# ETIOLOGY OF SPRING DEAD SPOT OF BERMUDAGRASS

By

FRANCISCO FLORES

Bachelor of Science in Biotechnology Engineering

Escuela Politécnica del Ejército

Quito, Ecuador

2008

Master of Science in Entomology and Plant Pathology

Oklahoma State University

Stillwater, Oklahoma

2010

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 2014

ETIOLOGY OF SPRING DEAD SPOT OF

BERMUDAGRASS

Thesis Approved:

Dr. Nathan Walker

Thesis Adviser

Dr. Stephen Marek

Dr. Jeffrey Anderson

Dr. Thomas Mitchell

ACKNOWLEDGEMENTS

I would like to acknowledge all the people who made the successful completion of this project possible. Thanks to my major advisor, Dr. Nathan Walker, who guided me through every step of the process, was always open to answer any question, and offered valuable advice whenever it was needed. Thanks to all the members of my advisory committee, Dr. Stephen Marek, Dr. Jeff Anderson, and Dr. Thomas Mitchell, whose expertise provided relevant insight for solving the problems I found in the way.

I also want to thank the department of Entomology and Plant Pathology at Oklahoma State University for being a welcoming family and for keeping things running smoothly. Special thanks to the members of the turfgrass pathology lab, Kelli Black and Andrea Payne, to Dr. Carla Garzón and members of her lab, to Dr. Stephen Marek and members of his lab, and to Dr. Jack Dillwith, who were always eager to help with their technical and intellectual capacities.

Thanks to my friends and family, especially to my wife Patricia, who helped me regain my strength several times during this process.

Finally, I want to thank the United States Golf Association and the Oklahoma Agricultural Experiment Station, which provided the funding for these projects and the Secretary of Science and Technology of Ecuador for their financial support.

Name: FRANCISCO FLORES

Date of Degree: DECEMBER, 2014

Title of Study: ETIOLOGY OF SPRING DEAD SPOT

Major Field: PLANT PATHOLOGY

Abstract: Spring dead spot (SDS) is the most devastating disease of bermudagrass (*Cynodon dactylon* and *C. dactylon* x *C. transvaalensis*) in the United States. The disease is caused by the fungus *Ophiosphaerella herpotricha*, *O. korrae,* or *O. narmari* that infect the grass root system. Control of the disease is often difficult requiring high fungicide application rates and subsequent herbicide inputs to control invasive weeds. A better understanding of the host-pathogen relationship can aid in the development of new bermudagrass varieties with increased resistance to SDS. The colonization and infection of different bermudagrass cultivars by *O. korrae*, the formation of reactive oxygen species during the infection process, and the production of phytotoxic compounds by *O. korrae* and *O. herpotricha* were evaluated. Additionally, several different plant species were evaluated for colonization and reaction to SDS-causing fungi. Superficial root colonization by *O. korrae* was similar for all cultivars tested; however, the fungus caused cortical necrosis in the interspecific hybrid cultivars Tifway 419 and Midlawn. In the common bermudagrass cultivar U3, and African accessions 3200 and Uganda symptoms of necrosis were minimal at 14 days after inoculation. The fungus was not able to penetrate the epidermis of intact stolons but wounding resulted on cortical colonization for all cultivars. Culture filtrates of *O. herpotricha* produced root discoloration of all bermudagrass cultivars while ROS levels during infection were significantly higher for U3 when compared to Midlawn and Tifway. Formation of ROS was observed consistently at the hyphal tips of colonizing fungi in all cultivar/fungi combinations tested and in vitro, underscoring the involvement of ROS during root infection and active metabolism. Both *Ophiosphaerella* spp. tested appear to have a broad host range showing diverse plant-fungus interactions where necrosis is not common at up to 14 days post infection for many species evaluated. The results suggest that root necrosis caused by SDS fungi is independent from HR associated with plant-generated ROS, and toxin production by the fungi may be a non-specific factor in root necrosis. Furthermore, infection and colonization of various bermudagrasses by *O. korrae* was found to be similar to that of *O. herpotricha*, suggesting that host genetic resistance may be used for effective management of SDS caused by both species.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

Figure Page

# CHAPTER I

## INTRODUCTION AND OBJECTIVES

The warm-season grass bermudagrass (*Cynodon dactylon* (L.) Pers. and *C. dactylon* × *C. transvaalensis* Burtt-Davy) is commonly used as a turfgrass in the southern United States where cool-season grasses are difficult to maintain. Limitations to the use of bermudagrass include shade, its susceptibility to winter injury, and the disease known as spring dead spot (SDS) (4). Spring dead spot is considered the most destructive disease of bermudagrass where it is cold enough during winter months for the grass to become dormant (9). The disease is often associated with high maintenance turf and is caused by one of three fungi from the genus *Ophiosphaerella*, namely *O. herpotricha*, *O. korrae*, or *O. narmari* (2; 5; 12; 15; 16) (Figs. 1 A-C). Even though there are indications of regional differences in the distribution of SDS pathogens in the United States (7), all three species may be found at a single location, probably due to the constant movement of soil, plants, and both together as sod (17). Symptomatic turf exhibits circular dead patches of up to 1 m in diameter that can lead to weed encroachment; patches appear in early spring when bermudagrass resumes growth after dormancy (Fig. 1 D). Management of SDS represents a great challenge, and knowledge about the etiology of the causal agents plays a major role for determining effective methods to control it.

Host-pathogen interactions at the cellular level have been previously observed in the SDS pathosystem (1). Roots and stolons of hybrid (*C. dactylon* × *C. transvaalensis*) and African (*C. transvaalensis*) were infected with transformed *O. herpotricha* strains that expressed either green fluorescent protein (GFP) or red fluorescent protein (tdTomato) which allowed tracking the colonization and infection of the fungus. Even though both hybrid and African stolons had fungal colonization, only hybrids exhibited necrosis suggesting an inherent tolerance of *C. transvaalensis* to *O. herpotricha* (1).

Even though several queries surrounding SDS at the cellular level have been elucidated, the mechanism that renders cells necrotic on susceptible plants is still unclear. It might be the product of an active hypersensitive response of bermudagrass, or a lytic cytotoxicosis in response to virulence factors or toxins secreted by the fungus (1). Testing the reaction of susceptible bermudagrass roots to *O. herpotricha* and *O. korrae* culture filtrates and for the presence of reactive oxygen species (ROS) on necrotic spots after infection, could reveal the mechanism that is acting on SDS caused cell death.

Several species have been reported as hosts of SDS pathogens. Besides turf-type bermudagrass, known hosts of *O. herpotricha* include zoysiagrass (*Zoysia japonica* Steud.) (6) and buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.) (11). While *O. korrae* can cause necrotic ring spot of cool season grasses including Kentucky bluegrass (*Poa pratensis* L), creeping red fescue (*Festuca rubra* L. subsp, *rubra* Smith), and annual bluegrass (*Poa annua* L) (3; 8; 18). Similar to *O. herpotricha, O. korrae* has also been reported causing SDS on zoysiagrass (14). The fungus has also been isolated from blanketgrass (*Axonopus compressus* Beauv.), St. Augustine grass (*Stenotaphrum*

*secundatum* Kuntze) and kikuyu grass (*Pennisetum clandestinum* Hochst) (10). If inoculated artificially, *O. korrae* and *O. narmari* can infect wheat, oats, barley, and rice (13; 16). Testing a panel of warm and cool-season grasses for their reaction to SDS pathogens may be important for identifying possible alternative hosts.

# OBJECTIVES

1. Compare and contrast necrosis and colonization of roots and stolons/rhizomes of resistant and susceptible interspecific hybrid, common, and African bermudagrasses by *O. korrae* that expresses fluorescent proteins.

2. Examine if reactive oxygen species (ROS) are produced in bermudagrass roots in response to *O. korrae* and *O. herpotricha* colonization.

3. Determine if phytotoxins are produced by *O. korrae* and *O. herpotricha* in culture.

4. Evaluate colonization in a panel of different grass and dicot species inoculated with *O. korrae* and *O. herpotricha* and subsequent host response.

**Literature cited**

1. Caasi, O. C., Walker, N. R., Marek, S. M., Enis, J. N., and Mitchell, T. K. 2010. Infection and colonization of turf-type bermudagrass by *Ophiosphaerella*

*herpotricha* expressing green or red fluorescent proteins. Phytopathology 100:415-423.

2. Dale, J. L., and Diaz, C. 1963. A new disease of bermudagrass turf. Arkansas Farm Research 12:6.

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

4. Dunn, J. H., Nelson, C. J., and Sebaugh, J. L. 1980. Characterization of thatch, rhizomes, carbohydrates, and spring deadspot in twenty cultivars of bermudagrass. Journal of the American Society for Horticultural Science 105:653-660.

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

6. Green, D. E. 1993. Pathogenicity of *Rhizoctonia solani* AG-2-2 and *Ophiosphaerella herpotricha* on zoysiagrass. Plant Disease 77:1040-1044.

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

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

9. Lucas, L. T. 1980. Spring deadspot of bermudagrass. Pages 183-187. in: Advances in Turfgrass Pathology B. G. Joyner, and P. O. Larsen, eds. Harcourt & Brace Jovanovich, Inc., Duluth, MN.

10. Smith, A. M. 1965. *Ophiobolus herpotrichus* a cause of spring dead spot in couch turf. Agricultural Gazette of New South Wales753-758.

11. Tisserat, N., Wetzel, H., Fry, J., and Martin, D. L. 1999. Spring dead spot of buffalograss caused by *Ophiosphaerella herpotricha* in Kansas and Oklahoma. Plant Disease 83:199.

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

13. Titone, P., Mocioni, M., Landschoot, P. J., and Gullino, M. L. 2004. Survey of ectotrophic root infecting fungi associated with turfgrass patch diseases in Italy Acta Horticulturae 491-498.

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

15. Wadsworth, D. F., and Young, H. C. 1960. Spring dead spot of bermudagrass. Plant Disease Reporter 44:516-518.

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

17. Wetzel, H. C., Skinner, D. Z., and 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.

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

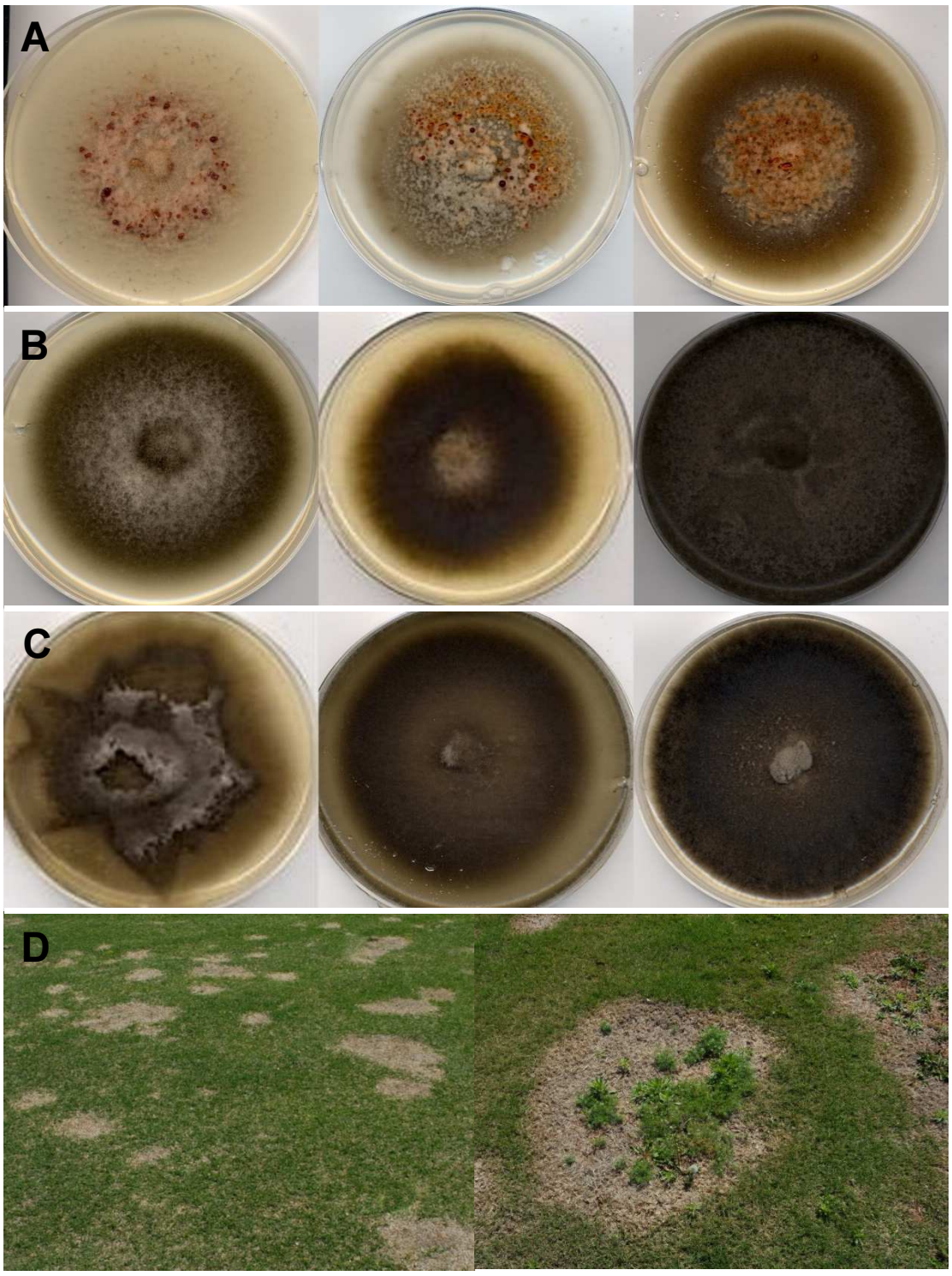


**Figure I-10** Characteristic morphologies of Ophiosphaerella spp. growing on potato dextrose agar. a) *O. herpotricha*, b) *O. korrae*, and c) *O. narmari*. d) Symptoms of spring dead spot on bermudagrass

# CHAPTER II

# LITERATURE REVIEW

## I -Turf type bermudagrass

Bermudagrass (*Cynodon dactylon* (L.) Pers. and *C. dactylon* × *C. transvaalensis* Burtt-Davy) is a warm-season, perennial, sod-forming species used widely for turf, forage, and soil stabilization world-wide (121). This versatile turfgrass is probably the best adapted to warm, humid regions of the United States, where it is used for residential lawns, commercial landscapes, athletic fields, and golf course fairways and roughs. The presence of both stolons and rhizomes provides the plant with an abundance of meristematic tissue to initiate new growth from following dormancy (28). Bermudagrass also has the highest growth rate among warm-season turfgrasses (6). These characteristics allow bermudagrass to establish quickly, recover rapidly from injury and wear, and regrow after adverse environmental conditions (88). Cultivars of bermudagrass are often drought tolerant and can produce extensive root systems (121).

The bermudagrass industry was initiated in the United States in the early 1940's when seed from North Africa was purchased by the United States government. In the 1960's, the first improved cultivar, Arizona common, was commercially made available.

The production of Arizona common was abandoned on the 1990's because of the development of NuMex Sahara, an improved seeded bermudagrass (76). Even though new seeded bermudagrass have characteristics that make them appealing for use as high-value turf (71), currently, the most improved cultivars are hybrids of common and African bermudagrass (*Cynodon transvaalensis* Burt-Davy) (59). The result of combining the tetraploid common bermudagrass and the diploid African bermudagrass is the production of sterile triploids, which typically do not produce viable seed. Therefore, vegetative propagation by sod or sprigs is required for reproduction of these cultivars. The combination of desirable characteristics from both parents provide selected hybrids with fine leaf texture, upright growth habit, excellent density, aggressive growth habit, and pest tolerance (88).

The zone of transition between warm-season and cool-season turfgrasses corresponds to the upper region of the southern United States. In the transition zone, bermudagrass enters a dormant condition in late fall and growth resumes in spring when soil temperatures are sufficiently warm for rhizomes and stolons to start developing shoots (88). One of the few fungal diseases that can cause damage to bermudagrass in the transition zone is spring dead spot (SDS) caused by fungi of the genus *Ophiosphaerella* (82).

**II - Spring Dead Spot**

Spring Dead Spot of bermudagrass and its hybrids is a disease that manifests itself as well defined, circular, dead spots that can be seen on spring as the grass resumes growth from dormancy (106). The disease may have been observed as early as 1936 in

Oklahoma, but it was not until the 1950's that it gained notoriety once the management of turfgrasses became more refined and hybrid cultivars of bermudagrasses became available (49; 106). By the early 1960's, the presence of the disease was reported from Nebraska, Pennsylvania, Missouri, Arkansas, and Kansas (15; 80; 106). In the United States, SDS is the most damaging disease in the northern range of adaptation of bermudagrass and is less severe in southern states where bermudagrass cold temperature induced dormancy is shorter or not present (55; 107). While spring dead spot has been observed in many states, is likely to expand in geographic presence as more cold-tolerant varieties are grown farther north, and now has been reported in South America (54), Asia (Y. Wu personal communication), and southern Europe (37).

