molecular Ecology Notes 4:137-138.

Smiley R.W., Dernoeden P.H., Clarke B.B. (2005) Compendium of turfgrass diseases APS Press The American Phytopathological Society, St. Paul, Minnesota.

Tisserat N.A., Hulbert S.H., Sauer K.M. (1994) Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiosphaerella korrae* and *O. herpotricha*. Phytopathology 84:478-482.

Tisserat N.A., Pair J.C., Nus A. (1989) *Ophiosphaerella herpotricha* , a cause of spring dead spot of bermudagrass in Kansas. Plant Disease 73:933-937.

Tredway L.P., Butler E.L. (2007) First report of spring dead spot of zoysiagrass caused by *Ophiosphaerella korrae* in the United States. Plant Disease 91:1684.

Tredway L.P., Tomaso-Peterson M., Perry H., Walker N.R. (2009) Spring dead spot of bermudagrass: A challenge for researchers and turfgrass managers, APSnet, Plant Health Progress. pp. 1-18.

Vos P., Hogers R., Bleeker M., Reijans M., Van De Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. (1995) AFLP: A new technique for DNA fingerprinting. Nucleic Acids Research 23:4407-4414.

Walker J., Smith A.M. (1972) *Leptosphaeria narmari* and *L. korrae* spp.nov., two long-spored pathogens of grasses in Australia. British Mycological Society 58:459-466.

Wetzel H.C. (1999) Geographic distribution and genetic diversity of three *Ophiosphaerella* species that cause spring dead spot of bermudagrass, Department of Plant Pathology, Kansas State University, Manhattan, Kansas.

Wetzel H.C., III, Hulbert S.H., Tisserat N.A. (1999a) Molecular evidence for the presence of *Ophiosphaerella narmari* n. comb., a cause of spring dead spot of bermuda grass, in North America. Mycological Research 103:981-989.

Wetzel H.C., III, Skinner D.Z., Tisserat N.A. (1999b) Geographic distribution and genetic diversity of three *Ophiosphaerella* species that cause spring dead spot of bermudagrass. Plant Disease 83:1160-1166.

CHAPTER FOUR

MANAGEMENT PRACTICES FOR SPRING DEAD SPOT OF BERMUDAGRASS CAUSED BY *OPHIOSPHAERELLA HERPOTRICHA* IN ARGENTINA

Introduction

The Buenos Aires Province is located on the Centre-East part of Argentina, right in the transition zone for the southern hemisphere, where neither cool season turfgrasses nor warm season turfgrasses have optimal growing conditions (McCarty, 2005). Bermudagrass (*Cynodon* spp.) and the interspecific hybrids *C. dactylon* × *C. transvaalensis* are frequently used on residential lawns, sports fields, and golf courses. Spring dead spot (SDS) disease is the most devastating disease of bermudagrass in North America, Australia and Argentina (Luc et al., 2005; Smiley et al., 2005), where it undergo complete dormancy (Martin and McCarty, 2005).

Symptoms of SDS are circular patches of bleached dead grass (Smith, 1971) and rings that are observed in the spring when bermudagrass resumes growth (Tredway et al., 2009b). Patches look sunken and more evident when temperature increases in the early spring. In Argentina patches are visible in August/September at the end of winter, and sometimes patches can be observed in early winter and July. Recovery of the turfgrass is very slow, mainly through the regrowth from healthy stolons that grow into the patches from its perimeter. Over the last decade, SDS has been recognized as the most important problem both in golf courses and polo fields in the Buenos Aires Province. This is because the diseased patches persist for several months of the spring season on the turf of those sports fields.

SDS is caused by three different species of fungi in the genus *Ophiosphaerella*: 1) *O. korrae* (J. C. Walker and A. M. Smith) Shoemaker and Babcock (synonym: *Leptosphaeria korrae* Walker and Smith), 2) *O. herpotricha* (Fr.:Fr.) J. C. Walker, and 3) *O. narmari* (J. C. Walker and A. M. Smith) Wetzel, Hulbert and Tisserat (synonym: *L. narmari* Walker and Smith). Spring dead spot is a well-known disease in bermudagrass in the U.S.: *O. korrae* is the predominant species in the south east, and *O. herpotricha* is the predominant species in the mid-west. *O. narmari was* also isolated in the U.S. but it is found with less frequency. Using molecular techniques and species specific primers (Tisserat et al., 1994), our team (Canegallo, Martin and Park, unpublished) recently identified *O. herpotricha* as the sole causal agent of SDS in Argentina..

The fungus colonizes the root surface, grows radially along the longitudinal axis, and produces a dark network of ectotrophic hyphae which appears to directly penetrate the epidermis. It colonizes then the root intercellularly and grows into the cortex, where some cells become necrotic. Vascular infection is produced only in advanced stages of disease development. Stolons are infected and colonized over the surface as well, and extensive colonization in the hollow center of the stolons was observed in Tifway and Midlawn cultivars (Caasi et al., 2010). Infection of all species of *Ophiosphaerella* in the field typically takes place during the autumn when daily temperatures are between 12-14 °C and soil temperatures are below 21°C (Crahay et al., 1988; Smiley et al., 2005; Walker et al., 2006). However, Walker et al. (2006) also demonstrated that new roots can be colonized and infected by *O. herpotricha* with cool soil temperatures during spring time in Oklahoma.

Chemical controls have been practiced in Buenos Aires, however the high cost of the fungicides make it necessary to explore alternative management practices that could be practical in Argentina to reduce the cost and improve the effectiveness of the control program. Current research on disease management of SDS agrees with the importance of an integrated management program. Integrating practices as improving soil conditions, proper nitrogen fertilization, reduction of soil pH, preventive fungicide programs, implemented over consecutive years would provide the best control, even though some disease may develop (Butler and Tredway, 2006; Vincelli, 2005).

The positive effect of acidic soil (rhizosphere) pH on reducing severity of patch diseases has been demonstrated in wheat (*Triticum aestivum* L)(Smiley and Cook, 1973), creeping bentgrass (*Agrostis stolonifera* Huds) (Kaminski and Dernoeden, 2005b) , and bermudagrass (*Cynodon spp*.) (Tredway et al., 2011). It appears not to be the direct effect of soil pH on the pathogen, but its indirect influence on the plant availability or deficiency of other nutrients, mainly manganese, and perhaps also on the population of microorganisms present in the rhizosphere acting as biological control agents (Hill et al., 2003). In the same way, it was reported that summer patch symptoms (caused by *Magnaporte poae* Landschoot and Jackson) in Kentucky bluegrass were more severe when nitrate sources of fertilizer were applied compared to ammonium sources (Thompson et al., 1995). Kaminski et al. (2005) also demonstrated that a reduction in pH using ammonium sulfate fertilizer produces a faster recovery of dead patches in creeping bentgrass infected with *Ophiosphaerella agrostis*. Cotrill et al. (2013) demonstrated in a field study in Missouri that pH manipulation may reduce severity of bermudagrass SDS

caused by *O. herpotricha* and *O. korrae* but this pH effect on disease reduction was not correlated with *in vitro* studies. Vincelli (2014) established that soil pH in the range of 5.0 to 5.3 in the rhizosphere significantly reduced SDS symtoms, however fungal growth of *Ophiosphaerella* species was not reduced at this low pH *in vitro*. The author speculated that when a more favorable environment is provided for the microorganisms that are antagonistic to the pathogens, then better conditions are given for the plant. During three years of study on previously SDS inoculated research plots, Tredway (2011) demonstrated that ammonium sulfate fertilization significantly reduced the SDS index (which combines disease severity and diameter of the patch) when *O. herpotricha* was the causal organism. However, a combined study of different sources of nitrogen and fungicides found no statistical differences on disease severity among plots receiving urea, ammonium sulfate, or calcium nitrate (Cottrill and Miller, 2012).

Aerification is a mechanical method of improving the exchange of air and water between the atmosphere and soil. Traffic over the turf causes soil compaction, and compacted soil conditions limit soil aeration and cause shallow rooting and reduced turfgrass vigor (Beard, 1973). Studies indicate that severe disturbance of the rootzone of symptomatic SDS bermudagrass, can reduce SDS damage caused by *O. herpotricha* (Tisserat and Fry, 1997). Cultivation by core aerification also removes some colonized roots, rhizomes and stolons and results in the production of new roots. Although these new roots will be re-colonized with the inoculum present in the soil, it would likely take a relatively longer period of time to disrupt the normal root functions. It was demonstrated that yearly removal of infected soil is the best spring dead spot cultural control, but

impractical for any golf course, so a combination of intense late summer core aerification and verticutting may promote the generation of new roots (Tisserat and Fry, 1997).

Many fungicides for SDS control have been tested since 1960 with variable results (Dernoeden, 1993). Reasons for the erratic outcomes are many, since epidemiology of SDS is not well known, and plant-fungus interaction occurs below ground and inside the roots. Varying aggressiveness of different causal organisms in different regions may partially explain the erratic outcomes in fungicide performance. Fungicide deliverance to the infected plant organs through irrigation prior to drying is important to transport the products into the root zone (Walker, 2009). Morning applications when dew is present and immediate activation of the irrigation system may increase the effectiveness of the fungicide treatment (Walker, 2009). To be effective for control, fungicides have to be applied in the fall, before bermudagrass plants go dormant. In a two-year study in North Carolina with fenarimol where application dates every 15 days were evaluated, differences were not detected among the treatments, applied at early August through early October. (Tredway et al., 2008).

Fenarimol was found as the only fungicide that reduced SDS incidence, in a test on Tifway bermudagrass (Wetzel, 2001). Similar results were obtained by Tredway and Butler (2006) with two fall applications of fenarimol on Tifway bermudagrass. In South Carolina on TifEagle putting greens, greatest reduction on plots affected with 2 or 3 preventive fall applications of fenarimol was observed. Meanwhile late winter applications although provided some disease control, turf quality was not acceptable (Luc et al., 2005). Walker found significant reduction of SDS severity in Oklahoma with

tebuconazole, propiconazole and fenarimol (Walker, 2005; Walker, 2009), but different results were obtained when tebuconazole was tested in 2012 and 2013. On the other hand, fenarimol and tebuconazole did not control a natural epidemic of SDS on Riviera bermudagrass trials conducted in 2011, and 2012 in Missouri (Earlywine and Miller, 2013; Earlywine and Miller, 2011).

The objective of this research was to develop best management practices in Argentina for spring dead spot control of bermudagrass caused by *O. herpotricha*. Field experiments were conducted to evaluate the individual and combined effects of different sources nitrogen fertilizers, the influence of core aerification, and fungicide applications on SDS.

Materials and Methods

Field Study Description

A field experiment was established in 2011 on a naturally infested 'Tifway' [*Cynodon transvaalensis* Burt-Davy × *C. dactylon* (L)] practice tee at Pilara Golf Club, Pilar, BA (Argentina) where symptoms of SDS were evident since 2010 (Figure 3.1). The experiment was repeated in 2013 and 2014. Pilar is located in the North East part of the Buenos Aires Province, coordinates 34°27'S 58°55'W.

The study was a three-factor factorial experiment with factor I: source of nitrogen (SN), factor II: aerification (A), and factor III: fungicides (F). The experimental design was a split-split plot randomized complete bock design (RCB), with 4 blocks conducted

with 3 treatments for factor I as the whole plot, 2 treatments for factor II as subplots, and 4 treatments for factor III as sub-subplots.

Plots for factor I: SN (source of nitrogen) measured 7 m × 8 m and treatments included urea (SN1), [water soluble, 46% of actual nitrogen (Profertil, Bahia Blanca, Argentina)], calcium nitrate (SN2) [$Ca(NO_3)_2$, water soluble, 15.5% of actual nitrogen, 19% calcium (YaraLiva CALCINT, Yara Argentina, Buenos Aires, Argentina], and ammonium sulfate (SN3), [$(NH_4)_2SO_4$, water soluble, 21% actual nitrogen, 24% SO_4, YPF Argentina]. The amount of actual nitrogen applied was 2.5 g $(m^2)^{-1}$ per month, in October, November, December, January, February and March (spring and summer time for the southern hemisphere, after bermudagrass resumed growth) divided in two applications per month. Fertilizers were granular applied with a Gandy 36 Variable Rate Drop Lawn Spreader (Gandy Company, Owatonna, MN 55060-0528). These SN treatments were divided in sub-plots measuring 3.5 m by 8 m with factor II (A).

Treatments for factor II: A (aerification) included a no aerification control (A1) and an aerification treatment (A2) that consisted of aerification two times in a year: the first week of December and the first week of February. Aerification was performed with a Toro Pro-Core 648 aerator (The Toro Company, Bloomington, MN 55420-1196), using 19.1 mm-inside diameter hollow tines in a 2×2 configuration of the machine, that resulted in a hole separation of 50.8 mm in both directions. Aerification were performed on December 6, 2011 and February 7, 2012 for the 2012 study; December 3, 2012 and February 8 for the 2013 study and December 3 and February 3 for the 2014 study. The aerification factor were further split with factor III: F (fungicides) in sub-sub plots

measuring 3.5 m by 2 m. Treatments for factor III included F1) non treated control, F2) two applications of tebuconazole 0.0875 g active ingredient m^{-1}, 28 days apart, F3) two applications of tebuconazole 0.175 g active ingredient m^{-1} 28 days apart, and F4) two applications of fenarimol 0.144 g active ingredient m^{-1} 28 days apart. No SDS symptoms were observed at the time of fungicide applications. Application timings were performed based on previous research on recommended fungicide application timing for SDS (Luc et al., 2005; Lucas, 1980; Tredway et al., 2008; Walker, 2009). Foliar fungicide treatments were applied with a custom made CO_2 pressurized push sprayer at 2 bar of pressure, calibrated to deliver 400 l ha^{-1}, equipped with two 11005 flat-fan nozzles (Spray Systems Co., Wheaton, IL) spaced 50 cm apart and 40 cm above the turfgrass canopy. Plots were immediately irrigated with 10 mm of water after fungicide application. Application dates are listed in Table 1.