The disease is more often associated with high maintenance turfgrass but it has also been observed on bermudagrass grown with less management and fewer inputs (17; 106). In California, the disease was found in a wide range of soil types and climates making it difficult to determine specific conditions for disease development (26). This was consistent with early observations that noted that soil with symptomatic turf didn't differ in pH, fertility level or organic matter content with soil in healthy turf areas (15). However, it has been suggested that soils with high clay content are more conducive for disease development than for soils with low clay content (17; 120).

**II –a- Causal agent**

Currently, three species of fungi in the genus *Ophiosphaerella* are known to cause SDS, namely *O. herpotricha, O. narmari,* and *O. korrae* and their taxonomic classification is defined as follows (12):

Kingdom: Fungi

Phylum: Ascomycota

Class: Dothidiomycetes

Subclass: Pleosporomycetidae

Order: Pleosporales

Family: Phaeosphaeriaceae

The cause of the disease was not known until 1965 when Smith (83) isolated a fungus from bermudagrass having symptoms and confirmed it as the cause of the disease by Koch's postulates. The fungus was first identified as *Ophiobolus herpotrichus* (Fr.) Sacc. based on perithecia, asci, and ascospore characteristics, but later was placed as an undescribed species in the genus *Leptosphaeria* and called *L. korrae*. *Leptosphaeria korrae* is also the cause of necrotic ring spot of Kentucky bluegrass (*Poa pratensis* L.), and SDS of bermudagrass in the southeastern and west coasts of the United States (14; 25; 83; 109; 119). Subsequently, Smith identified a more common cause of spring dead spot in New South Wales, Australia, and described a new species named *Leptosphaeria narmari*. *Leptosphaeria narmari* was also identified as the causal agent of SDS in southern Australia (39; 84; 109). Later examinations by Walker (108), Shoemaker and Babcock (79), and Wetzel et al. (116) placed all causal organisms of SDS within the genus *Ophiosphaerella,* and the clustering of these organisms on a single monophyletic group was supported by the analysis of the ITS region nucleotide sequence data (116).

*Ophiosphaerella herpotricha* was first found to be causing the disease in Kansas (93), while the presence of *O. narmari* was first confirmed in the US on California, Oklahoma and Kansas (116).

With the advancement of technology and the emergence of new molecular methods, the systematics of SDS causing fungi are being clarified. According to MAT protein sequence trees, there is no strong evidence that *O. korrae* belongs to the Phaeosphaeriaceae where it is currently placed based on ITS sequences (41). Amplified length polymorphism (AFLP) analysis of *O. herpotricha* and *O. korrae* showed that the former was represented by several haplotypes on a single location and host, while the latter comprised only a few haplotypes, that could be clustered in three different AFLP groups, from a wide range of locations and two different hosts (44; 74; 117).

**II –b- Morphology and Identification**

When grown on potato dextrose agar (PDA), *O. korrae* produces a distinctively dome-shaped mycelium (115). At first mycelium is hyaline, being abundant and aerial on the center while growing along the agar surface on the margins. Subsequently, aerial mycelium spreads to the whole colony, which starts to darken from the center of the colony, becoming dark gray in color. Many hyphae from the aerial mycelium can coalesce and form darker threads called runner hyphae. Under the microscope, two types of mycelium can be identified: one thick, light brown, strongly septate type averaging 3 µm to 4 µm wide, and a narrower, hyaline type averaging 2 µm in thickness. (83). On its host, *O. korrae* produces brown, septate, and branched hyphae 2.5-5 µm wide that can coalesce to form strands or flattened dark 50-400 µm diameter sclerotia. Closely packed

pseudothecia may also appear on infected plant tissue. The fruiting bodies are flask-shaped with a thick neck 400-600 µm tall and 300-500 µm wide. The neck of the pseudothecia can be 50-200 µm long and 200-250 µm wide with an ostiole of 80-100 µm and may have a thickened ridge. The pseudothecial wall is formed by several layers of brown cells. The neck canal is lined with hyaline periphyses that may present a reddish-brown colored material, in young pseudothecia. Asci contain eight spores and are bitunicate, cylindrical to clavate, 145-200 x 10-15 µm in size. Ascospores are filiform, twisted in a bundle and parallel to each other, pale brown in color, having up to 15 septa, and 100-235 x 4-5.5 µm in size, and rounded at the ends. There are numerous hyaline, septate pseudoparaphyses present inside the fruiting bodies (14; 109).

On potato dextrose agar, *O. narmari* aerial mycelium is initially white, will darken to buff, and after several days the buff colored hyphae will darken and resemble older colonies of *O. korrae* (109). 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 diam.) of flattened sclerotia-like structures. Pseudothecia may occur in clusters or singly in infected plant tissue. These can be 800 µm tall and 650 µm wide, black, and flask shaped with a thick neck. The neck can be 100-300 µm long and 300-450 µm wide often with two thick ridges and an ostiole > 150 µm wide lined with hyaline upwardly pointing periphyses containing a reddish-brown material. The pseudothecial wall is formed by several layers of flattened brown cells. Asci contain eight spores are bitunicate, clavate, narrowed towards the base, 100-155 x 11-13 µm in size. Ascospores are biseriate, elliptical to fusiform, pale brown in color, having up to 7 septa, 35-72 x 4-6 µm in size. There are numerous hyaline, septate pseudoparaphyses present inside the ascocarp (14; 109).

The mycelium of *O. herpotricha* isolates grown on PDA is initially white with the color darkening to tan or brown with age. After two weeks of growth, a brownish-black colored exudate is produced from the center the colonies (115). Pyriform pseudothecia are extremely rare and may form occasionally in infected host tissue. Based on limited specimens, fruiting bodies are 400-500 µm wide and 400-500 µm tall. The neck is 300-400 µm long and 200-250 µm wide with a 90-110 µm wide ostiole lined with hyaline 30-50 x 2-3 µm periphyses. The ascocarp contains numerous bitunicate, cylindrical asci 120-200 x 6-8 µm in size with eight ascospores each. Ascospores are 140-200 x 2.0-2.5 µm in size, having up to 20 septa. There are numerous 1 µm wide pseudoparaphyses inside the pseudothecia (79). No spores have been produced *in vitro* and no conidial stages have been found for any of the *Ophiosphaerella* species causing SDS (109).

Since fruiting structures are often absent in field infections, reliable identification can only be done in the laboratory (46). Several methods have been developed for the identification of SDS causing fungi. Protein band patterns generated by isoelectric focusing were used to differentiate between *O. narmari* and *O. korrae* and monoclonal antibodies have been developed for identification of the later (40; 68). Cloned DNA probes have been used for the identification of *O. narmari* and *O. herpotricha,* while specific primers that amplify the rDNA ITS region can be used for detection of *O. korrae* and *O. herpotricha* (77; 94; 95). A combination of random product amplification PCR primers have been used to distinguish the three *Ophiosphaerella* species; however, the time needed for the process (2-3 weeks) is very long (115).

**II –c- Symptoms**

There is normally no indication of the presence of the fungi in grasses or disease until symptoms start to appear typically in the spring (106). Common identification features in the field are bleached foliage with blackened, rotted roots and stems, in some cases, the presence of dark-colored, septate fungi with runner hyphae present in and around the necrotic tissues. Irregular sclerotia-like structures 5-25 μm in diameter have also been reported (46; 83). Affected areas appear as circular, dead patches ranging in size from a few centimeters to more than a meter in diameter that often increase in size each year. The margins of patches are usually even but may become irregular when patches have coalesced to form larger dead areas that can be a meter or more across. Occasionally turfgrass can survive within the center of the formerly dead zone resulting in a donut-like shape. Stolons and rhizomes from surrounding surviving bermudagrass are required to recolonize the dead areas, this may take more than one growing season if the patches are large. For large patches that can remain free of bermudagrass for longer periods of time, the establishment of non-desirable grasses and weeds can occur requiring herbicide applications to aid in bermudagrass re-colonization of these areas (15; 78; 102; 106). In addition, SDS is exclusively a disease of roots, rhizomes, and solons while most other diseases of turfgrasses are primarily foliar diseases (83) and once controlled, the plants recover rapidly from crowns.

**II –d- Biology**

Fungi that cause SDS are known as ectotrophic, root-infecting fungi meaning they colonize the surface of below-ground plant organs such as stolons and rhizomes prior to

infection. *Ophiosphaerella* species have been observed to colonize the surface of roots, rhizomes, and stolons and form dark runner hyphae (82). After colonization, the mycelium penetrates the epidermis at numerous points on the roots or through open wounds on stolons (11; 83). *O. herpotricha* enters the cortex with most hyphae growing intracellular and longitudinally along the root axis. Penetration of *O. korrae* is direct and, at later stages of penetration, the ectotrophic hyphae coalesce to form cushion-like aggregates. After infecting stolons, *O. korrae* forms mat-like hyphal aggregates that may act as survival structures (11). *Ophiosphaerella korrae* and *O. narmari* pseudothecia are commonly observed on infected stolons in Australia but are a rare occurrence under field conditions in the United States, *O. herpotricha* pseudothecia may be observed partially immersed in dead leaf, sheath and stolon tissues (46; 109). The absence of fruiting bodies in nature suggests that means for dispersal are likely to be through movement of infested root, crown, or stem tissues during planting and routine maintenance activities (50); *Ophiosphaerella herpotricha* may over-summer inside infected stolons (11). Nevertheless, the presence of several haplotypes of *O. herpotricha* in a single location in Oklahoma supports the occurrence of sexual recombination for this fungus (117). Such diversity does not occur on *O. korrae* (74; 117), which is a homothallic ascomycete belonging to the group where the two mating types idiomorphs (MAT1 and MAT2) are present in a single isolate (41).

*Ophiosphaerella narmari* grows better *in vitro* between 20 and 25 °C and in soil between 10 and 20 °C (85).The optimum growth temperature for *O. korrae* is 25 °C and there is no growth at 30 °C (14; 109). In a study by Crahay (14), the fungus caused 100% mortality on susceptible cultivars when infected at 15 °C, with all plants having stunted

brown to black roots. Runner hyphae accumulated into infection mats on the root surface (infection cushions) and there were no sclerotia found. At low temperatures the fungi have a competitive advantage and can outgrow the rate of bermudagrass root growth (14). *Ophiosphaerella herpotricha* grows better *in vitro* at temperatures between 20 and 25 °C. Optimal soil temperatures for plant colonization are between 15 and 25 °C were described by Tisserat et al. (93), but subsequent studies by Walker et al. (114) suggested that *O. herpotricha* had a minor effect on root growth at or above 25 °C. Severity of SDS caused by *O. herpotricha* in Oklahoma was correlated with freeze injury that occurred the first winter after planting (3). In general, optimal temperature for all SDS pathogens infection is similar and below 20 to 21 °C (114). The ability of *O. korrae* to grow at low water potentials may also play an important role in infection under drought stress situations. Even though high water potentials (-0.5 to -1.0 MPa) favor development of *O. korrae*, growth can still be detected at -5.0 MPa water potential when incubated at optimal temperatures (73). Soil-water may also be a factor influencing the activity of *O. herpotricha*; higher soil-water content creates conditions that are favorable for the fungus to cause disease (114).

The onset of SDS is associated with wet, cold weather during fall (85). Once *Ophiosphaerella* infects the plant, there is a gradual necrosis of the root system, which can be compensated by root regeneration during the warmer months, but root necrosis will overtake regrowth once root growth is retarded due to cooler weather (83). Damage to plants affected by SDS occurs during the late summer, autumn, and early winter; even though infection and colonization does not depend on dormancy of the plant cold-temperature induced dormancy is typically required for plant mortality (26; 83; 93).

Plants remain infected during summer and can be killed by the fungus while dormant throughout the winter possibly due to a combination of stolon necrosis, carbohydrate depletion, and secondary colonization by soil microbes (11; 110; 114). When high temperatures favor bermudagrass growth, *O. herpotricha* activity may decrease or cease entirely (102). Even though there is evidence that there is greatest disease activity during the fall, based on soil temperatures, spring and fall are equally important disease activity periods in Oklahoma (114).

**II -e- Etiology**

Infection of SDS-causing *Ophiosphaerella* species can occur on roots, stolons, or rhizomes and localized necrosis can be observed within days (102). After infection, susceptible bermudagrass varieties develop large necrotic lesions compared to an accession of *C. transvaalensis,* which exhibited a tolerant endophytic association with *O. herpotricha* in which the fungus colonized the stele of the plant and resulted in limited necrosis (11). The mechanism by which the fungi render plant tissue necrotic is still unknown. It might be the product of an active hypersensitive response of bermudagrass, or a lytic cytotoxicosis in response to virulence factors or toxins secreted by the fungus (11). The necrotrophic fungus *Sclerotinia sclerotiorum* is capable of altering the host cell redox machinery by producing a reducing environment during establishment of the pathogen, and then inducing ROS generation which triggers the hypersensitive response and dead of host cells in favor of the fungus (118). It has been suggested that the slow growth of bermudagrass into the affected area may be due to the presence of a toxin in the soil (49; 106). Leachates from SDS infected bermudagrass sod have been tested for their effect on healthy sod and a minimal reduction of foliar growth was observed. The

leachate contained a toxin that reduced ryegrass growth as well (49). Fermanian et al. (29) obtained methanol extracts from soil infested by SDS fungi; such extracts reduced shoot growth, root development and seed germination of bermudagrass. Furthermore, several metabolites, produced by *O. herpotricha* in liquid culture, produced necrosis when spotted onto leaves of different plant species including bermudagrass, although no correlation between necrosis and resistance to the disease was found (103).

Several genes associated the plant and fungal interaction, have been identified in the relationship of *O. herpotricha* with bermudagrass. Zhang et al. (121) generated cDNA libraries using suppression subtractive hybridization to identify putative SDS tolerance genes. Fifty-five percent of the transcripts did not match existing GenBank entries suggesting the presence of unique mechanisms in the *O. herpotricha*-bermudagrass interaction. When comparing the resistant cultivar Yukon with the susceptible cultivar Jackpot, most tolerant-related sequences with known function, corresponded to housekeeping genes. There was also a significant proportion of genes involved in cell signaling, besides genes in the oxidative burst, apoptosis, pathogen defense, antimicrobial, and low molecular weight signal categories. When comparing infected and non-infected Jackpot, a larger proportion of upregulated genes were involved in housekeeping suggesting little or no defense response by the susceptible cultivar. There were also significant differences in expression levels for both the susceptible and the resistant cultivars when examined in the fall and spring. The tolerant plant tends to retard symptom development despite the fact that it may be genetically compatible with the fungus; therefore, tissue damage may be controlled even under heavy infection. The

source of tolerance to *O. herpotricha* appears to be polygenic and may be associated with higher expression of defense response and signal transduction genes (121).

It has been observed that turfgrass crowns infected with *O. herpotricha* and *O. korrae* are more susceptible to cold damage that non-infected plants (69). Furthermore, the damage is exacerbated when there is acclimation before freezing occurs (45). During the acclimation process, the plant is exposed to low temperatures for long periods of time, during which, carbon sources can be depleted increasing susceptibility to freeze damage (22). Iriarte et al. suggested that SDS damage is a function of reduced freeze tolerance caused by pathogen colonization at temperatures above 10°C in late summer and fall and by carbon source depletion during winter (45).

**II –f- Host Range and Distribution**

The first bermudagrass varieties that were reported to be susceptible to SDS were the *C. dactylon* cultivars U-3 and common, African (*C. transvaalensis*), and hybrid (*C. dactylon* x *C. transvaalensis*) cultivars Tiffine and Tifgreen. U-3 was the cultivar most severely damaged by the disease (80; 106). Unfortunately, at this time *Ophiosphaerella* species were not yet identified as the cause of SDS, therefore no discrimination could be made among them.

Once the causal agents were identified, pathogenicity studies were conducted that focused on specific species. Baird et al. (3) tested several types of bermudagrass for their reaction to *O. herpotricha*. Varieties tested included twenty two *C. dactylon*, one *C. transvaalensis*, and eight hybrid entries. Although the African variety of bermudagrass (*C. transvaalensis*) showed the highest amount of living shoots within the diseased area

and a great potential to recover from the disease, grasses within all three types had some level of resistance to SDS caused by *O. herpotricha* (African, Guymon, Sundevil, Midlawn, Midfield, Ft. Reno, and Mirage).

Crahay et al. tested the *C. dactylon* varieties Tufcote and Vamont, and hybrids Tifgreen, Tifway, and Midiron for their susceptibility to *O. korrae*. Results from greenhouse experiments demonstrated different susceptibility levels depending on the fungal isolate used and field observations suggested that Vamont and Midiron had good resistance to SDS caused by *O. korrae*. *Ophiosphaerella korrae* can cause SDS on zoysiagrass (98) and necrotic ring spot of cold-season grasses including Kentucky bluegrass (*Poa pratensis* L), creeping red fescue (*Festuca rubra* L. subsp, *rubra* Smith), and annual bluegrass (*Poa annua* L) (18; 51; 119). The fungus has also been isolated from blanket grass (*Axonopus compressus* Beauv.), St. Augustine grass (*Stenotaphrum secundatum* Kuntze), and kikuyu grass *(Pennisetum clandestinum* Hochst) (83). If inoculated, *O. korrae* and *O. narmari* can infect wheat, oats, barley, and rice (96; 109). The bermudagrass hybrid, Tifdwarf, is especially susceptible to SDS caused by *O. narmari* (40). In addition to turf-type bermudagrasses, other known hosts of *O. herpotricha* include zoysiagrass (*Zoysia japonica* Steud.) (36) and buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.) (90).

Even though there are indications of regional differences in the distribution of SDS pathogens in the United States (44), all three species may be found at a single location, probably due to the movement of plants and soil (117). *Ophiosphaerella korrae* appears to be the most abundant SDS causing fungus in the southeastern United States

(44) and California (26); while *O. herpotricha* is more common in Oklahoma and Kansas but can be also found in North Carolina, Texas, and Tennessee (36; 44; 117). The presence of *O. narmari* has been confirmed in California, Oklahoma, Kansas, and North Carolina (44; 116). Distribution and host range of *O. herpotricha* suggests that the fungus could be native to the Great Plains in North America, but the fact that it has not been isolated from native buffalograss or other native grasses makes its origin unclear (44). The genetic variability of *Ophiosphaerella* samples examined from United States was found to be high for *O. herpotricha* and *O. narmari*, but in contrast was low for *O. korrae*, suggesting that the latter is an introduced species (117). From a study of the distribution of *Ophiosphaerella* isolates in North Carolina, Tredway et al. (101) concluded that the distribution of these fungi was not random and one species was usually dominate. The presence of SDS was also confirmed on New Zealand and Argentina but the specific species causing the disease was not identified (46; 54). A fungus from another genus, *Gaeumannomyces graminis*, was once associated with SDS in North Carolina and Alabama (64-66) but there is no further evidence of this association.

**II –g- Management**

Many attempts have been made to control SDS using fungicides. Small (81) suggested the continuous use of dithiocarbamates applications during the semi-dormant and dormant periods of bermudagrass. Concordantly, Smith (85) reported complete control in the field could be achieved with the use of 800 g/kg tetramethylthiuram disulfide (Thiram) at 1.31 g/$m^2$ or 300 g/l or disodium ethylene bisdithiocarbamate 19% (Nabam) at 5.2 ml/$m^2$ every four weeks from February through September. Sayed et al.

(78) did not obtain complete control for the fungicides they tested including dithiocarbamates. They observed a maximum control of 75% when four applications at weekly intervals of 4 g/l hydroxymercuric chlorophenol and 10% tetramethylthiuram disulfide 45% (Tersan OM) were applied during June and July. Kozelnicky (49) tested several chemicals and most caused some degree of reduction in SDS for the first two years and complete control by the third year. Greater disease reduction was achieved with monthly spring applications of n-trichloromethylthio-4-cyclohexene-1, 2-dicarboximide (Captan) and etridiazole (Terrazole), while fall applications were not favorable for disease reduction. Lucas (57) found that control of SDS could be achieved by five monthly applications of Benomyl, PCNB, or a combination of fungicides during July-November. But applications of Chloroneb, Nabam, Maneb, or Carboxin did not control the disease. Rates of benomyl application for total control were very high (1.56 g a.i./$m^2$) therefore its use was only recommended for golf course greens and tees. Presently, Benomyl and most products that contain PCNB are no longer available in the United States Control of SDS was achieved in California with five monthly applications starting on August of either 1.52 g/$m^2$ myclobutanil (Systhane), 1.22 g/$m^2$ propiconazole (Banner), 0.3 g $m^{-2}$ salicylic acid, benzalkonium chloride and propiconazole (Spotless), 1.22 g/$m^2$ fenarimol (Rubigan, Gowan Company, Yuma AZ), or 2.44 g/$m^2$ benomyl (Tersan) (70). Iriarte et al. (43) observed that three summer applications of trinexepac-ethyl at 0.61 g/$m^2$ improved turf quality and reduced diseased area up to 34% in plots where azoxystrobin was also applied (one September application at 0.06 g/$m^2$).

Fungicides labeled for control of SDS in the U. S. include, propiconazole (Banner MAXX, Syngenta Crop Protection, Inc. Greensboro NC), myclobutanil (Eagle, Dow

Agrosciences LLC, Indianapolis IN), thiophanate-methyl (3336, Cleary Chemical Coorporation, Dayton NJ), azoxystrobin (Heritage, Syngenta Crop Protection, Inc. Greensboro NC), and fluoxastrobin (Disarm, Arysta Lifescience North America LLC, Cary NC). Eagle is often used on golf course turfgrasses but control has not been consistent in trials (10; 54). Butler and Tredway found that watered-in (0.1 l/$m^2$) or surface (0.2 or 0.4 l/$m^2$) applications of fenarimol at 0.22 g/$m^2$ and propiconazole at 0.18 g/$m^2$ reduced SDS disease incidence (7; 97). They evaluated several fungicides for preventive control, and at first concluded that all were effective against SDS (8) but in subsequent trials indicated that azoxystrobin + propiconazole, fenarimol, myclobutanil, or tebuconazole provided effective control of both *O. herpotricha* and *O. korrae*. Application timing and rate didn't have a significant impact on SDS when fenarimol was applied between mid-August and mid-October (9). Walker (112) concluded that 0.3 ml/$m^2$ propiconazole, 0.67 g/$m^2$ tebuconazole and 1.8 g/$m^2$ fenarimol were effective at reducing SDS severity when applied three times in late summer but such rates may not be affordable for many turfgrass managers. Subsequent studies showed that two fall or one fall and one spring application of either tebuconazole or propiconazole were the most cost effective and at the same time effective controlling SDS (113). Walker (113) pointed out the importance of disease severity assessment before treatment when evaluating methods for controlling SDS.

Tredway et al. (99) tested several fungicides and fungicide combinations, showing significant control of SDS by azoxystrobin, propiconazole, fenarimol, thiophanate-methyl, and tebuconazole. According to studies by Tredway et al. (101) fenarimol was the only fungicide that exhibited consistent positive results in controlling SDS caused by

*O. korrae*. They recommend the application of a systemic fungicide prior to dormancy when soil temperatures are between 15 and 27 °C. Similar results were obtained by Luc et al. (54) showing that fenarimol was the most effective fungicide in controlling SDS. In general, fungicides should be delivered into the root zone by irrigation immediately after application (105). Butler and Tredway (9) showed that even though no significant difference between application methods were present, watered-in applications tended to provide more control than foliar applications.

Cultural management methods for SDS have also been studied. Susceptible turfgrasses can be replaced with more resistant species. Sayed et al. mentioned that renovation of the whole lawn or replacement of infected bermudagrass with zoysiagrass is the only possible means to control the disease but the later can also be infected by *O. korrae* (78; 98). Kozelnicky (49)) tried several cultural control methods including core aerification and/or verticutting, complete turf renovation, liming, soil removal, and replenishment but only complete renovation by rototilling to a depth of 30 cm was consistent in effective on reducing SDS. Lucas (55) recommended that aerification and weed control programs were necessary to help bermudagrass to recover. Verticutting should not be used once the bermudagrass begins to regrow over the dead patches to avoid removal of stolons present on the diseased area (55). Cultivation practices that disturb the upper root zone of bermudagrass such as sod removal or the combination of aeration and deep vertical mowing when the plant is entering the fall transitional period can be effective in reducing SDS and promote plant health (72; 92).

Higher mowing heights prior to cold-temperature induced dormancy may aid in preserving carbohydrate reserves and provide insulation during cold weather (105). It has been shown that SDS is less severe on cultivars that utilize stored carbohydrate and green-up early (22); Martin et al. (61) showed that *O. herpotricha* SDS necrotic patches were larger and winter kill was greater on bermudagrasses that were mowed at higher heights. The authors, however, didn't recommend reducing mowing heights as a method for managing SDS (60).

The relationship between SDS development and nitrogen application has also been explored for disease management. It was observed that the severity of SDS increased in plots without fungicides that received extra nitrogen in August and September (57). Nitrogen and potassium application rates and timing seemed to influence winter hardiness of bermudagrass. High nitrogen applications during late summer can exacerbate the disease, while low nitrogen can result in less disease (55; 56; 86). Even though it was thought that increased potassium applications would help control the disease, McCarthy et al. (66) demonstrated otherwise. The influence of potassium in the form of KCl and different sources of nitrogen ($NaNO_3$, $(NH_4)^2SO_4$ $NH_4Cl$ $CO(NH_2)^2$) have also been tested for SDS caused by *O. korrae*. The $NH_4$-N+KCl applications were found to reduce SDS, probably due to the acidification of the soil produced by these treatments (17). More recent studies by Tredway et al. (100) showed that the influence of nitrogen source is not the same for *O. herpotricha* and *O. korrae*. Calcium nitrate applications suppressed *O. korrae* while had no effect on *O. herpotricha*, for which, ammonium sulfate and sulfur coated urea were suppressive. It is not recommended to

apply excessive amounts of either nitrogen or potassium for the management of SDS (102).

Deployment of resistant cultivars can reduce disease severity. Several varieties of bermudagrass have been screened against *Ophiosphaerella* species causing SDS (3; 61). Among the varieties that showed increased resistance to *O. herpotricha* are African, Guymon, Sun devil, Midlawn, Midfield, Ft. Reno, Mirage (3), and Yukon (61). There are no turf-type cultivars known to be immune to the disease (62). Uncovering the mechanisms involved on plant-disease interaction has been the focus of recent studies since it could aid in the development of resistant cultivars. Bermudagrass cultivars with increased winter hardiness seem to also have increased resistance to SDS (1; 2; 62; 93). The dual role of chitinases in both disease and cold resistance may explain the correlation between winter hardiness and SDS resistance (3). After acclimation, Gatschet et al. (31; 32) found higher levels of chitinase in crowns of Midiron, a cold hardy cultivar, than in crowns of Tifgreen, a less cold hardy cultivar. Using real-time reverse-transcription PCR following infection of *O. narmari* to *C. dactylon*, McMaugh and Lyon (67) showed that a class II chitinase gene (CHTII) was overexpressed up to 26-fold compared to the non-infected control, indicating a potential role for CHTIIa in host plant defense.

Screening for SDS resistance can be done in the field by inoculating the soil with a known virulent strain of the fungus (61). However, using the field screening method typically requires two years after incubation before minor symptoms will appear and disease ratings need to be conducted for several growing seasons, making this method slow and expensive (91). There have been attempts to develop a faster method to screen

for SDS resistant cultivars. Baird et al. (3) performed greenhouse experiments where root discoloration area was rated after inoculation with *O. herpotricha*, but the results were not consistent with field experiments. Iriarte et al. (45) tested cold acclimation followed by freezing in order to identify SDS resistant cultivars but damage caused by *O. herpotricha* and *O. korrae* was indistinguishable when comparing resistant and susceptible cultivars. Walker et al, (111) developed an *in vitro* method that allows evaluation of differences in root colonization, which can be useful for screening for SDS resistant cultivars in less than a month. Furthermore, there is a potential for screening new genotypes for SDS resistance in a shorter period of time by using *Ophiosphaerella* fungi expressing fluorescent proteins (11).

**III Host-pathogen interaction**

Plants are generally resistant to most microbial pathogens due to a series of defense mechanisms that prevent infection. Successful pathogens have evolved to evade detection, deal with preformed molecules, or suppress host defense mechanisms (5). Incompatibility in the plant-pathogen interaction results in resistance, whereas a compatible plant-pathogen interaction results in a susceptible plant (47). There are multiple plant defenses against a pathogen, some are preformed like waxes, cell wall components, antimicrobial peptides, proteins, and non-proteinaceous secondary metabolites, while others, such as phytoalexins, are induced by microbial products known as elicitors (104). Current induced defense response models are highly influenced by the gene-for-gene interaction described by Flor (30) where resistance occurs when a pathogen's specific avirulence gene (Avr) and a corresponding specific resistance gene (R) of the plant are present. Avr proteins are part of a larger group of molecules called

effectors that include all proteins secreted by pathogens into host cells to enhance infection (21). In general, Avr genes, directly or indirectly, produce a target that a plant with the corresponding R gene recognizes, such recognition induces defense responses on the plant (104). The interaction between one specific Avr gene with a specific R gene is challenged by observations that suggest apparently unproductive associations (52), recognition of more than one Avr protein by a single R protein (35), and interference of avr/R protein interactions with one another (75). The recognition of effectors by plant intracellular receptors is known as effector-triggered immunity (ETI) and is a product of co-evolution of pathogen and host rendering a vast diversity of specific effectors and ETI receptors between and within species (21). Besides the highly pathogen-specific ETI, host defense can also be triggered by non-specific pathogen-derived elicitors such as oligosaccharides (20; 24), microbial proteins (27; 58), nucleic acids (87), and lipids (34). These molecules, which are conserved among pathogen species, are known as pathogen associated molecular patterns (PAMPs) and interact with pattern recognition receptors (PPRs) on the surface of the host cell giving rise to PAMP triggered immunity (PTI) (4; 21).

Even though ETI and PTI render similar responses, ETI is stronger and faster, and usually generates a hypersensitive response (HR), a form of localized cell death triggered by $H_2O_2$ that the plant undergoes to protect healthy cells from the pathogen (21; 53). Plant responses to specific and non-specific elicitors include ion influx, alkalinization of extracellular spaces, accumulation of reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), and reorganization of transcription (16; 104). The highly toxic ROS and RNI are by themselves a non-specific line of defense for plants against pathogens but

are also involved in transcriptional reprogramming in the vicinity of the infection site (104). Besides the HR, this reprogramming can result in synthesis of signaling intermediates including salicylic acid, ethylene, and jasmonic acid, altered cell walls, activation of downstream genes encoding for antimicrobial proteins, and synthesis of antimicrobial chemicals (16; 38). Antimicrobial compounds produced by the plants can be defined as phytoanticipins or phytoalexins. Some compounds may be a phytoanticipin in one plant while being a phytoalexin in other, the difference lies on the fact that phytoalexins are produced *de novo* in response to pathogen recognition while phytoanticipins are constitutive (19).

Plant pathogens can be classified broadly into necrotrophs, that kill the host in order to obtain nutrient, and biotrophs, that require a living host to complete their life-cycle (16). In general, necrotrophs produce phytotoxins and cell wall degrading enzymes capable of killing plant cells, while biotrophs have evolved to establish compatible interactions with the host that would result in disease (13; 16). HR is one of the most important reactions of the plant to deter the growth of biotrophic pathogens (13), but it has been observed that this response is a consequence of resistance and not the cause of it (48). Furthermore, necrotrophs can benefit from the HR in order to successfully infect host plants (33). It has been proposed that for coping with the HR necrotrophs use an array of enzymes including superoxide dismutase, peroxidase, catalase, laccases and polyphenol oxidases (63). ROS can originate either from the host or form the pathogen. Fungus-originated ROS may be instrumental in pathogenicity (42) or regulate endosymbiotic relationships (89). The production of ROS by the host may, in some cases, trigger the production of enzymes by necrotrophic pathogens that allow these

organisms to survive and grow in a ROS-rich environment (63). *Epichloë* endophytes are tightly regulated by their hosts (cool season grasses) to keep the fungus-plant relationship as mutualistic and ROS appears to play a major role in this regulation, when regulation fails the fungus can become pathogenic (23). The role of ROS during *Ophiosphaerella*-bermudagrass interactions is not known and understanding it will further our knowledge on how the fungus is able to cause disease.

**Literature cited**


1. Anderson, J. A., Kenna, M. P., and Taliaferro, C. M. 1988. Cold hardiness of Midiron and Tifgreen bermudagrass. Hortscience 23:748-750.
2. Anderson, J. A., Taliaferro, C. M., and Martin, D. L. 1993. Evaluating freeze tolerance of bermudagrass in a controlled environment. Hortscience 28:955.
3. Baird, J. H., Martin, D. L., Taliaferro, C. M., Payton, M. E., and Tisserat, N. A. 1998. Bermudagrass resistance to spring dead spot caused by *Ophiosphaerella herpotricha*. Plant Disease 82:771-774.
4. Boller, T., and Felix, G. 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annual Review of Plant Biology 60:379-406.
5. Borras-Hidalgo, O. 2004. Fundamentos de la interacción planta-patógeno. Biotecnologia Aplicada 21:1-4.
6. Busey, P., and Myers, B. J. 1979. Growth rates of turfgrasses propagated vegetatively. Agronomy Journal 71:817-821.
7. Butler, E. L., and Tredway, L. P. 2004. Development of effective strategies for control of spring dead spot in bermudagrass. Phytopathology 94:S167.

8. Butler, E. L., and Tredway, L. P. 2004. Preventative control of spring dead spot in bermudagrass athletic fields, 2002-2003. Fungicide and Nematicide Tests 59:T027.