Plots were maintained at a mowing height of 12 mm, weed free with one application of Equip WG (foramsulfuron 30 g + iodosulfuron 2 g) at 120 g ha^{-1} of the product in June and one application of 2,4-D at 500 g ai ha^{-1} plus dicamba 72 g active ingredient ha^{-1} in September.

Soil pH was measured using a 1:2.5 dilution of soil in water and pH was measured from the resulting solution in *Laboratorio Inagro*, Buenos Aires, Argentina. Every sub-plot was sampled at two different depths, at the root zone (0 to 8 cm. depth) and the bulk soil (8 to 20 cm depth). It was performed every year just before the first fungicide application.

Visual Evaluation of Turfgrass Quality and Disease Severity

Turfgrass quality (TQ) and disease severity (DSV) were visually evaluated every 14 days since bermudagrass resumes growth in late September. Turfgrass quality refers to the aesthetic and functional aspect of the turf and include a combination of color (indicates the general condition of the plant), density (number of aerial shoots per unit area), uniformity (estimate of the even appearance of the turf), texture (measure of width of the leaf blades), and disease or environmental stress (Morris, 1998). Turfgrass quality was evaluated using a scale from 1 to 10, where 1= poor non-acceptable turfgrass quality and 10=thick, dense, healthy turfgrass. Disease severity is defined as the percentage of symptomatic in a spring dead spot patch (Walker, 2013). Visual evaluations were taken over the patches present on each sub-sub plot. On a scale from 1 to 10, where 1=no disease, 5=50% of the turfgrass live into the patch and 10=no living turfgrass into the patch.

Digital Evaluation of Spring Dead Spot Incidence and Intensity

Spring dead spot incidence and intensity were also evaluated every 14 days using digital images. Disease incidence (DIn) is the proportion or percentage of diseased entities within a sampling unit (Seem, 1984). In this study the sampling unit was the sub-sub plot measuring 2×3.5 m. A digital photograph was taken for each sub-sub plot with a Nikon 1 J1 camera (Nikon Inc. Melville, NY 11747-3064, U.S.A.) on Oct 12 2012, Oct 15, 2013, and Oct 21, 2014, when bermudagrass resumed growth and was actively growing. The digital camera was mounted in a custom made monopod as described by Butler (2004), but modified at the vertical setting to 3.45 m and horizontal setting that was 1 m from the center of the monopod to reach the center of the sub-sub plot and

obtain a complete image of each sub-sub plot at the same exact location each time (Figure 3.2). Photographs were taken during morning hours to avoid shadows from the monopod on the sub-sub plots. The camera was set to fully automatic mode, and images were 1936 × 1296 pixels in size. Each image was transferred to a computer, resized and renamed using FastStone Photo Resizer 3.2 (FastStone Soft, www.faststone.org) program to 800 × 536 pixels and saved in the computer in jpeg (joint photographic expert group) format.

DIn was assessed using the computer program Sigma Scan 80 Pro v. 5.0 (SPSS, Inc., Chicago, IL 60611) for digital image analysis. The *Turf Analysis v 1-4* macro, capable of automated batch analysis for the high number of pictures, was used. (Karcher and Richardson, 2005). Settings to perform turfgrass cover analysis on the SigmaScan program for healthy bermudagrass were: hue 35 to 235; saturation 0 to 100 (Butler, 2004). Cover analysis was performed, and percentage of turfgrass coverage of the analyzed image was determined, by counting the number of green color pixels. Disease incidence was later calculated by the difference of 100% and the percentage of turfgrass coverage calculated by the SigmaScan program (Figure 3.3).

For the evaluation of disease intensity (DIt), digital photographs were taken every 14 days after bermudagrass resumed growth during 2013 and 2014. A custom made light box was built in Argentina similar to the light box made by NexGen Turf Research LLC, Maricopa, AZ 85139 (Schwartz, 2013) to obtain images with the same illumination on every evaluation date and an objective analysis of the images using SigmaScan computer program as described above. The light box size was 50 cm height × 50 cm width × 60 cm

depth (Figure 3.4). Illumination was provided by 4 equal 40 Watt spectrum daylight bulbs distributed uniformly in the box, which provided 67 lumens per Watt (Figure 3.5). Power was provided by a Honda portable power unit. Images were taken, resized for computer analysis, and saved as described above. Pictures were cropped to 37% of the original size to reach the infection center, and therefore measure disease intensity on the selected patch on each sub-sub plot (Figure 3.6). DIt is defined as the percentage of non-green pixels in a cropped photograph.

Results

This study shows the effects of fungicide treatments, nitrogen source, and aerification on spring dead spot of bermudagrass disease in a split-split plot randomized block design during three years in Buenos Aires Province (Table 2).

Spring dead spot disease pressure was very high in all three years of this study, and this had been typically observed at Pilara GC previously. Minimum mean temperature for March, April, and May (fall time in Southern hemisphere) were lower in 2012 and 2013 than in 2014 as well as during June, July, and August, winter time for southern hemisphere (Figure 3.7) (Sala, 2015). Therefore high disease incidence with severe symptoms developed every year of the duration of this study. For the year 2012, normal in terms of air temperature, severe symptoms of SDS impacted 9.57% on the total plot area (DIn). Disease was more severe in 2013, when the affected area showed DIn of 10.5%, with severe symptoms of 45.3% of dead turfgrass within the patch (DIt). Mean low air temperatures between June and October 2014 were higher than previous years,

and as expected, it was the least severe of the three years, with DIn of 5.5 % of the plot surface impacted by SDS. DIt was less as well, affecting 37.4% of the turf within the patches. Nevertheless, it was sufficient to allow good treatment evaluations (Figures 3.8 and 3.9).

Evaluation of this study started each year in late September, early spring in South America, when bermudagrass resumed growth and symptoms of spring dead spot appeared.

Visual observations and digital photographs were taken every 14 days, therefore expected differences were found as air temperature increased among the evaluation dates (ED). In 2012, the simple effect of source of nitrogen produced significant differences when DIn was evaluated (*P value* = 0.0405). Aerification did not result in any significant difference between the two treatments aerification vs no aerification for DIn during the same year. As expected, the main effect of fungicides showed significant differences for DIn ($P = 0.002$), turfgrass quality ($P < 0.0001$) and DSV ($P < 0.0001$), where DIt was not evaluated during this year. In 2013, the effect of nitrogen source (SN) showed significant differences between the treatments for DIn ($P = 0.002$), DIt ($P = 0.043$), TQ ($P < 0.0001$), and DSV ($P < 0.0001$). Aerification vs. no aerification showed a significant difference when DIt was evaluated ($P = 0.0001$), and the effect of fungicide showed significant differences for the entire evaluation: DIn ($P = 0.004$), DIt ($P < 0.001$), TQ ($P < 0.0001$), and DSV ($P < 0.0001$). During 2014, evaluations showed that simple effect of source of nitrogen significantly affected DIt ($P = 0.04$), aerification factor did not significantly impact on any of the effects evaluated, however the main effect of

fungicides produced significant differences among the treatments for DIn (P = 0.011), DIt (P < 0.001), TQ (P < 0.0001), and DSV (P < 0.0001) (Table 2).

When interactions were present, a series of profile plots were created to completely understand the nature of this interaction and to ensure that the simple effect of the factor were always reasonably consistent with the main effect (Tables 2 and 3). Fortunately in this study, the simple effect of the factors was reasonably consistent with the main effects which allowed the use and interpretation of the main effect to be valid.

Source of nitrogen effect on spring dead spot

The source of nitrogen applied had a significant effect on DIn in 2012, when plots treated with ammonium sulfate showed lower DIn (7.70%) followed by calcium nitrate (9.83%) and urea (11.18%) (Table 3). However, in 2013, DIn was best reduced with calcium nitrate (7.89%) when compared with urea (11.62%) and ammonium sulfate (12.14%). In 2014 no significant differences were found in symptomatic area among the three treatments (Figure 3.10).

During 2013, when ammonium sulfate was the source of nitrogen, DIt was 40.3%, significantly better than calcium nitrate (42.14%) and urea (43.77%). Similarly, in 2014, plots treated with ammonium sulfate resulted in DIt of 40.51%, significantly different from calcium nitrate treatments (45.27%) but not different from urea treated plots (43.41%). (Figure 3.11).

When turfgrass TQ and DSV were evaluated, SDS was significantly affected by source of nitrogen only in 2013, when plots treated with calcium nitrate had the highest TQ and the lowest DSV, followed by ammonium sulfate (Table 3). No significant

differences occurred among sources of nitrogen on TQ and DSV during 2012 and 2014 (Table 3). However, over the plots where ammonium sulfate was the source of nitrogen and no fungicide was applied (untreated control plots), TQ was enhanced and DSV was reduced. This effect was not shown on the plots where any of the three fungicide treatments were applied (figures 3.12 and 3.13)

As expected, the source of nitrogen significantly affected soil pH. This effect was observed in the three years of the study at both soil depths (the root zone = 0 to 8 cm depth, and the bulk soil = 8 to 20 cm depth). In 2012, at the root zone depth, the application of ammonium sulfate provided significant soil pH reduction (7.65) when compared with urea (7.9), and calcium nitrate (7.97) in 2012. The value of the pH was significantly lower in the rootzone (7.72) when compared to the bulk soil (7.96) (Figure 3.14). In 2013, ammonium sulfate significantly reduced pH to 7.68 when compared with urea (8.05) and calcium nitrate (8.04). pH at the two different soil depths was significantly lower at the root zone depth (7.74) than the bulk soil (8.1) (Figure 3.14). In 2014, soil pH over the plots fertilized with ammonium sulfate (7.67) were significantly lower than urea (8.22) and calcium nitrate (8.4), and again pH at the upper part of the soil (7.74) showed lower pH than the bulk soil (8.4) (Figure 3.14). Even though pH was reduced with ammonium sulfate, the pH values overall were still high and could be considered to continue to be a factor favoring increased severity of SDS.

Aerification effect on spring dead spot

Aerification did not affect DIn and TQ during the three years of the study (Table 3). When DIt was evaluated, there were significant differences (P=<0.0001) between

aerification treatments in 2013, with 2 aerifications reducing the intensity of spring dead spot (39.85 %), compared to no aerification (44.29 %). However it did not produce any significant difference in 2014. No aerification was also slightly more effective in reducing DSV in 2014 when compared 2-times aerification plots (2.83) with the untreated control (2.97), but no apparent benefits in reducing SDS were documented in 2012 and 2013 (Table 3). The aerification treatments did not impact the soil pH value during the three years of this study.

Fungicide effect on spring dead spot

The analysis of variance summary (Table 2) shows that preventive fungicide applications during the fall before bermudagrass dormancy were the most consistent and important factor evaluated in this experiment to reduce SDS. Fungicide applications consistently reduced disease incidence, disease intensity, and disease severity, and therefore increased general turf quality during each of the three years of study (Table 3). The effect of fungicides showed low p-values for every year of the effects evaluated (Table 2), however spring dead spot reduction was barely acceptable during 2012 and 2013, when disease pressure were higher. (Table 3).

In 2012, fenarimol (7.58 %) or tebuconazole at the low rate (7.62%) significantly reduced DIn, followed in performance by tebuconazole at the higher rate (DIn 10.06%), when compared to the untreated control plots (13.03%). (Table 3). In 2013 and 2014, all plots treated with the three different fungicide treatments significantly reduced disease incidence, but no differences were found comparing fungicides or tebuconazole rates (Figure 3.15) (Table 3).

Results showed that all fungicides significantly reduced DIt during 2013 when compared with untreated control plots (47.33% of diseased area) (Table 3). The high rate of tebuconazole with 35.9% of dead turf into the selected patch was more effective than fenarimol (42.4%) and tebuconazole at the low rate (42.75%), which did not differ with each other. In 2014, tebuconazole at the high rate (38.02%) had least DIt followed by tebuconazole at the low rate (40.6%) and fenarimol (43.49%), while untreated control plots showed a significant 50.51% of dead turfgrass (non-green pixels) into the infection center (Figure 3.16).

All the plots treated with fungicides showed lower DSV than the untreated control plots (Table 3). In 2012, DSV was significantly less in plots treated with the low rate of tebuconazole (3.46) or fenarimol (3.70) followed by tebuconazole at the high rate. In 2013, tebuconazole high rate (3.16) significantly reduced DSV, followed by fenarimol (3.61) and tebuconazole low rate (3.88) when compared with untreated control (4.95). In 2014, tebuconazole high rate (2.55) or fenarimol (2.7) best reduced DSV followed by tebuconazole low rate (2.98) and significantly higher the untreated control plots with a score of 3.38 of DSV.

In the plots where fungicides were applied, there was a significant improvement in general turf quality during the whole study. In 2012 there were no significant differences among the three fungicidal treatments but a significant response ($P<0.0001$) when compared with untreated control plots (Table 3). Nevertheless, significant differences were noted among the four treatments in 2013: plots treated with two applications of tebuconazole at the high rate (5.59) had the best TQ, followed by

fenarimol (5.36), tebuconazole low rate (5.01) and untreated control (4.2). In 2014 no significant differences were observed between plots treated with fenarimol (5.86) and tebuconazole at the high rate (5.95), followed by tebuconazole low rate (5.57) and untreated control plots (5.21) (Table 3).

Interactions between fungicide and source of nitrogen have been found on most of the effects evaluated during the three years of the study. However, although mathematically true, the simple effect of the factors, were reasonably consistent with the main effect. No interaction was found between fungicide treatments and evaluation dates when DIt was evaluated. The data indicate that preventive fungicide treatments did not improve the speed of healing of bermudagrass. Nevertheless, as the surface affected by SDS over untreated control plots were significantly higher (DIn, tables 3.2 and 3.3) than fungicide treated plots for the three years of the study, disease intensity (DIt) and disease severity (DSV) were lower in fungicide treated plots (Tables 3.2 and 3.3), complete recovery of bermudagrass turf was produced earlier than in untreated control plots (Figures 3.17 and 3.18).

Discussion

Results obtained in this study comparing the effects of source of nitrogen were not as conclusive as a three year field study in Maryland where the percent area affected by spring dead spot disease caused by *Leptosphaeria korrae* was less on every plot fertilized with any source of nitrogen compared with the non-treated control, but the best treatment observed were the plots treated with ammonium sulfate (Dernoeden et al., 1991).

Nevertheless, data obtained in this research suggests the positive effect of ammonium sulfate to reduce severity of spring dead spot when the causal agent is *Ophiosphaerella herpotricha*. When disease intensity was measured using digital image analysis, plots fertilized with ammonium sulfate had patches with more living turf inside (lower disease intensity) when compared with the other sources of nitrogen. Similar results were obtained by Tredway, et al (2009) who confirmed the positive affect of ammonium sulfate on disease control when the causal agent was *O. herpotricha.* However in this three-year study in Argentina, the influence of nitrogen source on percentage of plot area affected by SDS was not consistent, with one year (2012) where disease incidence was best reduced by ammonium sulfate, and another year (2013) where calcium nitrate ranked first for DIn reduction. The last year (2014) there was no differences among the three sources, similar to a study in Missouri (Cottrill and Miller, 2013), which showed no significant differences for disease severity reduction among the plots receiving the same three sources of fertilizers.

Soil pH was significantly reduced by ammonium sulfate, and in the plots where it was the source of nitrogen and no fungicide was applied (untreated control plots), TQ was enhanced and DSV was reduced. This effect was not shown on the plots where any of the three fungicide treatments were applied, perhaps because of a masking effect of the fungicides. This interaction would suggest that there would be a positive effect of reducing soil pH through the exclusive use of ammonium-based fertilizers like ammonium sulfate on SDS control, possible by enhancing plant resistance in some way or creating a more favorable environment for antagonistic microorganisms, as Vincelli

(2005) speculated. On the other hand, Cottrill, et al (2013) demonstrated in an *in-vitro* experiment, that growth of mycelium of *O. herpotricha* and *O. korrae* was significantly lower on pH 4 compared to pHs 5 to 9. Although no correlation was found with field studies, it would suggest that low pHs would delay the growth of *Ophiosphaerella* species mycelium. In a study by Kaminsky and Dernoeden (2005) on dead spot of bentgrass caused by *O. agrostis*, pH was reduced after one month of fertilization with ammonium nitrate to 6.36 and disease recovery was fastest, while when fertilized with urea, pH remained at 6.78 (Kaminski and Dernoeden, 2005a). Thompson, et al reported a reduction in soil pH from 6.6 to 5.8 and a significant reduction in summer patch lowering the pH during two consecutive years of applying ammonium sulfate as the source of nitrogen. Studies on take all disease of wheat demonstrated that the main effect of reducing soil pH is the increment of Mn availability (Huber and Mccaybuis, 1993). Previous field studies where ammonium sulfate was tested as a factor for SDS reduction, the decrease in the magnitude of pH was not registered (Cottrill and Miller, 2013; McCarty et al., 1992; Tredway et al., 2009a). In this study the pH was reduced but still remained relatively high (pH 7-7.5) which would have a large effect in nutrient availability, such as Mn, and could be considered to continue to be a factor favoring increased severity of SDS. Irrigation water quality may explain in part the small magnitude of pH reduction. Analysis of water quality in September 2012 showed high pH (8.6) and bicarbonates (308.7 mg l^{-1}), but low sodium (114.8 mg l^{-1}) and total dissolved salts (506.4 mg l^{-1}). Analysis was repeated in May and September 2013 when both pH and bicarbonates remained high (pH: 8.1/7.9; bicarbonates: 477.2/436.4 mg l^{-1}). Based on

McCarty (2005), water containing more than 4 meq l^{-1} may need to be acidified with sulfuric or phosphoric acid. It may be that in the site for this study at Pilara GC in Argentina, not only water treatment but also application of soil amendments like gypsum or sulfur are also means to help manage bicarbonate problems (McCarty, 2005), and additional years of fertilization with ammonium sulfate would be needed to lower the pH sufficiently for greater disease suppressive effects.

Effect of core aerification was not as consisted as demonstrated in a study by Tisserat and Fry, 1997. Removing the soil cores would remove some inoculum of the disease, and allow channels in the soil profile for the plant to generate new roots. It was also reported that in summer patch on annual and Kentucky bluegrass, a preventive aerification (in absence of symptoms) during the spring and fall is recommended to improve drainage, reduce soil compaction and aid in disease control (Smiley et al., 2005). However, only one of the three years under study, a positive effect of core aerification was observed. Core aerification, is a mechanical method to improve the exchange of air and water between the atmosphere and the soil, disrupt and destroy undesirable layers of different textures, and stimulate thatch decomposition (Beard, 1973), resulting in new shoot and root growth therefore plant health. It is important to maintain a balance of O_2 with CO_2 and other toxic gases in the root zone, not only for the roots but also for microorganisms which produce organic acids that increase soil acidity (Beard, 1973).

Several fungicides labeled in the US for SDS control have been tested since 1969 with variable results, and in general, fungicides have been less successful in the mid-western United States than in the southeastern United States (Tredway et al., 2009b). In

the mid-western US, the primary causal agent is *O. herpotricha*; and *O. herpotricha* has been the sole causal agent of SDS identified in Argentina. The results obtained in this study, however, showed conclusive benefits of preventive fungicide applications during the fall before bermudagrass goes dormant. During the years of 2012, 2013 and 2014 the application of fungicides consistently reduced the severity of the disease and enhanced the quality of a healthier turfgrass. Both fenarimol and tebuconazole performed well in this study, with the high rate of tebuconazole having comparable efficacy to fenarimol. Several studies in the US have shown the efficacy of preventive application of fenarimol and tebuconazole for SDS, with fenarimol being the most effective for reduction of disease incidence and severity either when *O. korrae* and *O. herpotricha* were the causal agents (Butler and Tredway, 2004; Luc and Martin, 2006; McCarty et al., 1992; Tredway and Butler, 2005; Walker, 2010; Walker, 2005; Wetzel, 2001). In addition, previous research conducted in Argentina showed excellent SDS control on 'TifEagle' putting greens with fenarimol (Luc et al., 2005), and with tebuconazole (Canegallo and Martin, unpublished). However in Missouri, no effect was seen from applied fungicides (Earlywine and Miller, 2013). These different outcomes may be due to the difference in aggressiveness of the species as was found in Oklahoma, where it is suggested that the most prevalent causal agent *O. herpotricha* has two primary periods of disease activity during a year (Walker et al., 2006) and in North Carolina, where the most prevalent *O. korrae* seems to be less aggressive than *O. herpotricha* (Tredway et al., 2008) Nevertheless, it appears that fungicides, including both fenarimol and tebuconazole, are a

viable and potentially valuable tool for managing SDS in Argentina, based on this study and previous trials in Argentina.

The method for assessing disease incidence using digital photography (Karcher and Richardson, 2005) was accurate and precise as reported by Butler (2004). The modification of this method by the use of the light box, removed the disadvantage presented by Butler (2004) concerning the effect of light quality and shadows over the plot, thus provided highly accurate results to detect differences among treatments under evaluation. These results indicate that incidence, intensity, and severity of spring dead spot of bermudagrass were consistently reduced by the use of fungicides, but barely to a level where the game of golf or polo can be played in acceptable conditions during early spring. The effect of nitrogen source and aerification were inconsistent. Moreover, neither a combination of two, nor the combination of the three factors produced a significant and constant effect of reducing SDS. Regardless, ammonium sulfate had an effect on the reduction of soil pH during the three years, what should have improved availability of micronutrients, therefore improved plant nutrition.

Although fungicide was consistently the best tool for reducing SDS incidence, intensity, and severity, spring fungicide applications may be tested to lower the colonization of the pathogen during this time of the year. Core aerification and thatch control during periods of active bermudagrass growth, to alleviate compaction, improve gas interchange with the atmosphere, increase water infiltration, and encourage the production of deeper and new roots need to be considered. In addition, the amount of inoculum of *O. herpotricha* that potentially colonize healthy roots during the spring

would be reduced (Walker et al., 2006). Finally, a program to upgrade the irrigation water quality and reduce soil pH in higher magnitude to increase nutrient availability would be beneficial for plant health and to expect better results on SDS control in Pilará GC the following year. Research performed in Pilar, Argentina, support the fact that control of spring dead spot of bermudagrass can be improved by the application of an integrated control program.

Table 4.1: Calendar and description of the experimental treatments for SDS management in 2012, 2013 and 2014 on the practice tee at Pilará GC, Pilar BA Argentina.

Factor and Treatment	Rate †	% a.i. ‡	Rate a.i. §	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Nitrogen Source ¶													
Urea	2.72	45	1.25			×	×	×	×	×	×		
Calcium Nitrate	8.07	15.5	1.25			×	×	×	×	×	×		
Ammonium Sulfate	6.25	20	1.25			×	×	×	×	×	×		
Aerification #													
No Aerification													
Aerification 2×								×		×			
Fungicide ††													
Untreated Control													
Tebuconazole	0.35	25	0.088								×	×	
Tebuconazole	0.7	25	0.175								×	×	
Fenarimol	1.2	12	0.144								×	×	

† Rates are in g $(m^2)^{-1}$ of formulated product per single application
§ Percentage of active ingredient of the formulated product
‡ Rates of active ingredients in g $(m^2)^{-1}$ per single application
¶ Fertilizers were applied the first and the third week of every month marked in 2011, 2012, 2013 and 2014.
# Aerification were performed December 6, 2011 and February 7, 2012 for the 2012 study; December 3, 2012 and February 8 for the 2013 study and December 3, 2013 and February 3, 2014 for the 2014 study.
†† Fungicides were applied on March 20th and April 17th, 2012; March 26th and April 23th, 2013; March 19th and April 16th, 2014

Table 4.2: Analysis of variance summary for the effects of source of nitrogen applied (SN), aerification practices (A), and fungicide applications (F) on SDS incidence measured by digital image analysis (DIn), disease intensity measured by digital image analysis (DIt), turfgrass quality measured by visual observations (TQ), and disease severity measured by visual observations (DSV) on the practice tee at Pilará GC, Pilar BA Argentina.

Factors and Interactions	2012					2013					2014				
	df	Digital Image Analysis		Visual Observations		df	Digital Image Analysis		Visual Observations		df	Digital Image Analysis		Visual Observations	
		DIn †	DIt ‡	TQ §	DSV ¶		DIn	DIt	TQ	DSV		DIn	DIt	TQ	DSV
		P>F					*P*>F					*P*>F			
Source of Nitrogen (SN)	2	0.0405	n.d.#	0.8701	0.051	2	0.002	0.043	< 0.0001	< 0.0001	2	0.151	0.044	0.3996	0.9247
Aerification (A)	1	0.794	n.d.	0.4019	0.377	1	0.0557	< 0.001	0.2175	0.8182	1	0.152	0.993	0.1927	0.0473
Fungicide (F)	3	0.002	n.d.	< 0.0001	< 0.0001	3	0.004	< 0.001	< 0.0001	< 0.0001	3	0.011*	< 0.001	< 0.0001	< 0.0001
Evaluation Date (ED)	3	n.d.	n.d.	< 0.0001	< 0.0001	6	n.d.	< 0.001	< 0.0001	< 0.0001	6	n.d.	< 0.001	< 0.0001	< 0.0001
F × SN	6	0.793	n.d.	0.0303	0.009	6	0.69	0.018	0.0022	< 0.0001	6	0.892	< 0.001	0.052	0.0006
F × A	3	0.892	n.d.	0.0409	0.5429	3	0.19	0.593	0.0706	0.4029	3	0.202	0.0011	0.0003	0.0167
F × ED	9	n.d.	n.d.	0.9104	0.6357	18	n.d.	0.555	0.8052	0.1076	18	n.d.	1.000	0.9994	0.8966

† Disease incidence evaluated using digital image analysis
§ Disease intensity evaluated using digital image analysis
‡ Turf quality evaluated through visual observations.
¶ Disease severity through visual observations
# n.d. non-determined

Table 4.3: Mean separation for the effects of source of nitrogen applied (SN), aerification practices (A), and fungicide applications (F) on SDS incidence measured by digital image analysis (DIn), disease severity measured by digital image analysis (DIt), turfgrass quality measured by visual observations (TQ), and disease severity measured by visual observations (DSV) on the practice tee at Pilará GC, Pilar BA Argentina.