9. Butler, E. L., and Tredway, L. P. 2006. Method and timing of fungicide applications for control of spring dead spot in hybrid bermudagrass. Plant Health Progress1-9 doi:10.1094/PHP-2006-0901-01-RS.

10. Butler, L., Tredway, L., Soika, M., and Bunting, M. 2007. Spring dead spot management in bermudagrass. Golf Course Management 75:100-104.

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

12. CABI, CBS, and Landcare Research 2011. Species Fungorum.

13. Coll, N. S., Epple, P., and Dangl, J. L. 2011. Programmed cell death in the plant immune system. Cell death and differentiation 18:1247-1256.

14. Crahay, J. N. 1988. Growth and pathogenicity of *Leptosphaeria korrae* in bermudagrass. Plant Disease 72:945-949.

15. Dale, J. L., and Diaz, C. 1963. A new disease of bermudagrass turf. Arkansas Farm Research 12:6.

16. Dangl, J. L., and Jones, J. D. G. 2001. Plant pathogens and integrated defense responses to infection. Nature 411:826-833.

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

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

19. Dixon, R. A. 2001. Natural products and plant disease resistance. Nature 411:843-847.

20. Doares, H., Syrovets, T., Weiler, E. W., and Ryan, C. A. 1995. Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proceedings of the National Academy of Sciences of the United States of America 92:4095-4098.

21. Dodds, P. N., and Rathjen, J. P. 2010. Plant immunity: towards an integrated view of plant–pathogen interactions. Nat Rev Genet 11:539-548.

22. Dunn, J. H., Nelson, C. J., and Sebaugh, J. L. 1980. Characterization of thatch, rhizomes, carbohydrates, and spring deadspot in twenty cultivars of bermudagrass. Journal of the American Society for Horticultural Science 105:653-660.

23. Eaton, C. J., Cox, M. P., and Scott, B. 2011. What triggers grass endophytes to switch from mutualism to pathogenism? Plant Science 180:190-195.

24. Ebel, J. 1998. Oligoglucoside elicitor-mediated activation of plant defense. BioEssays 20:569-576.

25. Endo, R. M., Ohr, H. D., and Krausman, E. M. 1984. The cause of spring dead spot disease (SDS) of *Cynodon dactylon* (L) Pers. in California. Phytopathology 74:812-812.

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

27. Felix, G., Duran, J. D., Volko, S., and Boller, T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. The Plant Journal 18:265-276.

28. Fermanian, T. W. 1980. The isolation of a toxin from spring dead spot areas in bermudagrass (*Cynodon* L. C. Rich) turf. Research, Agronomy PhD dissertation, Oklahoma State University, Stillwater, OK.

29. Fermanian, T. W., Ahring, R. M., and Huffine, W. W. 1981. The isolation of a toxin from spring dead spot areas in bermudagrass (*Cynodon* L.C. Rich) turf. International Turfgrass Research Journal 4:433-441.

30. Flor, H. H. 1956. The complementary genic systems in flax and flax rust. Advances in Genetics Incorporating Molecular Genetic Medicine 8:29-54.

31. Gatschet, M. J., Taliaferro, C. M., Anderson, J. A., Porter, D. R., and Anderson, M. P. 1994. Cold-acclimation and alterations in protein-synthesis in bermudagrass crowns. Journal of the American Society for Horticultural Science 119:477-480.

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

33. Govrin, E. M., and Levine, A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Current Biology 10:751-757.

34. Granado, J., Felix, G., and Boller, T. 1995. Perception of fungal sterols in plants: subnanomolar concentrations of ergosterol elicit extracellular alkalinization in tomato cells. Plant Physiology 107:485-490.

35. Grant, M. R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R. W., and Dangl, J. L. 1995. Structure of the *Arabidopsis* RPM1 gene enabling dual-specificity disease resistance. Science 269:843-846.

36. Green, D. E. 1993. Pathogenicity of *Rhizoctonia solani* AG-2-2 and *Ophiosphaerella herpotricha* on zoysiagrass. Plant Disease 77:1040-1044.

37. Gullino, M. L., Mocioni, M., and Titone, P. 2007. First report of *Ophiosphaerella korrae* causing spring dead spot of bermudagrass in Italy. Plant Disease 91:1200.

38. Hammond-Kosack, K. E., and Jones, J. D. G. 1996. Resistance gene-dependent plant defense responses. Plant Cell 8:1773-1791.

39. Hawkes, N. J. 1987. Spring dead spot of `Tifdwarf' turf in south Australia. Journal of the Sports Turf Research Institute 63:136-140.

40. Hawkes, N. J., and Harding, H. W. J. 1985. Isoelectric focusing as an aid to the identification of *Leptosphaeria narmari*, a cause of spring dead spot in turf. Australasian Plant Pathology 14:72-76.

41. Hsiang, T., Chen, F., and Goodwin, P. H. 2003. Detection and phylogenetic analysis of mating type genes of *Ophiosphaerella korrae*. Canadian Journal of Botany 81:307-315.

42. Hyon, G.-S., Ikeda, K.-i., Hosogi, N., Shinogi, T., and Park, P. 2010. Inhibitory effects of antioxidant reagent in reactive oxygen species generation and penetration of appressoria of *Alternaria alternata* japanese pear pathotype. Phytopathology 100:840-847.

43. Iriarte, F., Fry, J., and Tisserat, N. 2000. Evaluating best management practices for spring dead spot suppression in bermudagrass. Hortscience 35:390-391.

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

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

46. Jackson, N. 1993. Geographic distribution, host range, and symptomatology of patch diseases caused by ectotrophic fungi. Pages 161. in: Turfgrass patch diseases caused by ectotrophic root-infecting fungi B. B. Clarke, and A. B. Gould, eds. APS Press, Saint Paul, MN.

47. Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Annual Review of Genetics 24:447-463.

48. Kiraly, Z., Barna, B., and Ersek, T. 1972. Hypersensitivity as a consequence, not cause, of plant resistance to infection. Nature 239:456-458.

49. Kozelnicky, G. M. 1974. Updating 20 years of research: spring dead spot. USGA Green Section Record 12:12-15.

50. Landschoot, P. J. 1993. Ecology and epidemiology of ectotrophic root-infecting fungi associated with patch diseases of turfgrasses. Pages 161. in: Turfgrass patch diseases caused by ectotrophic root-infecting fungi B. B. Clarke, and A. B. Gould, eds. APS Press, Saint Paul, MN.

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

52. Leister, R. T., and Katagiri, F. 2000. A resistance gene product of the nucleotide binding site - leucine rich repeats class can form a complex with bacterial avirulence proteins *in vivo*. Plant Journal 22:345-354.

53. Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. 1994. $H_2O_2$ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79:583-593.

54. Luc, J. E., Canegallo, A., and Martin, B. 2005. Spring dead spot in bermudagrass greens in Argentina and South Carolina. Golf Course Management 73:92-95.

55. Lucas, L. T. 1980. Spring deadspot of bermudagrass. USGA Green Section Record 18:4-6.

56. Lucas, L. T. 1980. Spring deadspot of bermudagrass. Pages 183-187. in: Advances in Turfgrass Pathology B. G. Joyner, and P. O. Larsen, eds. Harcourt & Brace Jovanovich, Inc., Duluth, MN.

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

58. Luit, A. H. v. d., Piatti, T., Doorn, A. v., Musgrave, A., Felix, G., Boller, T., and Munnik, T. 2000. Elicitation of suspension-cultured tomato cells triggers the

formation of phosphatidic acid and diacylglycerol pyrophosphate. Plant Physiology 123:1507-1515.

59. Maples, P. J. 2001. Soaring with bermudagrass. A history of southern grasses. Golf Course management 69:33-36.

60. Martin, D. L., Taliaferro, C. M., Tisserat, N. A., Bell, G. E., Baird, J. H., Dobson, D. D., Anderson, J. A., and Kuzmic, R. M. 2001. Hardy bermudagrasses sought with resistance to spring dead spot. Golf Course Management 69:75-79.

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

62. Martin, D. L., Bell, G. E., Taliaferro, C. M., Tisserat, N. A., Baird, J. H., Dobson, D. D., Kuzmic, R. M., and Anderson, J. A. 2001. Spring dead spot resistance of inter-specific hybrid bermudagrasses. Pages 685-688. in: 9th International Turfgrass Research Conference, Toronto, Ontario, Canada, 15-21 July, 2001. International Turfgrass Society.

63. Mayer, A. M., Staples, R. C., and Gil-ad, N. L. 2001. Mechanisms of survival of necrotrophic fungal plant pathogens in hosts expressing the hypersensitive response. Phytochemistry 58:33-41.

64. McCarty, L. B., and Lucas, L. T. 1989. *Gaeumannomyces graminis* associated with spring dead spot of bermudagrass in the southeastern United States. Plant Disease 73:659-661.

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

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

67. McMaugh, S. J., and Lyon, B. R. 2003. Real-time quantitative RT-PCR assay of gene expression in plant roots during fungal pathogenesis. BioTechniques 34:982-986.

68. Nameth, S. T., Shane, W. W., and Stier, J. C. 1990. Development of a monoclonal-antibody for detection of *Leptosphaeria korrae*, the causal agent of necrotic ringspot disease of turfgrass. Phytopathology 80:1208-1211.

69. Nus, J. L., and Shashikumar, K. 1993. Fungi associated with spring dead spot reduces freezing resistance in bermudagrass. Hortscience 28:306-307.

70. Ohr, H. D., Murphy, M. K., Krausman, E. M., Endo, R. M., and Henry, J. M. 1987. Control of spring dead spot of bermudagrass California Agriculture 41:11.

71. Patton, A. J., Richardson, M. D., Karcher, D. E., Boyd, J. W., Reicher, Z. J., Fry, J. D., McElroy, J. S., and Munshaw, G. C. 2008. A guide to establishing seeded bermudagrass in the transition zone. Applied Turfgrass Science 5:1-20 doi:10.1094/ATS-2008-0122-01-MD.

72. Perry, H., and Tomaso-Peterson, M. 2007. Disease management strategies for controlling spring dead spot of bermudagrass. Phytopathology 97:S91-S92.

73. Plumley, K. A., Gould, A. B., and Clarke, B. B. 1997. Impact of temperature, osmotic potential, and osmoregulant on the growth of three ectotrophic root-infecting fungi of kentucky bluegrass. Plant Disease 81:873-879.

74. Raffle, V. L., and Hsiang, T. 1998. . Low level of DNA polymorphisms in isolates of *Leptosphaeria korrae* pathogenic on *Poa pratensis*. Canadian Journal of Plant Pathology-Revue Canadienne de Phytopathologie 20:48-54.

75. Ritter, C., and Dangl, J. L. 1996. Interference between two specific pathogen recognition events mediated by distinct plant disease resistance genes. The Plant Cell Online 8:251-257.

76. Rodgers, C. 2003. You've come a long way, bermuda. Golf Course management 71:71-91.

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

78. Sayed, M. Q., Droegemeier, H. K., and Bohm, M. J. 1969. Spring dead spot disease of bermudagrass and Its control. Transactions of the Kansas Academy of Science 72:203-209.

79. Shoemaker, R. A., and Babock, C. E. 1989. *Phaeosphaeria*. Canadian Journal of Botany 67:1500-1599.

80. Sill, W. H., Jr., Hansing, E. D., Kramer, C. L., and King, C. L. 1961. Kansas Phytopathological Notes: 1960. Transactions of the Kansas Academy of Science 64:292.

81. Small, W. A. 1964. Process for controlling spring dead spot. Pages 7 United States Patent Office. USA.

82. Smiley, R. W., Dernoeden, P. H., and Clarke, B. B. 2005. Compendium of Turfgrass Diseases. 3ed ed. The American Phytopatological Society, St. Paul, MN.

83. Smith, A. M. 1965. *Ophiobolus herpotrichus* a cause of spring dead spot in couch turf. Agricultural Gazette of New South Wales 76:753-758.

84. Smith, A. M. 1967. Spring dead spot of couch grass turf. Master in Agriculture Thesis, Agriculture, University of Sydney, Sydney.

85. 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.

86. Smith, J. D., Jackson, N., and Woolhouse, A. R. 1989. Fungal diseases of amenity turf grasses. E. and F. N. Spon, New York, NY.

87. Szittya, G., and Burgyan, J. 2001. Cymbidium ringspot tombusvirus coat protein coding sequence acts as an avirulent RNA. Journal of Virology 75:2411-2420.

88. Taliaferro, C., Roquette Jr., F., and Mislevy, P. 2004. Bermudagrass and stargrass. Pages 417-475. in: Warm-season (C4) grasses L. Moser, L. Sollenberger, and B. Burson, eds. American Society of Agronomy, Madison, WI.

89. Tanaka, A., Christensen, M. J., Takemoto, D., Park, P., and Scott, B. 2006. Reactive oxygen species play a role in regulating a fungus–perennial ryegrass mutualistic interaction. The Plant Cell Online 18:1052-1066.

90. Tisserat, N., Wetzel, H., Fry, J., and Martin, D. L. 1999. Spring dead spot of buffalograss caused by *Ophiosphaerella herpotricha* in Kansas and Oklahoma. Plant Disease 83:199.

91. Tisserat, N., Fry, J., Martin, D., Iriarte, F., and Wetzel, H., III. 2004. Identification, distribution, and aggressiveness of spring dead spot pathogens of bermudagrass. United States Golf Association. USGA Green Section record 42:15-18.

92. Tisserat, N. A., and Fry, J. 1997. Cultural practices to reduce spring dead spot (*Ophiosphaerella herpotricha*) severity in *Cynodon dactylon*. International Turfgrass Research Journal931-936.

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

94. Tisserat, N. A., Hulbert, S. H., and Nus, A. 1991. Identification of *Leptosphaeria korrae* by cloned DNA probes. Phytopathology 81:917-921.

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

96. Titone, P., Mocioni, M., Landschoot, P. J., and Gullino, M. L. 2004. Survey of ectotrophic root infecting fungi associated with turfgrass patch diseases in Italy. Acta Horticulturae 661:491-498.

97. Tredway, L., and Butler, L. 2005. Development of effective fungicide programs for spring dead spot control in hybrid bermudagrass. Phytopathology 95:S104.

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

99. Tredway, L. P., Butler, E. L., and Soika, M. D. 2007. Evaluation of fungicides for preventative control of spring dead spot in bermudagrass, 2005-2006. Plant Disease Management Reports 1:T030.

100. Tredway, L. P., Soika, M. D., and Butler, E. L. 2009. Response of spring dead spot caused by *Ophiosphaerella korrae* and *O. herpotricha* to fertilization programs and preventive fungicide applications. Phytopathology 99:S129.

101. Tredway, L. P., Butler, E. L., Soika, M. D., and Bunting, M. L. 2008. Etiology and management of spring dead spot of hybrid bermudagrass in North Carolina, USA. Acta Horticulturae 783:535-546.

102. Tredway, L. P., Tomaso-Peterson, M., Perry, H., and Walker, N. R. 2009. Spring dead spot of bermudagrass: a challenge for researchers and turfgrass managers. Plant Health Progress1-18. doi:10.1094/APSnetFeature-2008-0108.

103. Venkatasubbaiah, P., Tisserat, N. A., and Chilton, W. S. 1994. Metabolites of *Ophiosphaerella herpotricha*, a cause of spring dead spot of bermudagrass. Mycopathologia 128:155-159.

104. Veronese, P., Ruiz, M. T., Coca, M. A., Hernandez-Lopez, A., Lee, H., Ibeas, J. I., Damsz, B., Pardo, J. M., Hasegawa, P. M., Bressan, R. A., and Narasimhan, M. L. 2003. In defense against pathogens. Both plant sentinels and foot soldiers need to know the enemy. Plant Physiology 131:1580-1590.

105. Vincelli, P., and Williams, D. 1998. Managing spring dead spot of bermudagrass. Golf Course Management 66:49-53.

106. Wadsworth, D. F., and Young, H. C. 1960. Spring dead spot of bermudagrass. Plant Disease Reporter 44:516-518.

107. Wadsworth, D. F., Houston, B. R., and Peterson, L. J. 1968. *Helmintosporium spiciferum* a pathogen associated with spring dead spot of bermuda grass. Phytopathology 58:1658-1660.

108. Walker, J. 1980. *Gaeumannomyces, Linocarpon, Ophiobolus* and several other genera of scolecospored ascomycetes and *Phialophora* condial states, with a note on hyphopodia. Mycotaxon1-129.

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

110. Walker, N. 2006. Viability of dormant bermudagrass in spring dead spot patches caused by *Ophiosphaerella herpotricha* during winter months. Phytopathology 96:S119.

111. Walker, N., Marek, S., and Taliaferro, C. 2005. Colonization of bermudagrass roots produced from stolons by *Ophiosphaerella herpotricha*. Phytopathology 95:S107-S108.

112. Walker, N. R. 2005. Evaluation of fungicides for the management of spring dead spot of bermudagrass, 2003-2004. Fungicide and Nematicide TestsTO22.