Factors and Treatments	2012				2013				2014			
	Digital Image Analysis		Visual Observations		Digital Image Analysis		Visual Observations		Digital Image Analysis		Visual Observations	
	DIn †	DIt ‡	TQ §	DSV ¶	DIn	DIt	TQ	DSV	DIn	DIt	TQ	DSV
Source of Nitrogen												
Urea	11.18 a	n.d.††	6.19	4.22 a	11.62 a	43.77 a	4.77 b	4.18 a	6.27	43.41 ab	5.73	2.89
Calcium Nitrate	9.83 ab	n.d.	6.25	3.91 ab	7.89 b	42.14 ab	5.40 a	3.58 c	6.24	45.27 a	5.62	2.89
Ammonium Sulfate	7.70 b	n.d.	6.28	3.76 b	12.14 a	40.3 b	4.95 b	3.95 b	4.23	40.51 b	5.70	2.92
P > F	0.0405	n.d.	0.870	0.051	0.0002	0.0043	<0.0001	<0.0001	0.150	0.044	0.40	0.925
LDS (α = 0.05)	2.705		0.351	0.370	2.087	2.727	0.186	0.202	2.349	3.754	0.208	0.169
Aerification												
No Aerification	9.72	n.d.	6.18	3.89	11.39	44.29 a	4.99	3.89	4.87	43.07	5.71	2.97 a
Aerification 2×	9.43	n.d.	6.30	4.04	9.72	39.85 b	5.09	3.91	6.29	43.06	5.60	2.83 b
P > F	0.794	n.d.	0.402	0.359	0.056	<0.0001	0.216	0.818	0.152	0.99	0.19	0.047
LDS (α = 0.05)	2.209	n.d.	0.287	0.302	1.704	2.217	0.152	0.165	1.954	3.068	0.170	0.138
Fungicide												
Untreated Control	13.03 a	n.d.	5.67 b	4.71 a	13.74 a	47.33 a	4.20 d	4.95 a	8.44 a	50.15 a	5.21 c	3.38 a
Tebuconazole 0.0875 g $(m^2)^{-1}$	7.62 b	n.d.	6.52 a	3.46 c	10.51 b	42.75 b	5.01 c	3.88 b	5.32 b	40.6 bc	5.57 b	2.98 b
Tebuconazole 0.175 $(m^2)^{-1}$	10.06 ab	n.d.	6.29 a	4.00 b	8.76 b	35.9 c	5.59 a	3.16 d	4.09 b	38.02 c	5.95 a	2.55 c
Fenarimol 0.144 $(m^2)^{-1}$	7.58 b	n.d.	6.47 a	3.70 bc	9.2 b	42.4 b	5.36 b	3.61 c	4.47 b	43.49 b	5.89 a	2.70 c
P > F	0.002	n.d.	<0.0001	<0.0001	0.0004	<0.0001	<0.0001	<0.0001	0.0105	<0.0001	<0.0001	<0.0001
LDS (α = 0.05)	3.123	n.d.	0.401	0.424	2.410	3.130	0.241	0.234	2.783	4.338	0.239	0.195

† Disease incidence. Percentage of symptomatic plot area on sub-subplot image with SigmaScan Pro 5.0.
‡ Disease intensity. Percentage of non-green pixels on light box cropped photograph with SigmaScan Pro 5.0.
§ Turf quality scale 1=poor quality to 10=best quality
¶ Disease severity scale 1=no disease to 10=no growing turf into the patch
# 250 g of N/100 m2 separated in two monthly applications
†† n.d. non-determined
‡‡, Within columns and within main factors, means followed by the same letter are not significantly different according to LSD (P=0.05)

Figure 4.1: Location of the study. Practice tee at Pilará GC, Pilar BA, Argentina.

Figure 4.2: Digital photographs of entire sub subplot taken with a monopod for disease incidence assessment on the practice tee at Pilará GC, Pilar BA, Argentina.

Figure 4.3: Digital image analysis of the complete sub-subplots for SDS incidence (DIn) on the practice tee at Pilará GC, Pilar BA, Argentina. Data shows digital image analysis on a practice tee box of Tifway 419 bermudagrass at Pilara GC in 2013. SN=ammonium sulfate, A=aerification 2×, F=four fungicide treatments. Green = actual photography; red = SigmaScan 5.0 Pro software analysis.

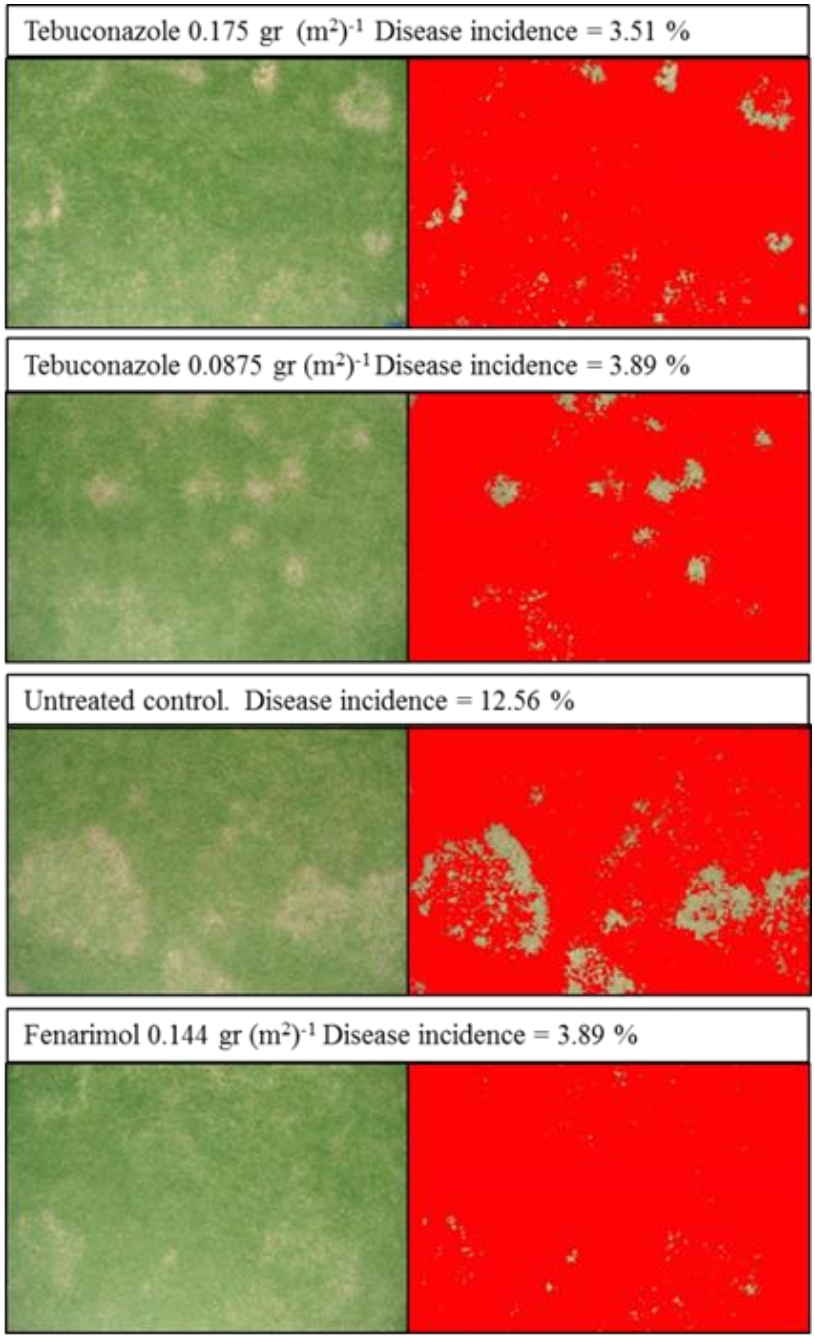


Figure 4.4: Light box used to take digital photographs for assessment of disease intensity (DIt) on the practice tee at Pilará GC, Pilar BA, Argentina.

Figure 4.5: Light arrangement inside of the light box used to take digital photographs for assessment of disease intensity (DIt) on the practice tee at Pilará GC, Pilar BA, Argentina.

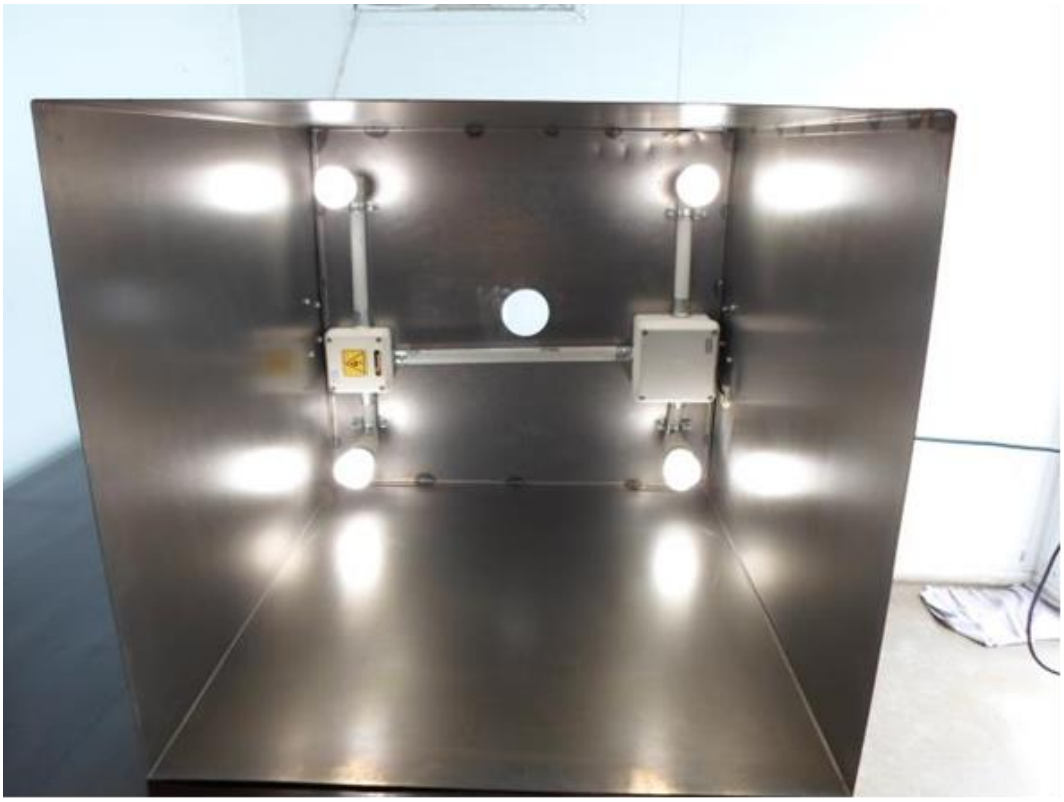


Figure 4.6: Example of digital image analysis of a set of photographs taken with the light box on a patch of spring dead spot for disease intensity (DIt) on the practice tee at Pilará GC, Pilar BA, Argentina. Plot # 42 SN=urea, A=aerification 2×, F=no fungicide (untreated control). Green = actual photography; red = SigmaScan 5.0 Pro software analysis.