113. 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.

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

115. Wetzel, H. C., Dernoeden, P. H., and Millner, P. D. 1996. Identification of darkly pigmented fungi associated with turfgrass roots by mycelial characteristics and RAPD-PCR. Plant Disease 80:359-364.

116. Wetzel , H. C., Hulbert, S. H., and Tissert, N. A. 1999. 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.

117. Wetzel, H. C., Skinner, D. Z., and 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.

118. Williams, B., Kabbage, M., Kim, H.-J., Britt, R., and Dickman, M. B. 2011. Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. PLoS Pathogens 7:e1002107.

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

120. Young, H. C., Sturgeon, R. V., and Huffine, W. W. 1973. Soil type associated with spring dead spot of bermudagrass. Phytopathology 63:450.

121. Zhang, Y., Guenzi, A. C., Anderson, M. P., Taliaferro, C. M., and Gonzales, R. A. 2006. Enrichment of bermudagrass genes associated with tolerance to the spring dead spot fungus *Ophiosphaerella herpotricha*. Physiological and Molecular Plant Pathology 68:105-118.

CHAPTER III

# INFECTION AND COLONIZATION OF BERMUDAGRASS SPECIES BY *OPHIOSPHAERELLA KORRAE*


## Abstract

Bermudagrass (*Cynodon* spp.) is the most commonly used turfgrass in the Southern United States where it can be severely affected by spring dead spot (SDS) caused by *Ophiosphaerella herpotricha*, *O. korrae*, and *O. narmari*. In this study, the infection of bermudagrass by *O. korrae* was characterized using a transformant that expressed a red fluorescent protein. Interspecific hybrid cultivars Midlawn and Tifway 419, *C. transvaalensis* accessions Uganda and 3200, and a *C. dactylon* cultivar U3 were inoculated and observed from 2 to 22 days post inoculation (DPI). For all cultivars tested, a similar rate of root colonization was observed; however, differences were observed for necrosis. Necrosis of Tifway 419 and Midlawn tissues was evident at 2 DPI, Uganda and 3200 at 8 DPI, and for U3 it was often not present at 14 DPI. The fungus rapidly penetrated the root epidermis and colonized the cortex of all cultivars by 4 DPI. *O. korrae* vasculature colonization of hybrid cultivars was rare but was common in *C. transvaalensis* and *C. dactylon* accessions. On intact stolons, the fungus did not penetrate the epidermis 22 DPI and necrosis was evident only on the surface of hybrid

bermudagrasses. Infection of wounded stolons resulted in necrosis for all cultivars. Infection and colonization of African and hybrid bermudagrasses by *O. korrae* was found to be similar to that of *O. herpotricha*, suggesting that host genetic resistance may be used for effective management of SDS caused by both species.

**Introduction**

Bermudagrass (*Cynodon dactylon* (L.) Pers. and *C. dactylon* x *C. transvaalensis* Burtt-Davy) is the most commonly used turfgrass in the Southern United States where cool-season grasses are difficult to grow. The two greatest limitations for northward bermudagrass utilization are its susceptibility to cold temperature injury and a disease known as spring dead spot (SDS) (7). Spring dead spot is considered the most destructive disease of bermudagrass where low temperatures induce grass dormancy (14). The disease is often associated with highly maintained bermudagrasses and is caused by one of three fungi from the genus *Ophiosphaerella*, namely *O. herpotricha* (Fr.) Walker, *O. korrae* (J. Walker and A. M. Smith) R. A. Shoemaker and C. E. Babcock, or *O. narmari* (J. Walker and A. M. Smith) Wetzel, Hubert and Tisserat (5; 8; 19; 20; 22; 25-27). *Ophiosphaerella herpotricha* is most common in the Midwestern United States and *O. korrae* more common in the southeast (27). The presence of both of these species and occasionally all three species at a single location can occur and is likely due to the movement of vegetative plant material or infested soil. Effective management of SDS can be costly and difficult therefore knowledge about the etiology of the pathosystem is critical for developing effective control methods.

Fungi that cause SDS and several other turfgrass diseases are described as ectotrophic, root-infecting fungi meaning they tend to colonize the surface of below-

ground organs such as rhizomes prior to infection. After colonization, hyphae will directly penetrate the epidermis or enter through wounds that can be common on rhizomes and stolons (3; 20). When colonizing a host, *Ophiosphaerella korrae* has previously been described to produce brown, septate, and branched hyphae (2.5-5 μm wide) that coalesce to form strands of runner hyphae, which accumulate into infection mats on the root surface (4; 26). All *Ophiosphaerella* species that cause SDS can infect roots, stolons, or rhizomes and localized necrosis can be observed within days (24). It is likely that *in vivo* and at low temperatures when bermudagrass growth slows, the fungus can rapidly colonize host tissues resulting in localized symptom development (4). As apparently healthy bermudagrass enters cold temperature-induced dormancy, additional events occur that cause plant death that results in the appearance of dead patches of grass once temperatures warm and the grass resumes growth.

Host-pathogen interactions at the cellular level have been previously been described for the *O. herpotricha* pathosystem on bermudagrasses (3). Roots and stolons of *C. dactylon* x *C. transvaalensis* (hybrid bermudagrass) and *C. transvaalensis* were infected with a transformed isolate of *O. herpotricha* that expressed green fluorescent (GFP) or red fluorescent proteins (tdTomato). Roots of two hybrid bermudagrass varieties, Midlawn on which SDS is generally less severe and Tifway 419, which is very susceptible both developed large necrotic lesions when colonized by *O. herpotricha*. For an accession of *C. transvaalensis*, a more tolerant endophytic-like association with *O. herpotricha* was observed and the fungus colonized the stele and caused limited necrosis (3). Even though both hybrid and *C. transvaalensis* stolons were colonized through wounds, *C. transvaalensis* exhibited little to no necrosis, suggesting an inherent tolerance

to *O. herpotricha* (3). It was suggested that host recognition of the fungus or response to infection and colonization may contribute to the events that cause plant death during dormancy (1).

Spring dead spot can occur on other warm-season grasses including zoysiagrass (*Zoysia japonica* Steud.) and buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.) (9; 21; 23). In addition, *O. korrae* is also the causal agent of necrotic ring spot on Kentucky bluegrass (*Poa pratensis* L.), a cool-season turfgrass species (6; 18).The process of infection, colonization, and pathogenicity of *O. korrae* in bermudagrass is limited (4; 8). It is also not known how the fungus interacts at a cellular level when colonizing susceptible and resistant bermudagrass varieties. Given that it can infect and cause disease on cool-season grasses, the interaction with different hosts may vary and may not be the same as *O. herpotricha*. The objectives of this study were to characterize the infection and colonization of several bermudagrass cultivars and accessions, with different levels of resistance, by an *O. korrae* isolate that expresses fluorescent proteins, evaluate host response, and compare these events those previously described for *O. herpotricha.* We believe that *O. korrae* and *O. herpotricha* infect bermudagrass roots similarly, readily colonizing the vasculature of tolerant cultivars and producing necrosis in the cortex of susceptible cultivars.

## Materials and Methods

**Fungal Transformation** Agrobacterium-mediated transformation (AMT) was conducted on twenty-three different isolates of *O. korrae* to express either the red fluorescent protein tdTomato (tdTom) or the green fluorescent protein (GFP), following

the protocol described by Caasi et al. (3). *Ophiosphaerella herpotricha* B6, a previously transformed isolate (3), was used as a positive control. Pathogenicity and growth rate of the transformed *O. korrae* isolates were compared to the wild type. A single transformant that most resembled the characteristics of the wild type was chosen for subsequent studies.

**Bermudagrass inoculation** The cultivars selected for study included the interspecific hybrids Tifway 419 and Midlawn, two *C. transvaalensis* accessions 3200 and Uganda, and a *C. dactylon* cultivar marketed as U3. Stolons were collected from container-grown plants and were surface sterilized with a 1.2% sodium hypochlorite, 38% ethanol solution for 2 to 4 minutes, rinsed with sterile, reverse osmosis (RO) water, and cut transversely into 2 to 4 cm segments, each containing at least one node. Sterile stolon segments were placed on potato dextrose agar (PDA) in Petri plates and incubated for up to 7 days at 25 °C in a vertical position to permit root growth. Stolons with roots and not exhibiting fungal contamination were transferred individually to sterile petri plates (100 × 15 mm) lined with a layer of wet paper towel and placed on a microscope slide (3 × 1 × 1 mm), aluminum foil was used to cover the roots to exclude light. Additional sterile RO water was added to saturate the paper towel. A single root from each stolon was inoculated with an agar plug (approximately 0.25 mm in diam.) containing mycelium from the edge of a 10 to14-day old culture of wild type or transformed *O. korrae*. One non-inoculated plant for every five inoculated replicates was used as a control. Plates were sealed with parafilm and incubated upright in a growth chamber at 17°C and 12 h photoperiod. The same inoculation method was used for

surface sterilized stolons that were either wounded (0.1-0.3 mm deep wounds made with a razor blade) or intact.

**Microscopy** Roots were observed using epifluorescence microscopy and stereomicroscopy at 2, 4, 8 and 14 day post-inoculation (DPI), and stolons at 3, 14, and 22 DPI. Surface colonization was observed directly and internal colonization observed using transverse sections made by hand-sectioning the plant organs with a razor blade. Three different roots or stolons were examined for each DPI stated previously. Whole and sectioned roots or stolons were mounted in water and observed with a Nikon Eclipse E800 microscope (Nikon Inc., Tokyo, Japan) equipped with mercury-arc using the UV-2E/C DAPI and G-2E/C TRITC epifluorescence filter sets. Digital images were captured using a monochrome QImaging Retiga 2000R charge-coupled device (CCD) camera (Quantitative Imaging Corp., Surrey, BC, Canada). Monochromatic grayscale images taken under each epifluorescent filter were artificially colored and combined as layers over a bright field image using QCapture Pro version 5.1.1.14 (Quantitative Imaging Corp., Surrey, BC, Canada). Composite images taken at 2 to 5 different depths were Z-stacked using AllFocus™ V1.06 (Saphicon LLC). Color images corresponding to the composites were captured using a Nikon SMZ-2T stereomicroscope (Nikon Inc., Tokyo, Japan) equipped with an Olympus DP71 digital camera (Olympus Imaging America Inc., Center Valley, PA). All images used for disease severity ratings were taken next to the point of inoculation.

**Disease severity ratings** Root colonization and necrosis were assessed for each root at the area next to the location of inoculation. Stereomicroscopic and microscopic

pictures were transformed to 3-bit (eight possible colors) images and the number of pixels corresponding to each color was determined using ImageMagick® (ImageMagick Studio, LLC). Root necrosis was assessed using digital stereomicroscopic images captured over a green background created by placing a clear polystyrene Petri plate tinted green with a permanent marker. The total root surface was obtained by subtracting lime and cyan pixels from the total number of pixels of the 3-bit image. The red and black pixels within the root surface were considered necrotic. Root colonization was assessed using composite pictures with bright background. Root surface was obtained by subtracting white pixels from the total number of pixels of the image. Red pixels within the root surface represented the percentage of root colonized by *O. korrae*. To determine differences ($P \leq 0.05$) in colonization and necrosis between cultivars at each DPI, analyses of variance was performed using the GLM procedure in SAS (version 9.3, SAS Institute, Cary, NC). Where significant, mean separation was conducted using Fishers Protected LSD. Spearman's' rank correlation coefficient between root colonization by *O. korrae* and necrosis was calculated for each cultivar using the CORR procedure in SAS.

**Results**

Out of 23 *O. korrae* isolates tested only OW6, isolated from West point, Mississippi, was successfully transformed using AMT. This isolate was transformed at an approximate rate of 1 transformant per 1,800 CFUs. Transformants that expressed either GFP or tdTom were obtained (Fig. A-1) with expression tdTom being the brightest and more stable of the two proteins. Transformant isolate 26bt2 was capable of producing necrosis on Midlawn at a rate similar to the wild type (Fig. A-3), therefore it was chosen for subsequent experiments.

The rate of colonization by *O. korrae* varied from 2 to 14 DPI among cultivars but no significant differences in colonization was observed on most DPI. One minor difference was for 3200 where initial colonization was slower when compared to the other cultivars (Fig. III-1). Even though differences were not always significant, at every DPI observed, Tifway 419 exhibited the greatest root surface necrosis followed by Midlawn, 3200, Uganda, and U3. Necrosis caused by *O. korrae* on U3 was always the least among all cultivars (Fig. III-1). The first appearance of necrosis was observed in roots of Tifway 419 and Midlawn by 2 DPI, 4 DPI for 3200, 8 DPI for Uganda, and for U3 by 14 DPI (Fig. III-1). The correlation between root necrosis and colonization was significant ($p < 0.05$) for Tifway 419, Midlawn, Uganda, and 3200 (Table III-1).

For all cultivars, most of the root surface area next to the inoculum was colonized by the fungus by 4 DPI (Fig. III-2A); with hyphae growing longitudinally along the root, mainly between the root cells (Fig. III-2B). *Ophiosphaerella korrae* penetrated bermudagrass roots directly, without specialized structures, and once inside grew inter

and intracellularly. For Midlawn and Tifway 419 cultivars, lateral roots were able to tolerate colonization before they ruptured the epidermis of the primary root (Fig. III-2C), but lateral roots that had already emerged from the primary root were readily colonized (Fig. III-2D). In contrast, for the cultivar U3 where the fungus had already colonized the vasculature, lateral roots were colonized through the vasculature before emergence (Fig. III-2E).

Fungal penetration was similar for all cultivars, when the fungus colonized the root surface it penetrated the epidermis directly and by 2 DPI, and colonization of the cortex was observed (Figs. III-3 and III-4). For Tifway 419 and Midlawn, cortical cell necrosis was apparent as the fungus colonized the tissue (Figs. III-3, A-5, and A-6). However, for 3200 and Uganda, the cortex was first extensively colonized before necrosis appeared (Figs. A-7 and A-8). In U3, surface colonization near the inoculum was slower, however, by 4 DPI the fungus usually colonized most of the root surface around the inoculum with necrosis rarely observed even up to 14 DPI (Figs. III-1, III-4, and A-9). Colonization of roots tissues stopped at the endodermis for Midlawn and Tifway 419, with vascular colonization occurring once for each cultivar, in short (< 1 cm) roots in which it appeared the fungus colonized this region through the root tip. For cultivars Uganda, 3200, and U3, vascular colonization was more common, especially in U3 (Fig. III-4). Ten out of fourteen roots in which the vascular tissues were colonized did not exhibit symptoms of necrosis. When *O. korrae* colonized the vasculature in U3 roots, cortical colonization appeared to decline while it continued to colonize the stele (Figs. III-4, and A-9). In contrast, for Uganda and 3200 the fungus continued to colonize the cortex and vasculature tissues (Figs. A-7 and A-8).

For all cultivars, the fungus extensively colonized the surface of stolons often forming mycelial aggregations or mats (Figs. III-5B, III-5D, and A-10-13). Midlawn and Tifway 419 stolons exhibited localized necrotic spots where dense colonization was present (Figs. III-5C, A-10, and A-11), while 3200, Uganda, and U3 stolons had only slight discoloration or exhibited no symptoms of necrosis (Figs. III-5A, A-12, and A-13). For all cultivars observed, the fungus did not penetrate beyond the intact epidermis of stolons when observed at 22 DPI. In contrast, vasculature colonization of stolons only occurred when stolon tissues were wounded and localized necrosis was evident by 15 DPI for all cultivars (Fig. A-14).

**Discussion**

Ninety six percent of the *O. korrae* isolates tested in this study were not readily transformed using AMT. In general the mycelium of *O. korrae* is more melanized than that of *O. herpotricha* and this may have reduced the number of isolates suitable for transformation. Isolate OW6 appeared less melanized, and showed greater sensitivity to hygromycin, and slower growth when compared to other *O. korrae* isolates. This isolate was transformed at a rate similar to *O. herpotricha* A55, which was previously transformed by Caasi et al. (3) and was used as positive control for the transformation experiments in this study. As previously reported by Caasi et al. (3) for *O. herpotricha*, *O. korrae* transformed isolates are indistinguishable from the wild type in terms of disease progression, but grew slower on PDA.

As described previously by Endo et al. (8), *O. korrae* hyphae colonized cortical cells but runner hyphae were not observed 14 DPI. The formation of sclerotia was not

observed on living plants or in dead and decayed tissues after prolonged colonization. The absence of sclerotia is consistent with the findings of Crahay et al. (4) where *O. korrae* resting structures were not observed 3 months after inoculation. In this study, secondary lateral roots were infected and became necrotic as they emerged and these may have been misidentified as cortical sclerotia by Endo (8). The formation of cushion-like hyphal aggregates by *O. herpotricha* and *O. korrae* were previously reported (3; 4) at later stages of infection. In this study, such aggregates were not evident on the roots using bright field microscopy; however, a few, small mycelial mats were observed using fluorescence microscopy. On the surface of inoculated stolons, mycelial mats were formed frequently and were more evident, but did not appear to be associated with penetration of the hyphae into the cortex of the stolon.