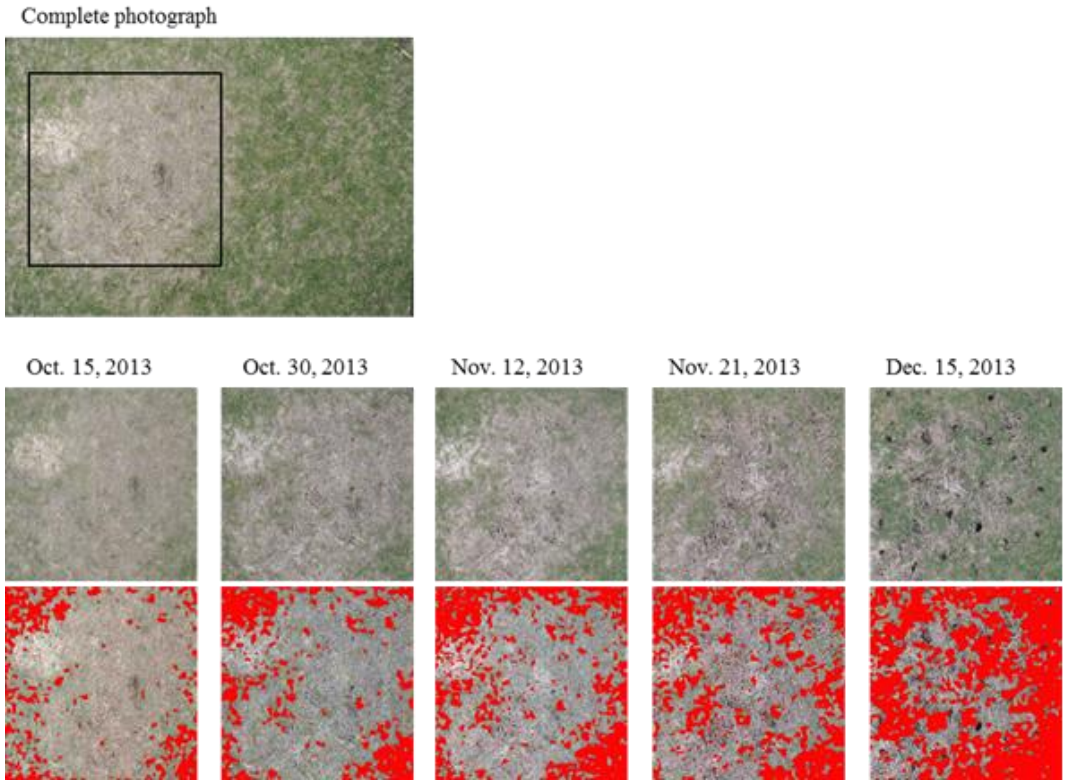
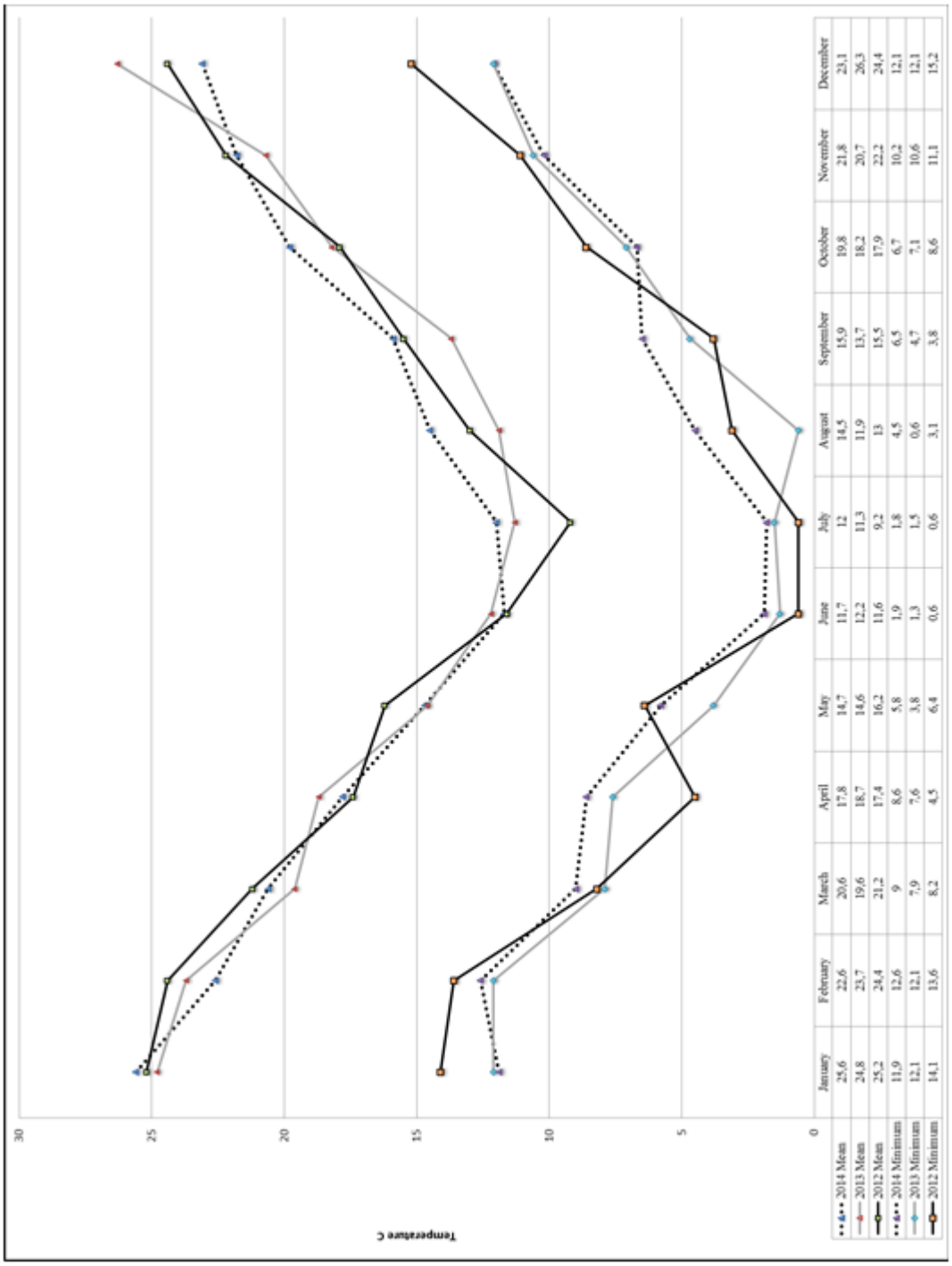
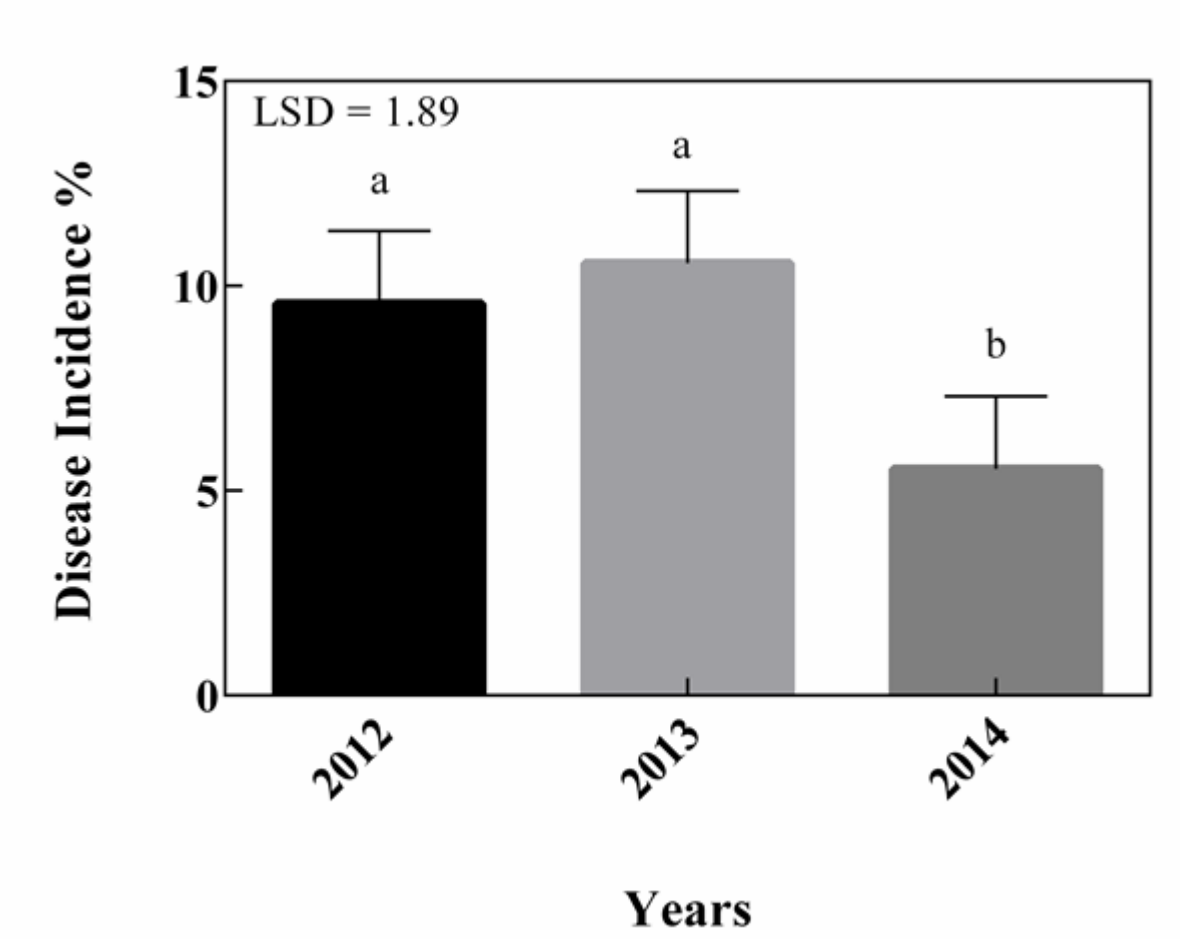


Figure 4.7: Monthly mean and monthly minimum mean temperatures for Pilar area during 2012, 2013, and 2014.



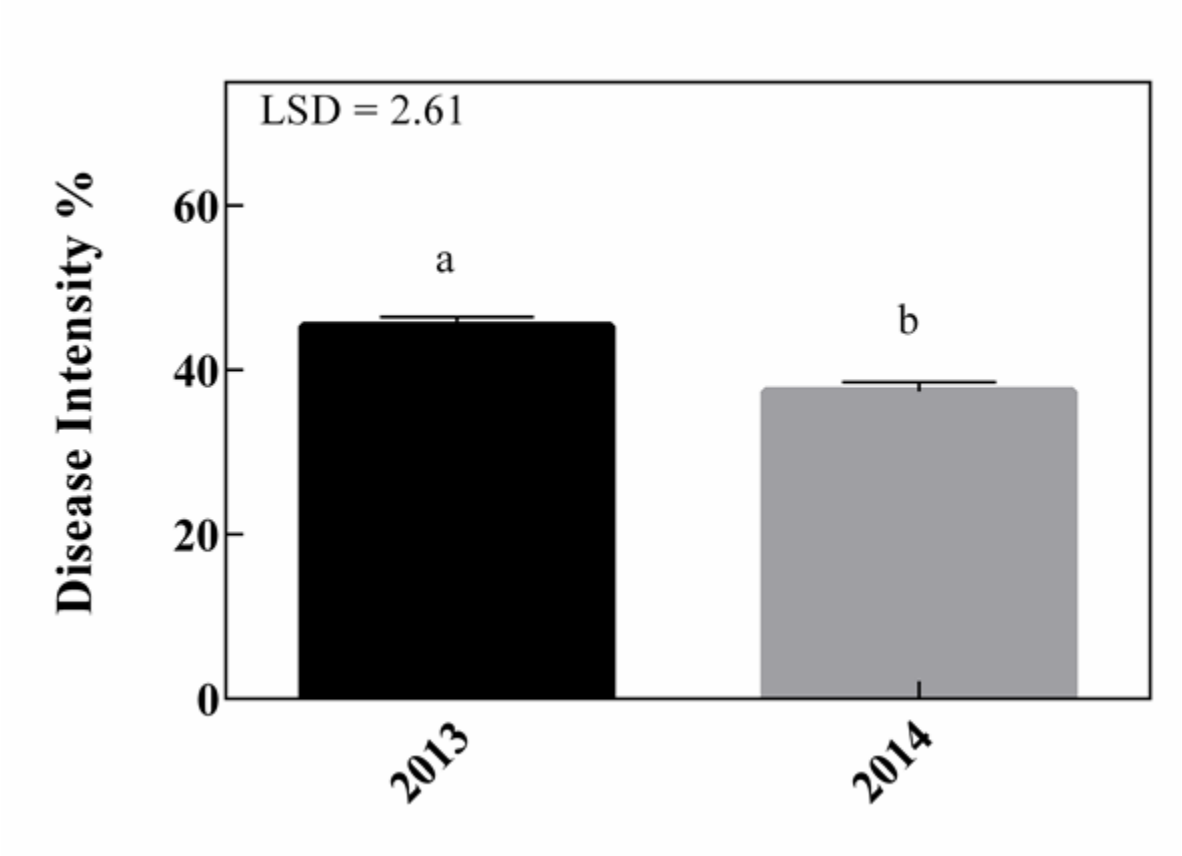
	January	February	March	April	May	June	July	August	September	October	November	December
2014 Mean	25,6	22,6	20,6	17,8	14,7	11,7	12	14,5	15,9	19,8	21,8	23,1
2013 Mean	24,8	23,7	19,6	18,7	14,6	12,2	11,3	11,9	13,7	18,2	20,7	26,3
2012 Mean	25,2	24,4	21,2	17,4	16,2	11,6	9,2	13	15,5	17,9	22,2	24,4
2014 Minimum	11,9	12,6	9	8,6	5,8	1,9	1,8	4,5	6,5	6,7	10,2	12,1
2013 Minimum	12,1	12,1	7,9	7,6	3,8	1,3	1,5	0,6	4,7	7,1	10,6	12,1
2012 Minimum	14,1	13,6	8,2	4,5	6,4	0,6	0,6	3,1	3,8	8,6	11,1	15,2

Figure 4.8: Means and standard errors of disease incidence (DIn) measured through digital image analysis on the practice tee at Pilará GC, Pilar BA, Argentina during 2012, 2013, and 2014.



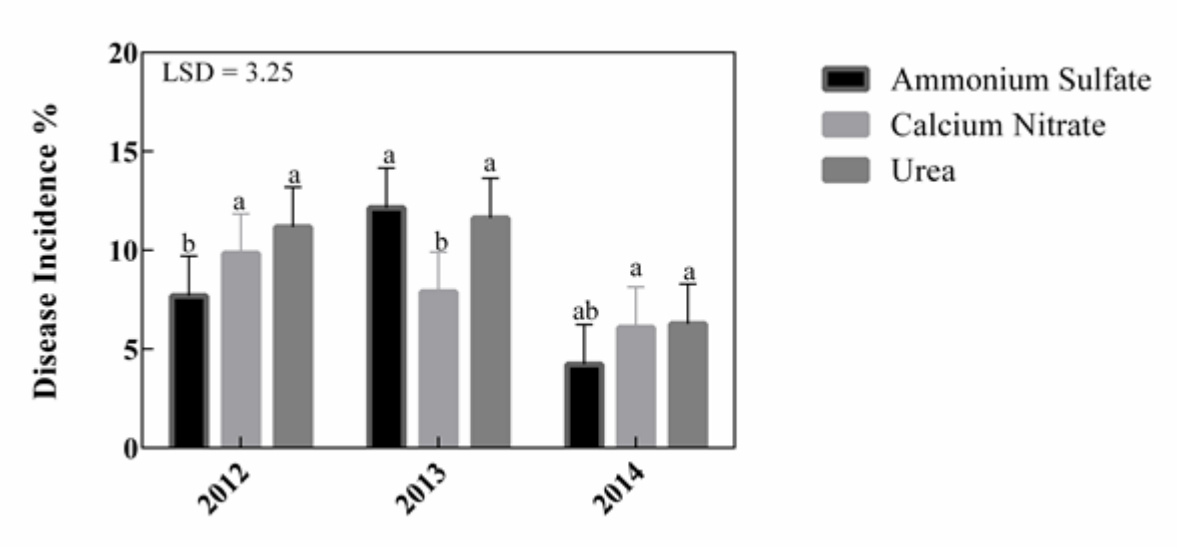
DIn (Disease incidence): percentage of diseased entities within a plot.
Scale Means followed by the same letter are not significantly different according to LSD (0.05).

Figure 4.9: Means and standard errors of disease intensity (DIt) measured through digital image analysis on the practice tee at Pilará GC, Pilar BA, Argentina during 2013, and 2014.



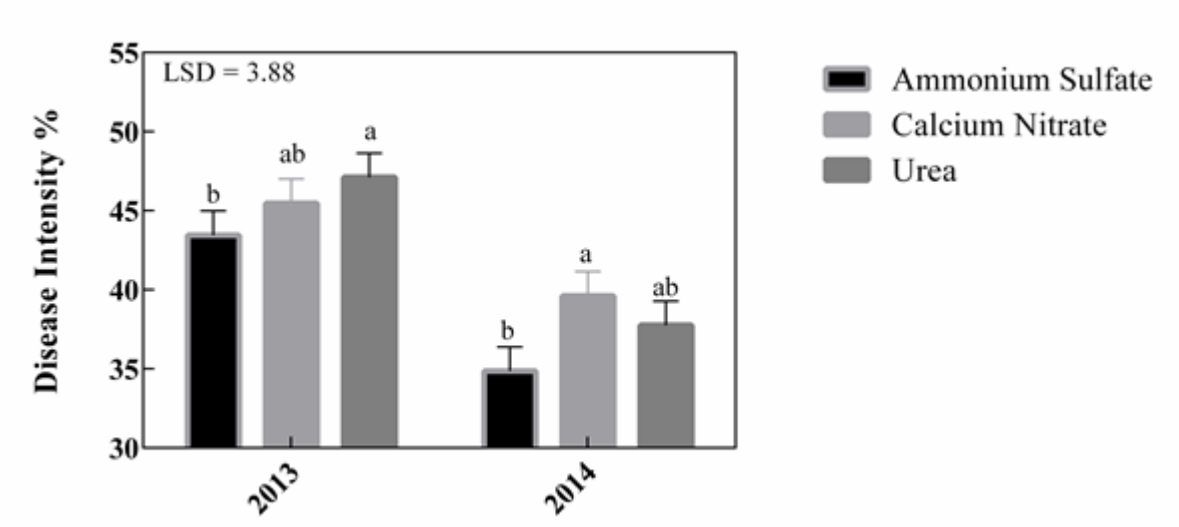
DIt (Disease intensity): percentage of non-green pixels in a cropped photograph.
Means followed by the same letter are not significantly different (LSD, P=0.05).

Figure 4.10: Effect of source of nitrogen (SN) on SDS incidence (DIn) measured trough digital image analysis on the practice tee at Pilará GC, Pilar BA, Argentina during 2012, 2013, and 2014.



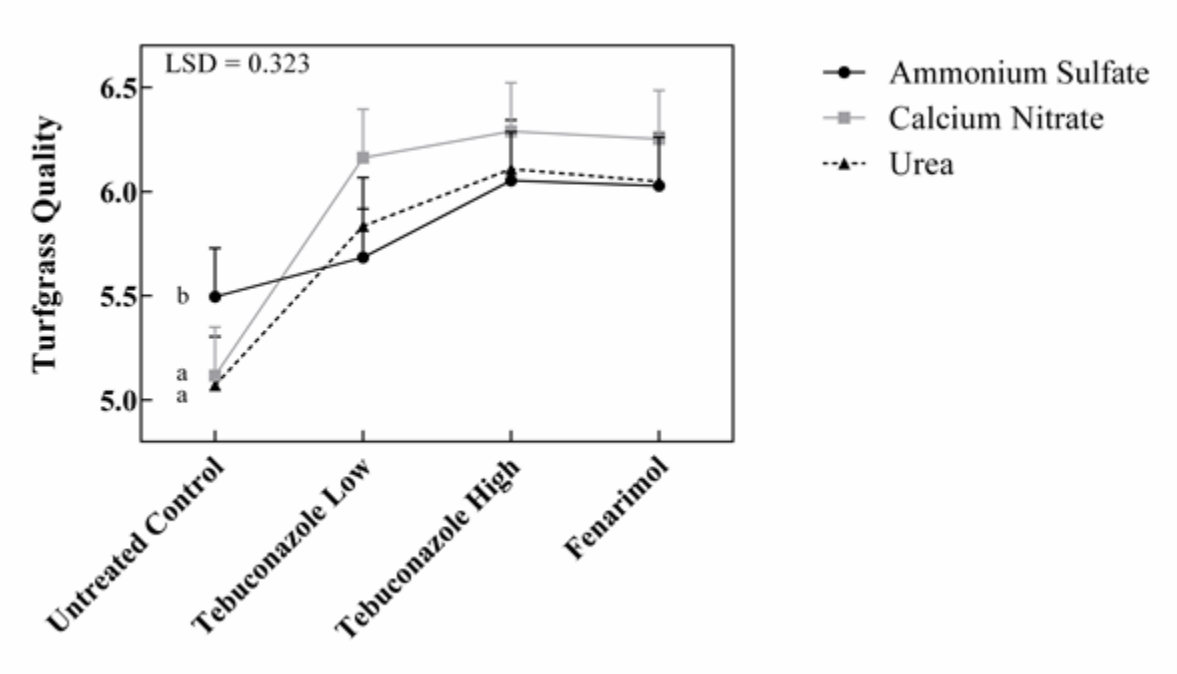
DIn (Disease incidence): percentage of diseased entities within a plot.
Within years, means followed by the same letter are not significantly different according to LSD (0.05)

Figure 4.11: Effect of source of nitrogen (SN) on disease intensity evaluated through digital image analysis (DIt) on the practice tee at Pilará GC, Pilar BA, Argentina during 2013 and 2014.



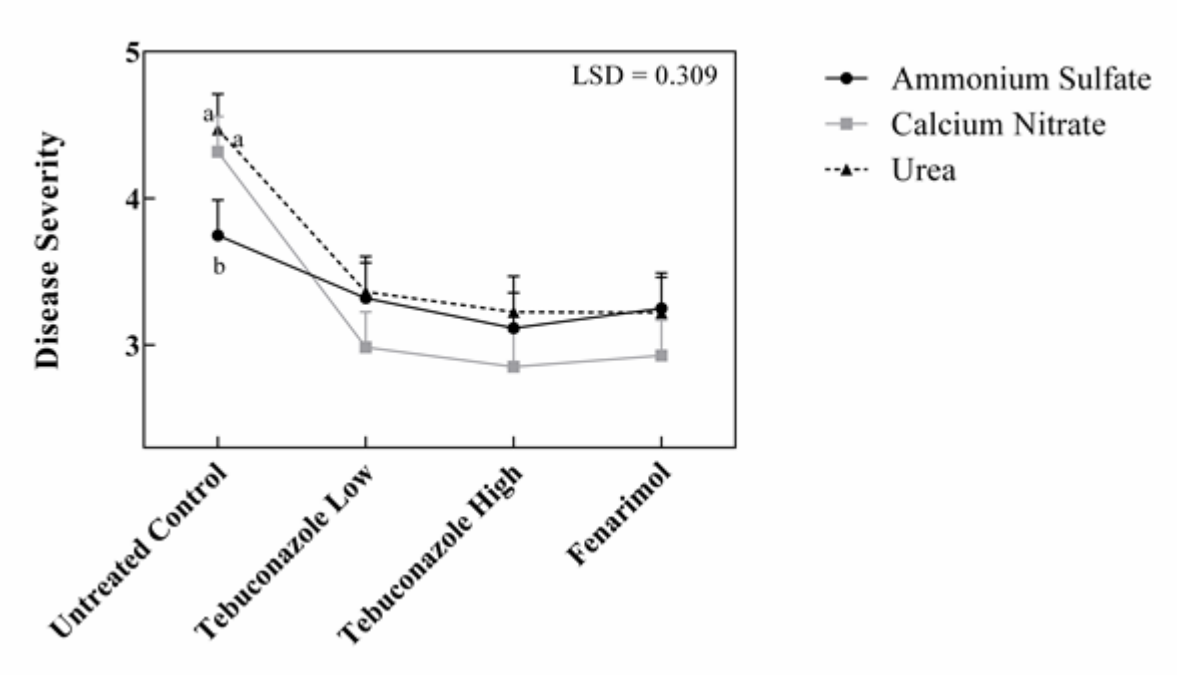
DIt (Disease intensity): percentage of non-green pixels in a cropped photograph.
Within years, means followed by the same letter are not significantly different (LSD, P=0.05).

Figure 4.12: Interaction fungicide (F) × source of nitrogen (SN) on turfgrass quality (TQ) on the practice tee at Pilará GC, Pilar BA, Argentina during 2012, 2013, and 2014. Significant effect of ammonium sulfate for turfgrass quality (TQ) on the untreated control plots is observed.



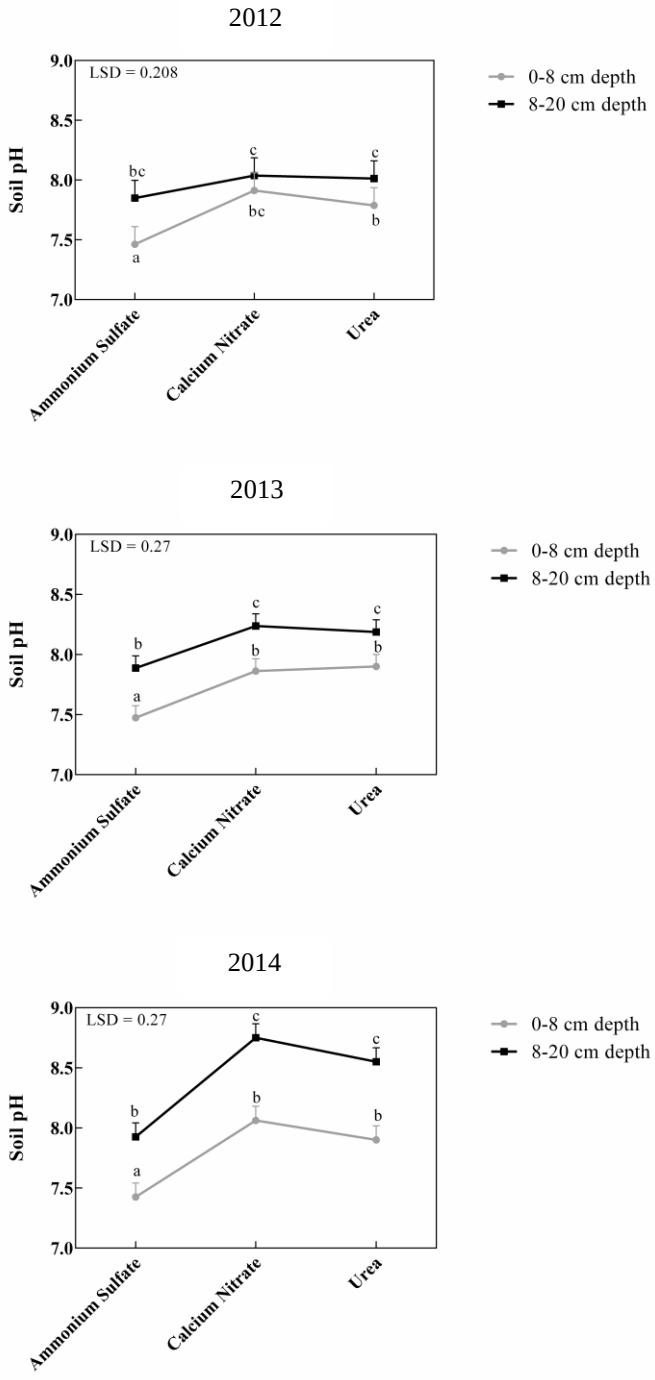
Turfgrass Quality Scale: 1=poor non-acceptable turfgrass quality, 10= thick dense healthy turfgrass
Means followed by the same letter are not significantly different (LSD, P=0.05).

Figure 4.13: Interaction fungicide (F) × source of nitrogen (SN) on disease severity (DSV) on the practice tee at Pilará GC, Pilar BA, Argentina during 2012, 2013, and 2014.



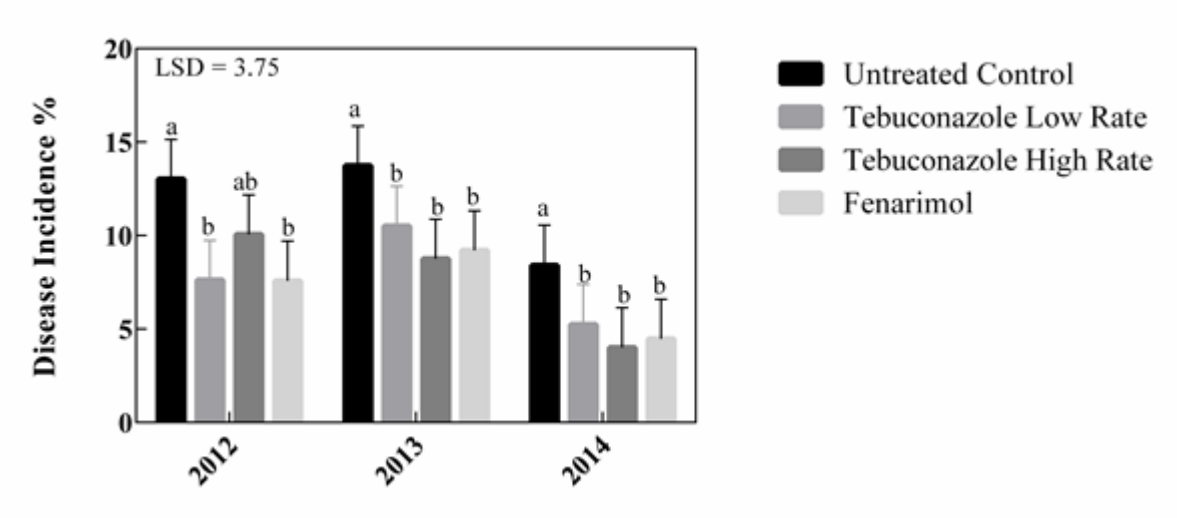
Disease Severity Scale: 1= No disease, 5= 50 % alive turf into a patch, 10= no alive turf into a patch.
Means followed by the same letter are not significantly different (LSD, P=0.05).

Figure 4.14: Means of soil pH at two different soil depths (root zone = 0 to 8 cm and bulk soil = 8 to 20 cm) tested before fungicide applications on the practice tee at Pilará GC, Pilar BA, Argentina during 2012, 2013, and 2014.