Caasi et al. (3) demonstrated that *O. herpotricha* directly penetrated into the cortex of bermudagrass roots with intracellular hyphae growing longitudinally along the root axis. *O. korrae* also penetrated the root directly and moved along the root axis. A similar infection process has been described for *Fusarium oxysporum* on tomato, where the fungus preferentially colonizes the grooves between epidermal cells, has no specific infection sites, and lacks appressoria (12). But, in contrast with this study, *F. oxysporum* pathogenicity has been associated with colonization of the stele on tomato roots (12; 17). Colonization of the vasculature was common in U3 and often seen in *C. transvaalensis* cultivars, while on hybrid cultivars it was only present after the root was completely colonized. The common bermudagrass used in this study designated as U3 is not true to type of the original U3 cultivar and its origin is unclear; however, in field studies where this grass was intensively inoculated with *O. herpotricha,* very little disease has occurred,

or SDS patches were small (data not shown). Endo (8) observed vascular colonization only in advanced stages of disease development, when the whole root is colonized, which suggests that the fungus may penetrate into the vasculature of certain cultivars at the root tip.

The most severe necrotic response in roots was observed in Tifway 419, a cultivar that is very susceptible to SDS. In hybrid cultivars Midlawn and Tifway 419, hyphae completely colonized the cortex of the roots growing inter and intracellularly; however, the vasculature was seldom colonized as growth stopped at the endodermis. These findings are similar to those of Caasi et al. (3) for these two cultivars. While colonization and necrosis were similar between these cultivars, *in vivo* the severity of SDS on Midlawn is significantly less than Tifway 419 (15). Even though greenhouse experiments have failed to relate winter hardiness to SDS resistance for bermudagrass (10), field studies suggest that the greater tolerance to SDS by Midlawn is linked to this cultivars' greater cold tolerance (1; 2; 15).

Caasi et al. (3) described *C. transvaalensis* as expressing less necrosis and having vasculature colonization. Here, two varieties of *C. transvaalensis* were examined and were colonized similarly to the two hybrid cultivars but had significantly less necrosis compared to Tifway. No necrosis was found on *C. transvaalensis* when *O. korrae* colonized the vasculature, and this is consistent to what was observed with *O. herpotricha* (3). A striking difference in response to *O. korrae* infection was observed between Tifway 419 and U3; while *O. korrae* would colonize mostly the cortex of Tifway 419 roots and render infected cells necrotic as soon as 2 DPI, the fungus would preferentially colonize the vasculature of U3 where no necrosis was observed in most

plants up to 14 DPI. Common bermudagrass (*C. dactylon*) was not included in the studies conducted by Caasi et al. (3) as these cultivars are often utilized in situations where management inputs do not encourage SDS or the cultivars widely grown in the Midwestern United States are more resistant to SDS *in vivo*.

Pseudothecia of *O. korrae* and *O. narmari* are commonly observed on infected stolons in Australia and appear to be rarely produced under field conditions in the United States (11; 26). Crahay et al. (4) produced fruiting bodies *in vitro* using non-sterile plant material. In this study, efforts were not successful in inducing pseudothecia production by either wild-type or transformed isolates in sterile bermudagrass plants (data not shown). The absence of fruiting bodies and sclerotia suggests that a means for dispersal is likely through the movement of infested plant material (13). As Caasi et al. (3) reported for *O. herpotricha*, in this study stolons were only colonized by *O. korrae* superficially, unless a wound was present. *Ophiosphaerella korrae* was recovered from dead stolon tissues nine months after infection (data not shown). This further suggests that infected stolons could be both a source of inoculum and where the fungus can survive during summer months when bermudagrass growth in favored as suggested by Caasi et al. (3).

While *O. korrae* readily colonized the roots of all cultivars tested, widespread necrosis was only associated with cultivars that prevented vascular colonization. The mechanisms used by the plant to impede endodermis penetration by the fungus is still unknown but represents a promising target for future research. There might be specific genes in bermudagrass that enable vascular colonization; in tomato, the resistance gene I-2 has been described as a factor preventing vascular colonization of roots by *Fusarium* where colonization of the stele represents susceptibility (16). Overall, infection and

colonization of bermudagrass by *O. korrae* resembles that of *O. herpotricha* for those cultivars examined suggesting both fungi are homologous. Therefore, similar management strategies such as breeding for cultivars with better field resistance may be used for both species. These breeding efforts may be enhanced through the use of evaluating the *in vitro* responses of different bermudagrass cultivars to infection and colonization by *O. korrae* or *O. herpotricha.*

**Acknowledgements**

Published with the approval of the Director, Oklahoma Agricultural Experiment Station, Stillwater, OK. This project was supported in part by the United States Golf Association and Oklahoma Agricultural Experiment Station project 2833.

**Literature cited**

1. Anderson, J. A., Taliaferro, C. M., and Martin, D. L. 1993. Evaluating freeze tolerance of bermudagrass in a controlled environment. Hortscience 28:955.
2. Baird, J. H., Martin, D. L., Taliaferro, C. M., Payton, M. E., and Tisserat, N. A. 1998. Bermudagrass resistance to spring dead spot caused by *Ophiosphaerella herpotricha*. Plant Disease 82:771-774.
3. Caasi, O. C., Walker, N. R., Marek, S. M., Enis, J. N., and Mitchell, T. K. 2010. Infection and colonization of turf-type bermudagrass by *Ophiosphaerella herpotricha* expressing green or red fluorescent proteins. Phytopathology 100:415-423.
4. Crahay, J. N. 1988. Growth and pathogenicity of *Leptosphaeria korrae* in bermudagrass. Plant Disease 72:945-949.

5. Dale, J. L., and Diaz, C. 1963. A new disease of bermudagrass turf. Arkansas Farm Research 12:6.

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

7. Dunn, J. H., Nelson, C. J., and Sebaugh, J. L. 1980. Characterization of thatch, rhizomes, carbohydrates, and spring deadspot in twenty cultivars of bermudagrass. Journal of the American Society for Horticultural Science 105:653-660.

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

9. Green, D. E. 1993. Pathogenicity of *Rhizoctonia solani* AG-2-2 and *Ophiosphaerella herpotricha* on zoysiagrass. Plant Disease 77:1040-1044.

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

11. Jackson, N. 1993. Geographic distribution, host range, and symptomatology of patch diseases caused by ectotrophic fungi. Pages 161. in: Turfgrass patch diseases caused by ectotrophic root-infecting fungi B. B. Clarke, and A. B. Gould, eds. APS Press, Saint Paul, MN.

12. Lagopodi, A. L., Ram, A. F. J., Lamers, G. E. M., Punt, P. J., Van den Hondel, C. A. M. J. J., Lugtenberg, B. J. J., and Bloemberg, G. V. 2002. Novel Aspects of

Tomato Root Colonization and Infection by Fusarium oxysporum f. sp. radicis-lycopersici Revealed by Confocal Laser Scanning Microscopic Analysis Using the Green Fluorescent Protein as a Marker. Molecular Plant-Microbe Interactions 15:172-179.

13. Landschoot, P. J. 1993. Ecology and epidemiology of ectotrophic root-infecting fungi associated with patch diseases of turfgrasses. Pages 161. in: Turfgrass patch diseases caused by ectotrophic root-infecting fungi B. B. Clarke, and A. B. Gould, eds. APS Press, Saint Paul, MN.

14. Lucas, L. T. 1980. Spring deadspot of bermudagrass. Pages 183-187. in: Advances in Turfgrass Pathology B. G. Joyner, and P. O. Larsen, eds. Harcourt & Brace Jovanovich, Inc., Duluth, MN.

15. Martin, D. L., Bell, G. E., Taliaferro, C. M., Tisserat, N. A., Baird, J. H., Dobson, D. D., Kuzmic, R. M., and Anderson, J. A. 2001. Spring dead spot resistance of inter-specific hybrid bermudagrasses. International Turfgrass Society Research Journal 9:685-688.

16. Mes, J. J., Van Doorn, A. A., Wijbrandi, J., Simons, G., Cornelissen, B. J. C., and Haring, M. A. 2000. Expression of the Fusarium resistance gene I-2 colocalizes with the site of fungal containment. The Plant Journal 23:183-193.

17. Olivain, C., and Alabouvette, C. 1999. Process of tomato root colonization by a pathogenic strain of *Fusarium oxysporum* f. sp. *lycopersici* in comparison with a non-pathogenic strain. New Phytologist 141:497-510.

18. Raffle, V. L., and Hsiang, T. 1998. Low level of DNA polymorphisms in isolates of *Leptosphaeria korrae* pathogenic on *Poa pratensis*. Canadian Journal of Plant Pathology-Revue Canadienne de Phytopathologie 20:48-54.

19. Smiley, R. W., Dernoeden, P. H., and Clarke, B. B. 2005. Compendium of Turfgrass Diseases. 3ed ed. The American Phytopatological Society, St. Paul, MN.

20. Smith, A. M. 1965. *Ophiobolus herpotrichus* a cause of spring dead spot in couch turf. Agricultural Gazette of New South Wales753-758.

21. Tisserat, N., Wetzel, H., Fry, J., and Martin, D. L. 1999. Spring dead spot of buffalograss caused by *Ophiosphaerella herpotricha* in Kansas and Oklahoma. Plant Disease 83:199.

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

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

24. Tredway, L. P., Tomaso-Peterson, M., Perry, H., and Walker, N. R. 2009. Spring dead spot of bermudagrass: a challenge for researchers and turfgrass managers. Plant Health Progress1-18. doi:10.1094/APSnetFeature-2008-0108.

25. Wadsworth, D. F., and Young, H. C. 1960. Spring dead spot of bermudagrass. Plant Disease Reporter 44:516-518.

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

27. Wetzel, H. C., Skinner, D. Z., and 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.

**TABLE:**

**Table III-1** Spearman's' correlation coefficients (ρ) and probability (P) values for the relationship between *O. korrae* colonization[x] and necrosis[y] of roots of different bermudagrass cultivars.

| Cultivar | $\rho^z$ | $P^z$ |
|---|---|---|
| Midlawn (*Cynodon dactylon* x *C. transvaalensis*) | 0.75 | 0.005 |
| Tifway 419 (*C. dactylon* x *C. transvaalensis*) | 0.75 | 0.005 |
| Uganda (*C. transvaalensis*) | 0.76 | 0.004 |
| 3200 (*C. transvaalensis*) | 0.60 | 0.039 |
| U3 (*C. dactylon*) | 0.52 | 0.086 |

[x] Root colonization was assessed using 3-bit images of composite microscopic pictures with bright background. Root surface was obtained by subtracting white pixels from the total number of pixels of the image. Red pixels (hyphae) within the root surface represented the percentage of root colonized by *O. korrae*.

[y] Root necrosis was assessed using 3-bit images of digital stereomicroscopic pictures captured over a green background. The total root surface was obtained by subtracting lime and cyan pixels from the total number of pixels of the 3-bit image. The red and black pixels within the root surface were considered necrotic.

[z] Calculated using the CORR procedure in SAS using quantitative data

**FIGURES:**

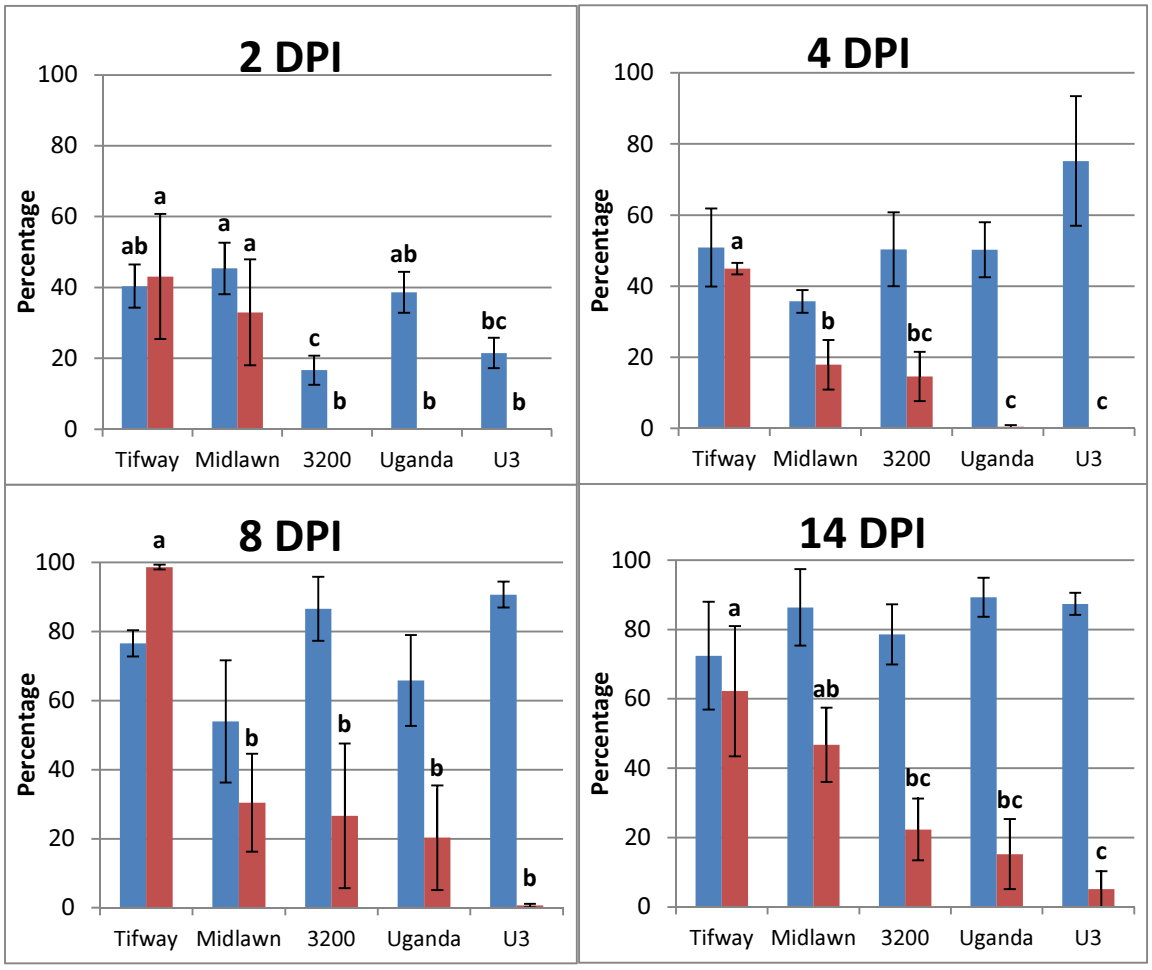


**Figure III-1** Colonization (blue) and necrosis (red) caused by *O. korrae* on bermudagrass roots at 2, 4, 8 and 14 days post inoculation (DPI). Bars represent one standard error of the mean, Columns with the same letter are not different according to Fisher's Protected LSD, ($P \leq 0.05$).

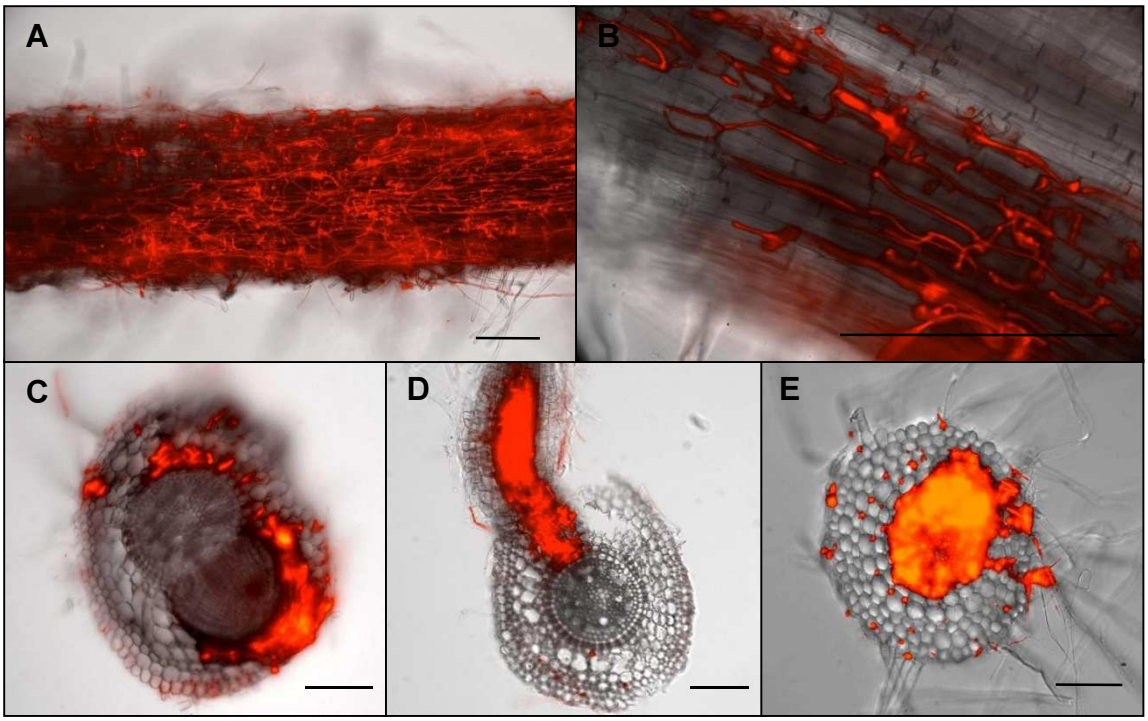


**Figure III-2** *Ophiosphaerella korrae* colonizing roots of bermudagrass cultivars A) 3200 at 4 days post infection (DPI), B-C) Tifway 419 8 DPI, D) Midlawn 14 DPI, and E) U3 22 DPI. A and B show root surfaces near inoculation points and C-E show transverse sections through lateral root emergence sites. Bars represent 100 µm.