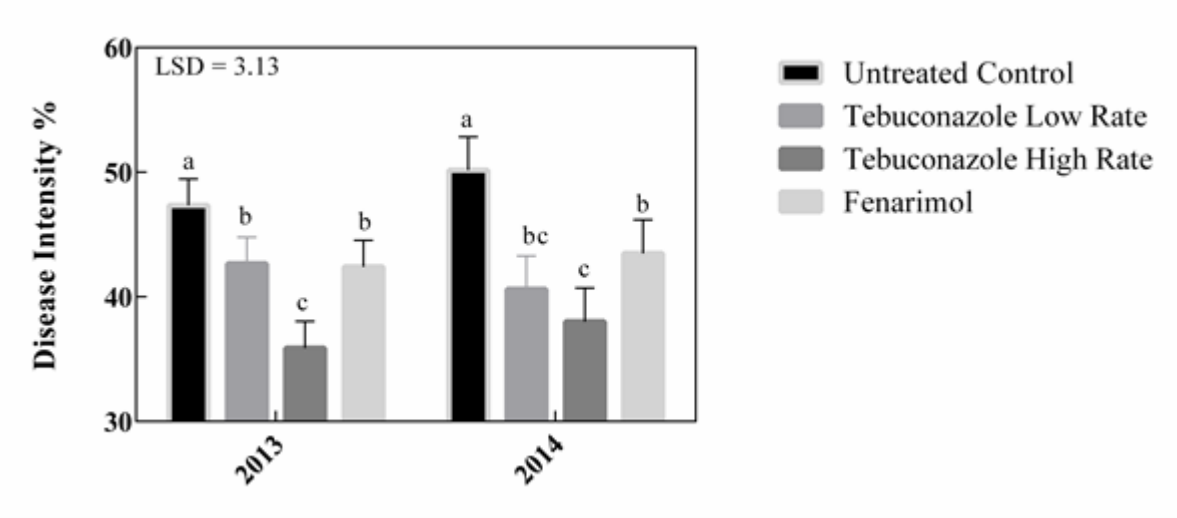
Means followed by the same letter are not significantly different according to LSD (0.05)

Figure 4.15: Effect of fungicides (F) on DSD incidence (DIn) evaluated through digital image analysis on the practice tee at Pilará GC, Pilar BA, Argentina during 2012, 2013, and 2014.



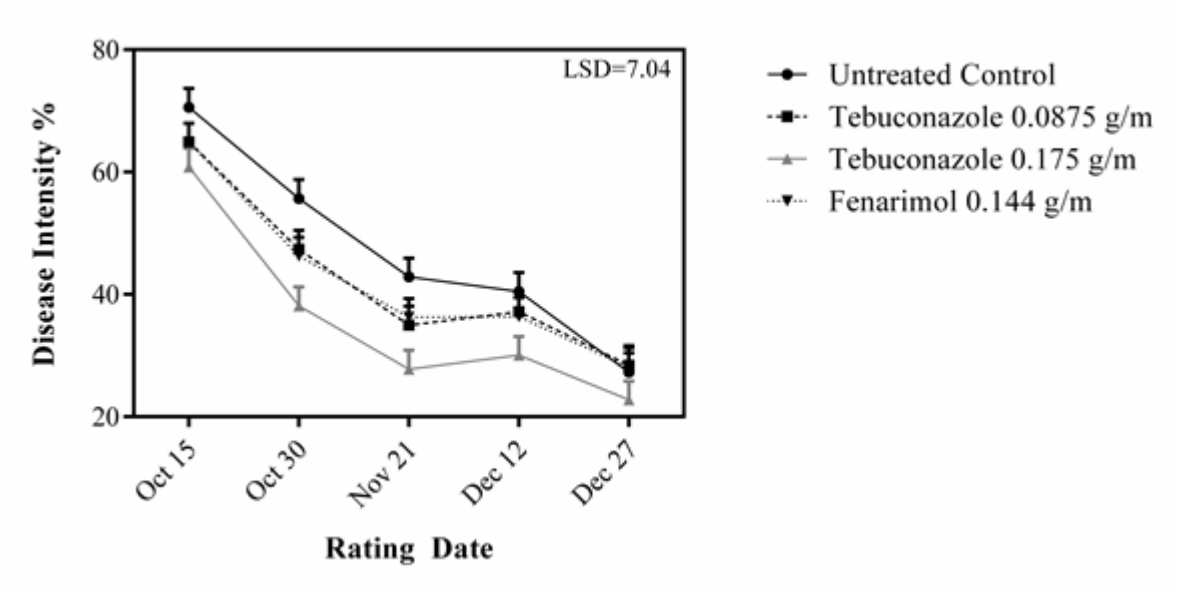
DIn (Disease incidence): percentage of diseased entities within a plot.
Means followed by the same letter are not significantly different according to LSD (0.05)

Figure 4.16: Effect of fungicides (F) on SDS intensity evaluated through digital image analysis (DIt) on the practice tee at Pilará GC, Pilar BA, Argentina during 2013 and 2014.



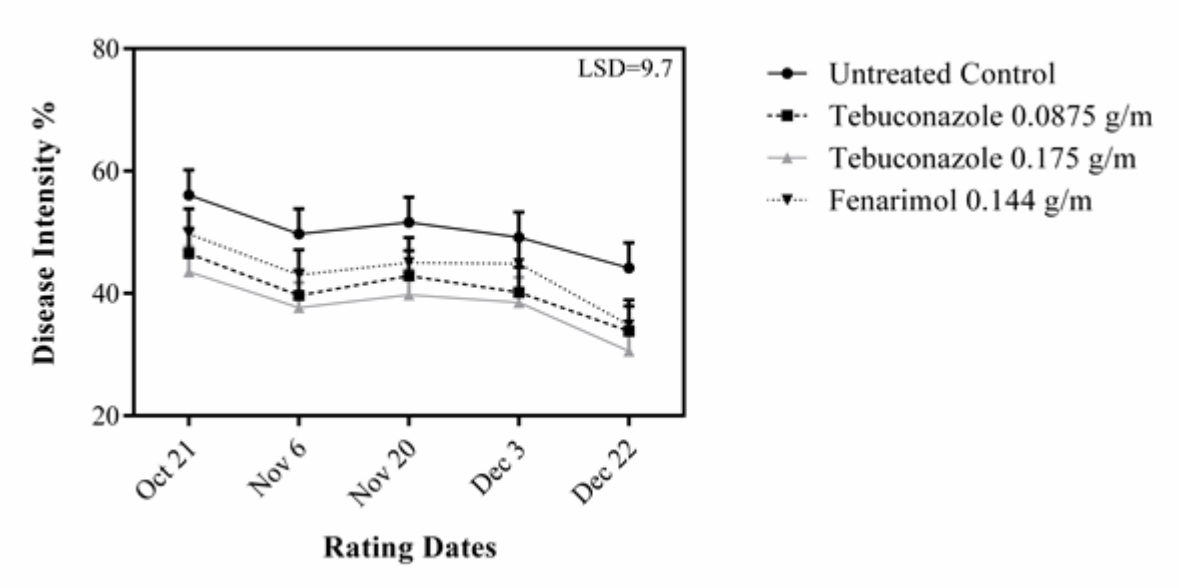
DIt (Disease intensity): percentage of non-green pixels in a cropped photograph.
Within years, means followed by the same letter are not significantly different (LSD, P=0.05).

Figure 4.17: Interaction fungicide treatments (F) × evaluation dates (ED) on disease intensity evaluated through digital image analysis (DIt) on the practice tee at Pilará GC, Pilar BA, Argentina during 2013.



DIt (Disease intensity): percentage of non-green pixels in a cropped photograph.

Figure 4.18: Interaction fungicide treatments (F) × evaluation dates (ED) on disease intensity evaluated through digital image analysis (DIt) on the practice tee at Pilará GC, Pilar BA, Argentina during 2014.



DIt (Disease intensity): percentage of non-green pixels in a cropped photograph.

Literature Cited

Anonymous. (2011) Los primeros pasos, Asociacion Argentina de Golf http://www.aag.com.ar/aag2011-dev/articulo051120110002225.asp.

Baird J.H., Martin D.L., Taliaferro C.M., Payton M.E., Tisserat N.A. (1998) Bermudagrass resistance to spring dead spot caused by *Ophiosphaerella herpotricha*. Plant Disease 82:771-774.

Beard J.B. (1973) Turfgrass Science and Culture Prentice Hall Inc. Englewood Cliffs, NJ 07632.

Beck L.L., Cooper T., Hephner A., Straw C., Henry G. (2013) Effect of pre-emergence herbicides on bermudagrass recovery from SDS, Golf Course Management. pp. 80-85.

Berendsen R., Pieterse C.M.J., Bakker P.A.H.M. (2012) The rhizosphere microbiome and plant health. Trends in Plant Science 17:478-486.

Burke M.J., Anderson J.A., Buchanan D.W., Rajashekar C.B., George M.F. (2000) Evidence for large negative pressure potentials in plant cells during freezing.

Butler E.L. (2004) Development of novel strategies for control of spring sead spot of bermudagrass, Department of Plant Pathology, North Carolina State Unversity, Raleigh, NC.

Butler E.L., Tredway L.P. (2004) Development of effective strategies for control of spring dead spot in bermudagrass. Phytopathology 94.

Butler E.L., Tredway L.P. (2006) Method and timing of fungicide application for control of spring dead spot in hybrid bermudagrass, Plant Health Progress, doi: 10.1094/PHP-2006-0901-01-RS.

Caasi O.C., Walker N.R., Marek S.M., Enis J.N., Mitchell T.K. (2010) Infection and colonization of turf-type bermudagrass by *Ophiosphaerella herpotricha* expressing green or red fluorescent proteins. Phytopathology 100:415-423.

Camara M.P.S. (1999) Systematics of *Ophiosphaerella, Paraphaeosphaeria and Phaeosphaeria*: a molecular and morphological approach, Plant Pathology, The Pennsylvania State University.

Canegallo A., Martin S.B., Park W., Agudelo P., McCarty L., Kerrigan J., Tredway L., Cantoro R. (2015) Characterization of *Ophiosphaerella* species causing spring dead spot disease of bermudagrass in South Carolina and Buenos Aires, Argentina., in: T. A. P. Society (Ed.), APS Annual Meeting, Pasadena, CA.

Cottrill D., Miller L. (2012) Suppressing spring dead spot of bermudagrass with an integrated approach of cultural and chemical practices, Turfgrass and Ornamental Field Day, Division of Plant Sciences - CAFNR. University of Missouri, Columbia, Missouri. pp. 15-16.

Cottrill D.J., Miller G.L. (2013) Impact of nitrogen source and pH on mycelial growth of the spring dead spot pathogens, *Ophiosphaerella herpotricha* and *O. korrae*, 2013 APS Meeting.

Crahay J.H., Dernoeden P.H., O'Neill N.R. (1988) Growth and pathogenicity of *Leptosphaeria korrae i*n bermudagrass. Plant Disease 72:945-949.

Chen, Han, Zhou. (2002) The rhizosphere pH change of *Pinus koraiensis* seedlings as affected by N sources of different levels and its effect on the availability and uptake of Fe, Mn, Cu, and Zn. Journal of Forrestry Research 13:37-40.

Dernoeden P.H. (1993) Integrating strategies for the management of patch diseases caused by ectotrophic fungi, Turfgrass Patch Diseases, APS Press The American Phytopathological Society - St Paul, Minnesota. pp. 123-161.

Dernoeden P.H., Crahay J.N., Davis D.B. (1991) Spring dead spot and bermudagrass quality as influenced by nitrogen source and potassium. Crop Science 31:1674-1680.

Dernoeden P.H., Zhang M., Wetzel H.C. (1995) First report of necrotic ring spot (*Leptosphaeria korrae*) in creeping red fescue in Maryland. Plant Disease 79:966.

Douhan G.W., Olsen M.W., Herrell A., Winder C., Wong F., Entwistle K. (2009) Genetic diversity of *Labyrinthula terrestris*, a newly emergent plant pathogen, and the discovery of new Labyrinthulid organisms. Mycological Research 113:1192-1199.

Dunn J.H., Nelson C.J., Sebaugh J.L. (1980) Characterization of thatch, rhizomes, carbohydrates, and spring dead spot in twenty cultivars of bermudagrass (Abstract). Journal of the American Society for Horticultural Science 105:653-657.

Earlywine, Miller. (2013) Evaluation of fungicides for spring dead spot control on bermudagrass, Division of Plant Science University of Missouri Columbia, MO 65201.

Earlywine D., Miller G. (2014) Evaluation of Torque and 3336f for spring dead spot control on bermudagrass, 2012-2013, Plant Disease Management Reports 8:T021.

Earlywine D., Miller G.L. (2011) Evaluation of fungicides for spring dead spot control on bermudagrass 2010-2011, Plant Disease Management Reports 7:T008.

Endo R.M., Ohr H.D., Krausman E.M. (1985) *Leptosphaeria korrae*, a cause of the spring dead spot disease of bermudgrass in California. Plant Disease 69:235-237.

Flores F., Walker N., Mitchell T., Marek S., Anderson J. (2012) Infection and colonization of bermudagrass by *Ophiosphaerella korrae*, a causal fungus of spring dead spot of bermudagrass. Phytopathology 102:39-40.

Garret S.D. (1970) Pathogenic root-infecting fungi Cambridge University Press.

Gatschet M.J., Taliaferro C.M., Porter D.R., Anderson M.P., Anderson J.A., Jackson K.W. (1996) A cold-regulated protein from bermudagrass crowns is a chitinase. Crop Science (Abstrtact) 36:712-718.

Graham L.S., Striclen M.B. (1994) Plant chitinases (abstract). Canadian Journal of Botany 72:1057-1083.

Gullino M., Mocioni, M., Tittone P. (2007) First report of spring dead spot of bemrudagrass in Italy 91:1200.

H. C. Wetzel I., Skinner D.Z., Tisserat N.A. (1999) Geographic distribution and genetic diversity of three *Ophiosphaerella* species that cause spring dead spot of bermudagrass. Plant Disease 83:1160-1166.

Hill W.J., Heckman J.R., Clarke B.B., Murphy J.A. (2003) Summer patch disease severity on Kentucky bluegrass in response to fertilizer source. Journal of Plant Nutrition July. 26:1499-1512.

Hou Y., Lou A. (2011) Population genetic diversity and structure of a naturally isolated plant species, *Rhodiola dumulosa* (crassulaceae). PLoS ONE 6.

Huber D.M., Mccaybuis T.S. (1993) A multiple component analysis of the take-all disease of cereals. Plant Disease 77:437-447.

Iriarte F.B., Fry J.D., Martin D.L., Todd T.C., Tisserat N.A. (2005) Effect of cold acclimation and freezing on spring dead spot severity in bermudagrass. Hortscience 40:421-423.

Iriarte F.B., Wetzel H.C., III, Fry J.D., Martin D.L., Tisserat N.A. (2004) Genetic diversity and aggressiveness of *Ophiosphaerella korrae*, a cause of spring dead spot of bermudagrass. Plant Disease 88:1341-1346.

Kaminski J., Dernoeden P. (2005a) Nitrogen source on dead spot (Ophiosphaerella agrostis) recovery in creeping bentgrass. International Turfgrass Society 10:214-233.

Kaminski J., Dernoeden P.H. (2005b) Nitrogen source impact on dead spot (*Ophiosphaerella agrostis*) recovery in creeping bentgrass. . InternationalTurfgrassSociety Research Journa 10.

Kaminski J.E., Dernoeden P.H., Mischke S., O'Neill N.R. (2006) Genetic diversity among *Ophiosphaerella agrostis* strains causing dead spot in creeping bentgrass. Plant Disease 90:146-154.

Karcher D.E., Richardson M.D. (2005) Batch analysis of digital images to evaluate turfgrass characteristics. Crop Science:1536-1539.

Kaur A.J., Sadana U.S. (2010) Nitrogen source and manganese application effects on manganese dynamics in the rhizosphere of wheat cultivars grown on manganese-deficient soils. Journal of Plant Nutrition 33:831-845.

Kozelnicky G.M. (1974) Updating 20 years of research: Spring dead spot on bermudagrass, USGA Green Section Record, USGA. pp. 12-15.

Landschoot P.J. (1994) Taxonomy and biology of ectotrophic root-infecting fungi associated with patch diseases of turfgrasses, in: B. B. Clarke and A. B. Gould (Eds.), Turfgrass Patch Diseases: Caused by Ectotrophic Root-Infecting Fungi, The American Phytopathological Society., St. Paul, Minnesota. pp. 41-71.

Landschoot P.J., June P.D. (1996) First report of necrotic ring spot on *Poa annua* putting greens in Pennsylvania. Plant Disease 80:712.

Luc J.E., Canegallo A., Martin S.B. (2005) Spring dead spot in bermudagrass greens in Argentina and South Carolina, Golf Course Management. pp. 92-95.

Luc J.E., Martin S.B. (2006) Control of spring dead spot in bermudagrass ptting greens in South Carolina, F&N Tests Vol 61:T010.

Lucas L.T. (1980) Control of spring dead spot of bermudagrass with fungicides in North Carolina. Plant Disease 64:868-870.

LynksMagazine. (2015) History of golf in South Carolina, Lynks Magazine http://www.linksmagazine.com/special_sections/history-of-golf-in-south-carolina.

MacDonald B. (1997) The population genetics of fungi: tools and techiques. Phytopathology P1997:448-453.
Martin B., McCarty B. (2005) Best Pest Management and Control-Turfgrass Diseases, in: L. B. McCarty (Ed.), Best Golf Course Management Practices, Pearson Prentice Hall. pp. 588-589.
Martin D.L., Bell G.E., Baird J.H., Taliaferro C.M., Tisserat N.A., Kuzmic R.M., Dobson D.D., Anderson J.A. (2001) Spring dead spot resistance and quality of seeded bermudagrasses under different mowing heights. Crop Science 41:451-456.
McCarty L.B. (2005) Best golf course management practices Pearson Prentice Hall - Upper Saddle RIver, NJ 07458.
McCarty L.B., DiPaola J.M., Lucas L.T. (1991) Regrowth of bermudagrass infected with spring dead spot following low temperature exposure. Crop Science 31:182-184.
McCarty L.B., Lucas L.T., Dipaola J.M. (1992) Spring dead spot occurrence in bermudagrass following fungicide and nutrient applications. Hortscience 27:1092-1093.
Milla-Lewis S.R., Zuleta M.C., van Esbroeck G.A., Quesenberry K.H., Kenworthy K.E. (2013) Cytological and molecular characterization of genetic diversity in *Stenotaphrum*. Crop Science 53:296-308.
Morris K.N. (1998) A guide to NTEP turfgrass ratings, http://www.ntep.org/reports/ratings.htm.
Nus J.L., Shashikumar K. (1993) Fungi associated with Spring Dead Spot reduces freezing resistance in bermudagrass. HortScience 28:306-307.
O'Donnell K. (1992) Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). Current Genetics 22:213-220.
Owens C.B., Szalanski A.L. (2005) Filter paper for preserving insect DNA samples.
Pair J.C., Crowe F.J., Willis W.G. (1986) Transmission of spring dead spot disease of bermudagrass by turf/soil cores. Plant Disease 70:877-878.
Perry D.H., Tomaso-Peterson M., Baird R. (2010) Seasonal variation in frequency of isolation of *Ophiosphaerella korrae* from bermudagrass roots in Mississippi and pathogenicity and optimal growth of selected isolates. Mycopathologia 169:395-402.
Perry H., Tomaso-Peterson M. (2006) Frequency of occurrence of Ophiosphaerella korrae on Tifway bermudagrass roots in Mississippi. Phytopathology 96:S92.
Raaijmakers J.M., Paulitz T.C., Steinberg C., Alabouvette C., Moenne-Loccoz Y. (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant and Soil 321:341-361. DOI: Doi 10.1007/S11104-008-9568-6.
Raffle V.L., Hsiang T. (1998) Low level of DNA polymorphisms in isolates of *Leptosphaeria korrae* pathogenic on Poa pratensis. Can. J. Plant Path 20:48-54.
Rice W.R. (1989) Analyzing tables of statistical tests. Evolution 43:223-225.
Rosenberg N.A. (2004) Distruct: a program for the graphical display of population structure. Molecular Ecology Notes 4:137-138.

Sala D. (2015) Datos historicos. clima sur GBA, http://www.climasurgba.com.ar/historia.php.
Sauer K.M., Hulbert S.H., Tisserat N.A. (1993) Identification of *Ophiosphaerella herpotricha* by cloned DNA probes. Phytopathology 83:97-102.
Schoch C.L. (2006) A multigene phylogeny of the Dothideomycetes using four nucrear loci. Mycologia 98:1041-1052.
Schoch C.L., Seifert K.A., Huhndorf S., Robert V., Spouge J.L., Levesque C., Chen W. (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences, USA 109:6241-6246.
Schwartz B. (2013) Using the light box on assesing turfgrass quality.
Seem R.C. (1984) Disease incidence and severity relationships. Annual Review of Phytopathology:133-150.
Smiley R.W., Cook R.J. (1973) Relationship between take-all of wheat and rhizosphere ph in soils fertilized with ammonium vs nitrate-nitrogen. Phytopathology 63:882-890.
Smiley R.W., Dernoeden P.H., Clarke B.B. (2005) Compendium of turfgrass diseases APS Press The American Phytopathological Society, St. Paul, Minnesota.
Smiley R.W., Fowler M.C., Hummel N.W., Petrovic A.M., Pidduck H.M., Strang C. (1986) Necrotic ring spot and summer patch of inoculated Kentucky bluegrasses 1:58.
Smith A.M. (1971) Spring dead spot of couch grass turf in New South Wales. The Journal of the Sports Turf Research Institute 47:54-59.
Taylor J., Spatafora J., Barbee M., Schoch C. (2015) Tree of life web project, tolweb.org/Ascomycota/20521.
Thompson D.C., Clarke B.B., Heckman J.R. (1995) Nitrogen form and rate of nitrogen and chloride application for the control of summer patch in Kentucky bluegrass. Plant Disease 79:51-56.
Thompson D.C., Kobayashi D.Y., Clarke B.B. (1998) Suppression of summer patch by rhizosphere competent bacteria and their establishment on Kentucky bluegrass. Soil Biology & Biochemistry 30:257-263. DOI: Doi 10.1016/S0038-0717(97)00114-4.
Tisserat N., Fry J. (1997) Cultural practices to reduce spring dead spot *(Ophiosphaerella herpotricha)* severity in Cynodon dactylon. Int. Turfgrass Soc. Res. J. 8:931-936.
Tisserat N., Iriarte F.B., Wetzel III H.C., Fry J., Martin D.L. (2004) Identification, distribution, and aggresiveness of spring dead spot pathogens of bermudagrass, USGA Green Section Record, USGA, USGA. pp. 15-18.
Tisserat N., Kennelly. (2008) Spring dead spot, K-State Turfgrass Research, www.plantpath.ksu.edu/doc985.ashx.
Tisserat N.A., Hulbert S.H., Sauer K.M. (1994) Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiosphaerella korrae* and *O. herpotricha*. Phytopathology 84:478-482.
Tisserat N.A., Pair J.C., Nus A. (1989) *Ophiosphaerella herpotricha* , a cause of spring dead spot of bermudagrass in Kansas. Plant Disease 73:933-937.

Tredway, Soika, Butler. (2011) Impact of fertilization programs and preventive fungicide applications on spring dead spot in hybrid bermudagrass. , http://www.slideshare.net/turfpathology/impact-of-fertilization-programs-and-preventive-fungicides-on-spring-dead-spot.

Tredway L. (2011) Spring dead spot of zoysiagrass, NCSU, Turf Diseases: Golf Course Disease Updates by University Professors. pp. 1.

Tredway L., Butler L. (2005) Development of effective fungicide programs for spring dead spot control in hybrid bermudagrass. Phytopathology 95.

Tredway L.P., Butler E.L. (2007) First report of spring dead spot of zoysiagrass caused by *Ophiosphaerella korrae* in the United States. Plant Disease 91:1684.

Tredway L.P., Butler E.L., Soika M.D., Bunting M.L. (2008) Etiology and management of spring dead spot of hybrid bermudagrass in North Carolina, USA. Proceedings of the Iind International Conference on Turfgrass Science and Management for Sports Fields:535-545.

Tredway L.P., Soika M.D., Butler E.L. (2009a) Response of spring dead spot caused by *Ophiosphaerella korrae* and *O. herpotricha* to fertilization programs and preventive fungicide applications, Phytopathology. pp. S129.

Tredway L.P., Tomaso-Peterson M., Perry H., Walker N.R. (2009b) Spring dead spot of bermudagrass: A challenge for researchers and turfgrass managers, APSnet, Plant Health Progress. pp. 1-18.

Vincelli P. (2005) Managing spring dead spot of bermudagrass. , University of Kentuky. Cooperative Extension Service, http://www2.ca.uky.edu/agc/pubs/id/id130/id130.htm.

Vos P., Hogers R., Bleeker M., Reijans M., Van De Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. (1995) AFLP: A new technique for DNA fingerprinting. Nucleic Acids Research 23:4407-4414.

Wadsworth D.F., Young H.C. (1961) Spring dead spot of bermudagrass. USGA Journal and Turf Management, Reprint from Plant DIsease Reporter 44 (7) July 15, 1960.

Walker. (2013) Evaluation of post fungicide application irrigation on the control of spring dead spot of bermudagrass in Oklahoma, 2012-2013, Plant Disease Management Reports.

Walker J., Smith A.M. (1972) *Leptosphaeria narmari* and *L. korrae* spp.nov., two long-spored pathogens of grasses in Australia. British Mycological Society 58:459-466.

Walker N. (2007) Root colonization of several species of native grasses by *Ophiosphaerella herpotricha*, 46th Annual Meeting of the Society of Nematologists and Annual Meeting of the American Phytopathological Society (SON/APS 2007).

Walker N. (2010) Timing fungicide applications for spring dead spot, Golf Course Management. pp. 84-92.

Walker N.R. (2005) Evaluation of fungicides for the management of spring dead spot of bermudagrass, 2003-2004, F&N Tests Vol 60:T022.

Walker N.R. (2009) Influence of fungicide application timings on the management of bermudagrass spring dead spot caused by *Ophiosphaerella herpotricha*. Plant Disease 93:1341-1345.

Walker N.R., Mitchell T.K., Morton A.N., Marek S.M. (2006) Influence of temperature and time of year on colonization of bermudagrass roots by *Ophiosphaerella herpotricha*. Plant Disease 90:1326-1330.

Wetzel H.C. (1999) Geographic distribution and genetic diversity of three *Ophiosphaerella* species that cause spring dead spot of bermudagrass, Department of Plant Pathology, Kansas State University, Manhattan, Kansas.

Wetzel H.C. (2001) Evaluation of fungicides for control of spring dead spot of bermudagrass, 2000, F&N Tests 56:T3.

Wetzel H.C., III, Hulbert S.H., Tisserat N.A. (1999a) Molecular evidence for the presence of *Ophiosphaerella narmari* n. comb., a cause of spring dead spot of bermuda grass, in North America. Mycological Research 103:981-989.

Wetzel H.C., III, Skinner D.Z., Tisserat N.A. (1999b) Geographic distribution and genetic diversity of three *Ophiosphaerella* species that cause spring dead spot of bermudagrass. Plant Disease 83:1160-1166.

Worf G.L., Stewart J.S., Avenius R.C. (1986) Necrotic ring spot disease of turfgrass in Wisconsin. Plant Disease 70:453-458.

Yeh S., Moffatt B.A., Griffith M., Xiong F., Yang D.S.C., Wiseman S.B., Sarhan F., Danyluk J., Xue Y.Q., Hew C.L., Doherty-Kirby A., Lajoie G. (2000) Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. Plant Physiology 124:1275-1284.