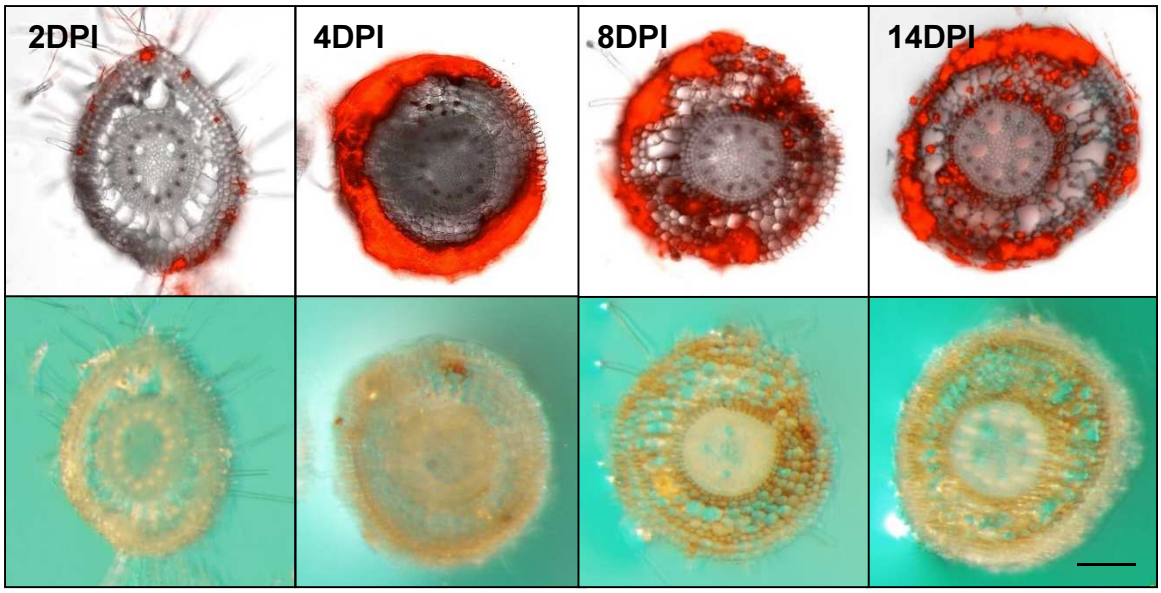


**Figure III-3** Transverse sections of Tifway 419 roots exhibiting colonization (red, top row) and necrosis (brown, bottom row) by *Ophiosphaerella korrae* at 2, 4, 8 and 14 days post inoculation (DPI). Top row, fluorescence images, and bottom row, bright field. Bar represents 100 µm.

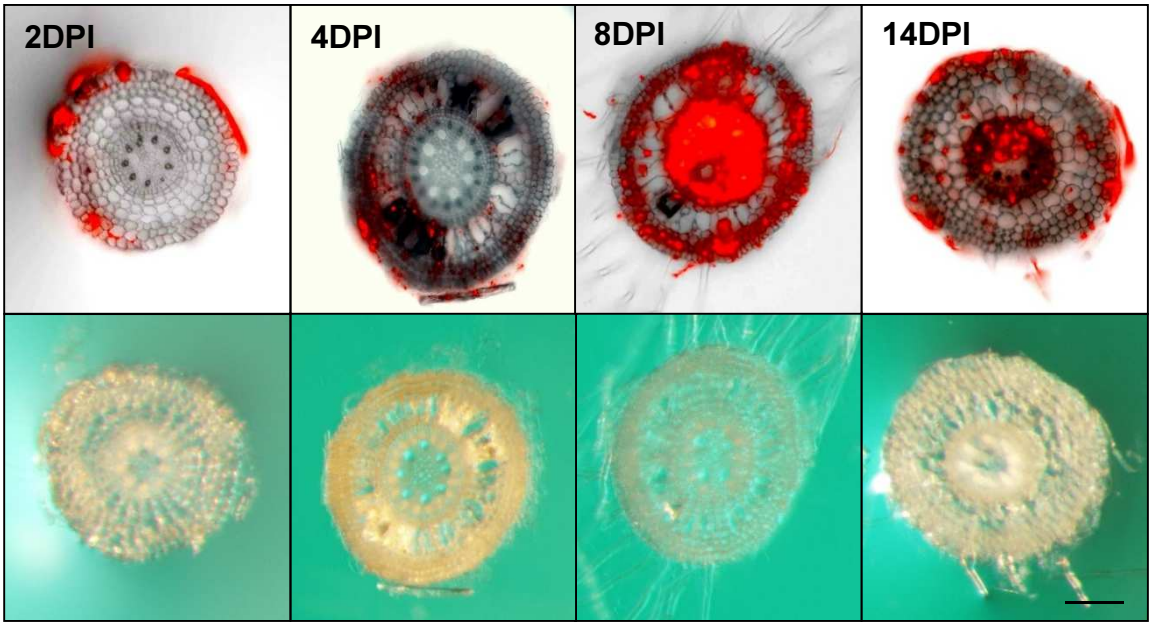


**Figure III-4** Transverse sections of U3 roots exhibiting colonization (red, top row) and necrosis (brown, bottom row) by *Ophiosphaerella korrae* at 2, 4, 8 and 14 days post inoculation (DPI). Top row fluorescence images bottom row bright field. Bar represents 100 µm.

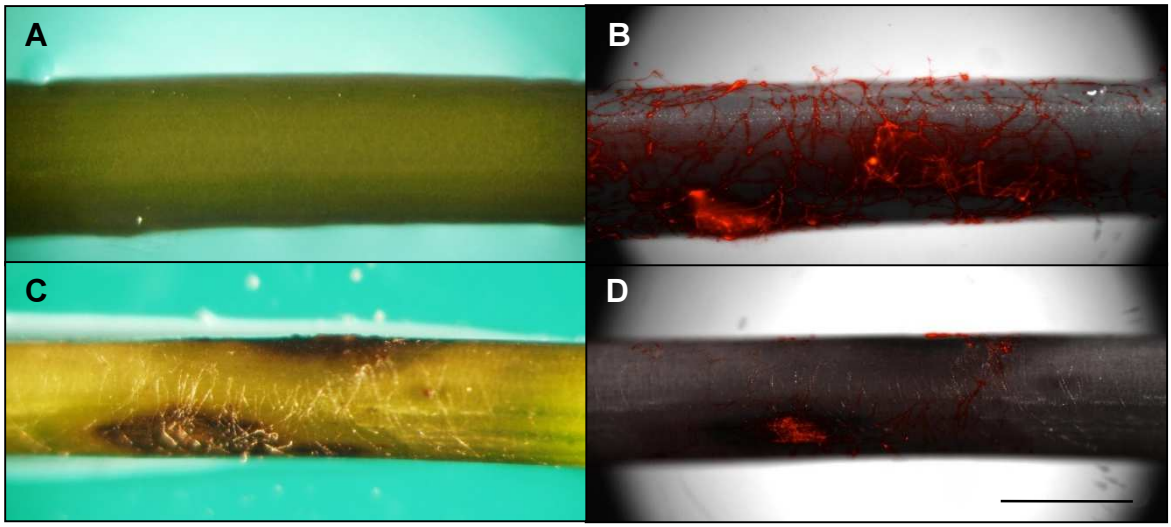


**Figure III-5** Stolons of bermudagrass cultivars A-B) Uganda, and C-D) Midlawn, 18 days post inoculation with *Ophiosphaerella korrae*. Bar represents 1 mm.

CHAPTER IV

# MECHANISMS CAUSING NECROSIS IN THE SPRING DEAD SPOT PATHOSYSTEM


## Abstract

Bermudagrass (*Cynodon dactylon, C. transvaalensis* and *C. dactylon x C. transvaalensis*) is a widely used sports turfgrass world-wide. One of the most damaging diseases of bermudagrass is spring dead spot (SDS), caused by *Ophiosphaerella herpotricha*, *O. korrae,* and *O. narmari.* These fungi are characterized as necrotrophic but the mechanism by which they render host cells necrotic remains unknown. Necrotrophs can produce toxins and are able to exploit the host hypersensitive response (HR) to colonize and infect host cells. The objectives of these studies were twofold: (1) to determine if toxins produced by SDS-causing fungi cause necrosis of plant cells, and (2) to investigate the formation of reactive oxygen species (ROS) during fungal infection of plant cells which is a common feature of the HR. For toxin detection via necrosis, concentrated extracts of culture filtrates of *O. herpotricha* and *O. korrae* grown in different types of media were tested on roots of creeping bentgrass and bermudagrass cultivars Tifway, Midlawn, and U3. Concentrated extracts of filtrates of *Ophiosphaerella herpotricha* grown on Fries medium resulted in root necrosis of all bermudagrasses

tested. For ROS detection, bermudagrass roots were inoculated with a tdTom expressing transformed isolate of *O. herpotricha* or *O. korrae* and stained with 2', 7'-dichlorofluorescein diacetate at four to seventy-four hours after inoculation. Roots were examined using bright field and fluorescence microscopy. Based on positive staining, root cells formed ROS in response to fungal infection. In *C. dactylon* cultivar U3, which is partially resistant to SDS, levels of ROS were significantly higher when compared to the interspecific hybrid cultivars Midlawn and Tifway. Formation of ROS was observed consistently at the hyphal tips of colonizing fungi in all cultivar/fungi combinations tested, with *O. herpotricha* being a significantly stronger ROS generator than *O. herpotricha*. These studies suggest that root necrosis caused by SDS fungi is independent from plant-generated ROS and toxin production by the fungi can be a non-specific factor in root necrosis. In addition, the increased production of ROS by SDS fungi at the time of root penetration underscores the role of these molecules during plant pathogen interactions.

**Introduction**

Spring dead spot (SDS) of bermudagrass (*Cynodon dactylon* (L) Pers*., C. dactylon* × *C. transvaalensis* Burtt-Davy) is caused by the fungi *Ophiosphaerella herpotricha*, *O. korrae,* or *O. narmari,* which infect roots, stolons, and rhizomes causing conspicuous dead patches, in some cases larger than 1 m in diameter following winter dormancy (27; 30; 31). Fungi that cause SDS are regarded as necrotrophs, a designation for pathogens that kill host cells usually through production of pathogenic toxins that kill host cells (19). Non-specific phytotoxic compounds have been previously isolated from *O. herpotricha* (29), but these were not tested on roots, the primary organ colonized by SDS-causing fungi.

Necrotizing capabilities of these fungi have been shown to be variable among cultivars (2; 8). In partially resistant bermudagrass varieties, the fungi typically colonize the vasculature of the roots without causing necrosis, but for susceptible varieties necrosis rapidly develops as the fungus colonizes cortical cells (2; 8). The differences observed in cell necrosis *in vitro* for cultivars that vary in disease severity *in vivo* may suggest that cell death in response to the presence of SDS-causing fungi is important in the pathosystem.

Fungal strategies for plant infection are diverse and often include a component of reactive oxygen species (ROS) that are major factors in plant pathogen interactions (10). In above-ground plant organs, such as leaves, the hypersensitive response (HR), initiated by the generation of ROS, can prevent pathogen colonization of resistant cultivars, but can also promote infection by necrotrophs (26). Furthermore, there is evidence showing that induction of host-cell death pathways is required for plant infection by necrotrophic fungi (4). The HR is not a well-studied phenomenon in below-ground plant organs (18). Light is often a requirement for HR development, nevertheless there are alternative HR pathways that do not require light nor ROS to progress (9; 16; 24). Fungal-generated ROS can be produced when the fungi interact with plant cells and this ROS production may be instrumental in pathogenicity (12; 25). Conversely, ROS also appear to be involved in maintaining the endosymbiotic relationship of *Epichloë festucae* and *Lolium perenne* (23). The objectives of this study were, i) to evaluate the production of host specific toxins by SDS-causing fungi, and ii) to analyze ROS production during the interaction between *Ophiosphaerella* species and bermudagrass cultivars that are susceptible or partially resistant to spring dead spot. The hypotheses of this study were that *Ophiosphaerella* spp.

are capable of producing a phytotoxin in culture and that there is an oxidative burst generated by susceptible cultivars in response to *Ophiosphaerella* spp. colonization.

## Materials and Methods

**Plant preparation** The bermudagrass cultivars selected for study included the interspecific hybrids (*C. dactylon* × *C. transvaalensis*) cv Tifway 419 and Midlawn, and a *C. dactylon* cultivar identified as U3. A non-host, cool-season creeping bentgrass (*Agrostis stolonifera* L.) cv SR1020 also was evaluated. Stolons of the turfgrasses were washed with distilled water and cut into 2 to 5 cm segments, each containing at least one node. Segments were surface sterilized with a 1.2% sodium hypochlorite, 38% ethanol solution for 2 to 4 minutes, rinsed with sterile, reverse osmosis (RO) water, placed on Petri plates containing potato dextrose agar (PDA), and incubated for up to 7 days at 25 °C in the dark to permit root growth and confirm cuttings were axenic.

**Culture filtrates and bioassay** Five, 5 mm diam. agar plugs containing mycelium of *O. korrae* isolate OW6 or *O. herpotricha* isolate A55 grown on PDA under artificial light for seven days at room temperature were placed into flasks containing either 50 or 500 ml of sterile toxin-inducing media. Media used in this experiment include Fries medium (for 1 L: 5 g of ammonium tartrate, 1.0 g of ammonium nitrate, 0.5 g of magnesium sulfate, 1.3 g of potassium phosphate [dibasic], 2.6 g of potassium phosphate [monobasic], 30.0 g of sucrose, 1.0 g of yeast extract) (15), *Alternaria alternata* toxin production medium (ATM for 1 L: 0.75 g of glycine, 0.1 g of sodium chloride, 1.31 g of potassium phosphate [dibasic] trihydrate, 0.5 g of magnesium sulfate heptahydrate, 0.13 g of calcium chloride dihydrate; 0.5 g of yeast extract, 0.67 g of L-malic acid; and 20.7 g of or glucose)

(17), and *Alternaria alternata* Asparagine L-malic acid medium (ALM for 1 L: 0.67 g of L-malic acid, 1.2 g of L-asparagine, 1 g of sodium chloride, 1 g of potassium phosphate [dibasic] trihydrate, 0.5 g of magnesium sulfate heptahydrate, 1.3 g of calcium chloride dihydrate, 0.5 g of yeast extract and 21.6 g of glucose) (3). Fries media with sucrose content reduced to either 17 % (5.1 g/L) or 50 % (15 g/L) was also tested. Glucose solutions at a 10 X concentrations were prepared and autoclaved separately. L-malic acid was added to autoclaved media using a stock (67 g/L) solution which was sterilized by filtration through a 0.2 μm syringe filter (Acrodisc, Pall corporation, Ann Arbor, MI) (3). Inoculated and non-inoculated control flasks were incubated at 21°C in the dark on an orbital shaker at 80 rpm for 2 days followed by stationary growth for 19 days. Crude culture filtrates were prepared by vacuum filtration through 90 mm diam. ashless filter paper (Whatman, GE Healthcare Life Sciences, Pittsburgh, PA).

Two techniques were used for concentrating culture filtrates. In the first, 50 ml culture filtrates were lyophilized. In the second, 500 ml filtrates were used for liquid-liquid extractions. To perform liquid-liquid extractions, the aqueous filtrates were reduced to a 100 ml volume in a rotary evaporator under reduced pressure (<1 kPa) at 45°C for 5 h at 200 rpm and either extracted twice using 300 ml of ethyl acetate each time or used for a sequential extraction with 300 ml hexane, 300 ml chloroform and 300 ml ethyl acetate. The aqueous layer from the extracts was discarded and the solvent layer was evaporated to dryness at 45°C and reduced pressure (<1 kPa) in a rotary evaporator. Lyophilized samples were resuspended in 2 ml sterile water while residues of extraction were resuspended in 2 ml ethyl acetate. All resuspended samples were filtered through sterile 0.8 μm syringe filters (Acrodisc, Pall Corporation, Ann Arbor, MI).

Resuspended samples were assayed on intact roots of all the grass by spotting them at one cm from the stolon with 10 µl of resuspended sample using a micropipette. For each assay, a minimum of three roots were treated. Extracted filtrates of non-inoculated media and ethyl acetate alone were used as negative controls. The treated roots were incubated at 17 ºC on a layer of moistened paper towel in sterile petri plates lined with aluminum foil, to prevent light exposure. Whole and sectioned roots were observed from 3 to 8 days after treatment with the extracts.

**Reactive oxygen species detection** Under sterile conditions, rooted stolon segments that were free of fungal contamination were transferred individually to Petri plates lined with moistened paper towel and aluminum foil as described previously. Roots were inoculated with a 1 mm diam. PDA plug from the margin of a 10- to14-day old culture of *O. korrae* isolate 26bt2 or *O. herpotricha* isolate B6, which were previously transformed to express the red fluorescent protein tdTOM (2; 8), and covered with a second layer of moistened paper towel and aluminum foil. In preliminary studies, the presence of ROS in the elongation zone and tips of non-infected control roots was observed and roots were not inoculated near these regions. Roots were inoculated at 1 cm from the node. One non-inoculated plant for every five inoculated replicates was used as control. Plates were sealed with parafilm and incubated upright in a growth chamber at 17°C and a 12 h photoperiod.

Roots were sampled and stained at either 4, 24, 48 or 72 hours post inoculation (hpi) with 25 µM 2', 7'-dichlorofluorescein diacetate (DCF-DA) (21) to reveal ROS. Roots were either hand-sectioned with a razor blade or left intact and were observed with a Nikon Eclipse E800 microscope (Nikon Inc., Tokyo, Japan) equipped with mercury-arc using the G-2E/C TRITC and GFP-3535B epifluorescence filter sets and bright field illumination.

Roots from six to nine plants were examined per treatment. A categorical rating scale was used to evaluate the presence and localization of root-produced ROS or fungus-produced ROS expressed as green fluorescence. Among root-produced ROS, 0 = no green fluorescence present in the root, 1 = weak green fluorescence present, 2 = strong green fluorescence present. For fungus-produced ROS, 0 = green fluorescence present in hyphal tips only, 1 = green fluorescence extensively present in the hyphae. *Ophiosphaerella herpotricha* and *O. korrae* growing on PDA for five days were also stained for ROS detection as described above and examined.

A total of 167 individual data points were recorded for each response variable. Statistical analysis was conducted with SAS (version 9.3, SAS Institute, Cary, NC). For root-produced ROS, PROC LOGISTIC under a proportional odds cumulative logit model for polytomous data was used. For fungus-produced ROS, a Poisson regression analysis was performed using PROC GENMOD. Goodness-of-fit tests were performed on the proposed models using Pearson chi-square criteria.

**Results**

**Phytotoxin production** Tifway 419 roots treated with lyophilized Fries and ALM filtrates from *O. herpotricha,* exhibited browning at the treatment site*;* a milder reaction was observed with lyophilized ATM filtrates from *O. korrae* on Tifway 419 intact roots (Table IV-1). Direct ethyl acetate liquid-liquid extraction of culture filtrates of *O. herpotricha* grown in Fries and ALM media resulted in 50 µl of an oily brown product from Fries medium and 20 mg of clear crystals from the ALM medium (Fig. IV-1). Fries medium extracts resulted in darker and more extensive discoloration of Tifway 419 roots

than ALM medium extracts. For extracts of *O. herpotricha* culture in Fries medium the discoloration of Tifway 419 and U3 roots at 3 days was less than that of Midlawn but discoloration increased with time (Fig. IV-2C, IV-2D, IV-2G, and IV-2H). Creeping bentgrass exhibited less discoloration which only occurred in the root tips (Fig. IV-2E). Collapse and browning of cells were present in Midlawn root cross sections and emerging lateral roots which exhibited the darkest discoloration response to extracts (Table IV-1; Fig. IV-3A). No necrosis was observed on roots treated with extracts of non-inoculated Fries medium (Fig. IV-2A and IV-3B). Extracts of *O. herpotricha* grown in Fries media with reduced sucrose produced no reaction on Midlawn roots (Fig. IV-2F). Evaluation of extracts obtained by sequential liquid-liquid extraction (hexane-chloroform-ethyl acetate) for *O. herpotricha* grown in Fries medium resulted in a minimal reaction on Midlawn roots for the ethyl acetate extract only (Table IV-1).

**Root-generated ROS** Staining of ROS in the roots was not consistent when DAB was used, therefore only DCF-DA staining data was used for the analyses. Intracellular root-generated ROS was observed during fungal colonization with significant differences occurring among cultivars and over time (hpi) ($P = 0.0006$ and 0.0184 respectively) but not between fungal isolates (Table IV-2). Midlawn and Tifway 419 roots generated less ROS, expressed as DCF green fluorescence, compared to U3, with the lowest amounts of fluorescence present in Tifway 419 roots (Table IV-3 and Fig. IV-4). Green fluorescence in colonized root cells was seldom observed in Tifway 419 at all evaluation time points (Fig. IV-4). Similar to Tifway 419, green fluorescence in Midlawn roots was rare and inconsistent (Fig. IV-4). U3 was the most likely to exhibit strong green fluorescence among the three cultivars evaluated (Table IV-3 and Fig. IV-4). Symptoms of necrosis

were rarely observed in roots of U3 and were more common on Tifway 419 and Midlawn by 72 hpi (results not shown, see chapter III).

The incidence of root cells exhibiting green fluorescence associated with fungal colonization was higher at 48 and 72 hpi than 24 hpi (Table IV-3). Root colonization by *O. herpotricha* was rare at 4 hpi for either Midlawn or U3 making the determination of ROS formation in response to the fungi difficult at this time period. However when observed, fungal colonization at 4 hpi resulted in a higher likelihood of green fluorescence of the root cells than cells at 24 hpi for all fungus-plant combinations tested (Table IV-3).

**Fungus-generated ROS** For all cultivars, DCF green fluorescence indicating ROS formation was significantly more prevalent in *O. herpotricha* hyphae compared with *O. korrae* (Table IV-2). In hyphae growing on solid media *O. herpotricha* formed a more widespread green fluorescence compared to *O. korrae* (Fig. IV-5) suggesting that this difference is intrinsic to the fungal isolates used. Intense green fluorescence was observed in hyphal tips that appeared to be penetrating roots for all cultivar and fungal combinations and it was occasionally observed in the rest of the mycelium of colonizing fungi. When using 72 hpi as reference, widespread green fluorescence was most likely to occur in fungal hyphae at 24 hpi for *O. korrae* and at 48 hpi for *O. herpotricha* but the differences were not significant (Table IV-4). Even though hyphae of *O. herpotricha* were rarely observed on roots by 4 hpi, inoculum placed next to the roots displayed green fluorescence in hyphae (Fig. IV-4). The likelihood of exhibiting extensive green fluorescence by either one of the two fungal isolates used was lower, but not significantly different, when infecting U3 compared to Midlawn and Tifway 419 (Table IV-4).

## Discussion

**Phytotoxin production** Prior to the identification of the causal agents of SDS it was observed that leachates obtained from diseased grass had a negative effect on the growth of healthy bermudagrass, possibly indicating the presence of a toxin in the soil (13). Fungal media used in previous studies in necrotrophic fungi to induce toxin production appear to have induced *Ophiosphaerella* to produce toxins that elicited symptoms of necrosis on bermudagrass roots. The lack of significant discoloration of creeping bentgrass roots suggests that the putative toxic compounds present in the extracts are host specific. However, no clear specificity was observed between susceptible and partially resistant bermudagrass cultivars. Different media influenced the production of toxic compounds by *O. herpotricha* and toxin production was favored by higher concentrations of sucrose. *Ophiosphaerella* spp. are capable of producing toxic compounds in growth media, and toxins may also be produced during infection of roots causing plant cell death. Emerging lateral roots exhibited significant necrosis that was similar to the necrosis observed as blackened circular structures previously (6; 8). Based on the results of this study, *Ophiosphaerella* spp. are capable of producing phytotoxic compounds. Previous studies on the isolation of metabolites from *O. herpotricha* culture filtrates resulted in the identification of seven different compounds (29). Of these seven metabolites, six caused necrosis when infiltrated in turfgrass leafs; however, no evaluation was conducted on roots (29). In this study, culture filtrate extracts caused root discoloration consistent with the necrosis produced during infection. But, further investigation is required to identify the specific compounds that are responsible for necrosis and to prove their direct relationship to plant cell death during fungal infection.

**Reactive oxygen species** Of the three cultivars tested in this study, Tifway 419 is the most susceptible to SDS in the field, followed by Midlawn and U3. The lack of ROS in the roots of Tifway 419 and Midlawn is congruent with other well studied pathosystems where disease interaction results in no ROS accumulation *in planta* during compatible pathogen colonization (5; 32). Tifway 419 and Midlawn show similarities in their interaction with *Ophiosphaerella* spp. showing root necrosis as early as 2 days post inoculation (dpi), while U3 is often not necrotic up to 14 dpi (2; 8). Cultivar U3 is partially resistant in the field and inoculated roots can remain non-necrotic despite being heavily colonized by *O. korrae* (8). The association of ROS generation by U3 root cells but not by Tifway 419 root cells during fungal colonization implies ROS is not involved in root cell necrosis. However a HR cannot be excluded as a cause of root cell death, although ROS production is a common occurrence during HR it is not a universal feature of this response (28). It is possible that in the SDS pathosystem, plant cell death pathways responsible for cell necrosis are independent from plant-generated ROS.

Even though the first report of ROS involvement in the HR was reported in potato tuber tissue infected by *Phytophthora infestans* (5), it appears that light is an important requirement for ROS-related HR in above ground tissues. Alternative HR pathways can take place in the absence of light (9; 16; 24) and may be more likely to occur in the root system. When testing compatible and incompatible interactions between *Arabidopsis* and *Hyaloperonospora parasitica*, on leaves and roots Hermanns et al. (11) found that the oxidative burst followed by cell death was only present when infection occurred in leaves and not in roots. There seems to be marked differences in the way HR

functions in above-ground compared to below-ground organs and ROS accumulation during the initial stages of infection may be a hallmark only in above-ground infection.

Reports of plant-generated ROS during below-ground plant-microbe interactions are generally associated with symbiotic relationships (10) where ROS arises intracellularly while HR-related ROS is generated in the apoplast (7). Production of ROS has previously been observed in roots of plants interacting with *Rhizobium* (22) or arbuscular mycorrhizae (7; 20). The greatest intracellular ROS signal detected in this study was for the cultivar U3, which is tolerant to SDS. Even though a sustained oxidative burst is characteristic of above-ground incompatible interactions resulting in HR, fungal colonization of U3 roots does not seem to be hindered by high ROS production but rather is promoted by it, allowing vasculature colonization. Therefore, ROS generation during this interaction appears to be analogous to early stages of below-ground symbioses.

Production of ROS by *Ophiosphaerella* hyphae coincides with the time at which fungal penetration occurred in the spring dead spot pathosystem (2; 8). It has been shown that the role of ROS generated by fungi can be critical during plant-pathogen interactions, but it appears to be diverse across pathosystems (10). In other systems, ROS is important for establishment and preservation of symbiotic relationships (1; 23), as a regulator of development (14), or produced to oxidize the plant surface (25). The presence of intracellular ROS in non-infecting hyphal tips indicates that they may be involved in saprotrophic fungal growth, while increased ROS generation during penetration suggests a more direct involvement of these molecules during plant colonization.

**Conclusions** Based in the findings reported in this study, root necrosis in susceptible cultivars resulting from the infection by SDS-causing fungi is likely to be caused by a combination of non-specific toxins produced by the fungi and ROS-independent cell death mechanisms of the plant.

**Acknowledgements**

Published with the approval of the Director, Oklahoma Agricultural Experiment Station, Stillwater, OK. This project was supported in part by the United States Golf Association and Oklahoma Agricultural Experiment Station project 2833. The authors would like to acknowledge Mauricio Moreno at KU Leuven in Belgium and Andrea Payne at Oklahoma State University for assistance in statistical analyses.

**Literature Cited**

1. Baptista, P., Martins, A., Pais, M., Tavares, R., and Lino-Neto, T. 2007. Involvement of reactive oxygen species during early stages of ectomycorrhiza establishment between *Castanea sativa* and *Pisolithus tinctorius*. Mycorrhiza 17:185-193.
2. Caasi, O. C., Walker, N. R., Marek, S. M., Enis, J. N., and Mitchell, T. K. 2010. Infection and colonization of turf-type bermudagrass by *Ophiosphaerella herpotricha* expressing green or red fluorescent proteins. Phytopathology 100:415-423.
3. Clouse, S. D., Martensen, A. N., and Gilchrist, D. G. 1985. Rapid purification of host-specific pathotoxins from alternaria alternata f. sp. lycopersici by solid-phase adsorption on octadecylsilane. Journal of Chromatography A 350:255-263.

4. Dickman, M. B., Park, Y. K., Oltersdorf, T., Li, W., Clemente, T., and French, R. 2001. Abrogation of disease development in plants expressing animal antiapoptotic genes. Proceedings of the National Academy of Sciences 98:6957-6962.

5. Doke, N. 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiological Plant Pathology 23:345-357.

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

7. Fester, T., and Hause, G. 2005. Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. Mycorrhiza 15:373-379.

8. Flores, F. J., Marek, S. M., Anderson, J. A., Mitchell, T. K., and Walker, N. R. 2014. Infection and colonization of several bermudagrasses species by *Ophiosphaerella korrae* (submitted).

9. Guo, A., Reimers, P. J., and Leach, J. E. 1993. Effect of light on incompatible interactions between *Xanthomonas oryzae* pv *oryzae* and rice. Physiological and Molecular Plant Pathology 42:413-425.

10. Heller, J., and Tudzynski, P. 2011. Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. Annual Review of Phytopathology 49:369-390.

11. Hermanns, M., Slusarenko, A. J., and Schlaich, N. L. 2003. Organ-Specificity in a Plant Disease Is Determined Independently of R Gene Signaling. Molecular Plant-Microbe Interactions 16:752-759.

12. Hyon, G.-S., Ikeda, K.-i., Hosogi, N., Shinogi, T., and Park, P. 2010. Inhibitory effects of antioxidant reagent in reactive oxygen species generation and penetration of appressoria of *Alternaria alternata* japanese pear pathotype. Phytopathology 100:840-847.

13. Kozelnicky, G. M. 1974. Updating 20 years of research: spring dead spot. USGA Green Section Record 12:12-15.

14. Lara-Ortíz, T., Riveros-Rosas, H., and Aguirre, J. 2003. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in *Aspergillus nidulans*. Molecular Microbiology 50:1241-1255.

15. Liu, Z. H., Faris, J. D., Meinhardt, S. W., Ali, S., Rasmussen, J. B., and Friesen, T. L. 2004. Genetic and Physical Mapping of a Gene Conditioning Sensitivity in Wheat to a Partially Purified Host-Selective Toxin Produced by Stagonospora nodorum. Phytopathology 94:1056-1060.

16. Montillet, J. L., Chamnongpol, S., Rusterucci, C., Dat, J., van de Cotte, B., Agnel, J. P., Battesti, C., Inze, D., Van Breusegem, F., and Triantaphylides, C. 2005. Fatty acid hydroperoxides and $H_2O_2$ in the execution of hypersensitive cell death in tobacco leaves. Plant Physiology 138:1516-26.

17. Morisseau, C., Ward, B. L., Gilchrist, D. G., and Hammock, B. D. 1999. Multiple epoxide hydrolases in *Alternaria alternata* f. sp. *lycopersici* and their relationship

to medium composition and host-specific toxin production. Applied and Environmental Microbiology 65:2388-95.

18. Okubara, P., and Paulitz, T. 2005. Root defense responses to fungal pathogens: A molecular perspective. Pages 215-226. in: Root Physiology: from Gene to Function H. Lambers, and T. Colmer, eds. Springer Netherlands.

19. Prell, H. H., and Day, P. R. 2001. Plant-Fungal Pathogen Interaction: A Classical and Molecular View. Springer Verlag, Berlin.

20. Salzer, P., Corbière, H., and Boller, T. 1999. Hydrogen peroxide accumulation in Medicago truncatula roots colonized by the arbuscular mycorrhiza-forming fungus Glomus intraradices. Planta 208:319-325.

21. Sandalio, L. M., Rodríguez-Serrano, M., Romero-Puertas, M. C., and del Río, L. A. 2008. Imaging of reactive oxygen species and nitric oxide in vivo in plant tissues. Pages 397-409. in: Methods in Enzymology C. Enrique, and P. Lester, eds. Academic Press.

22. Santos, R., Hérouart, D., Sigaud, S., Touati, D., and Puppo, A. 2001. Oxidative burst in alfalfa-*sinorhizobium meliloti* symbiotic interaction. Molecular Plant-Microbe Interactions 14:86-89.

23. Tanaka, A., Christensen, M. J., Takemoto, D., Park, P., and Scott, B. 2006. Reactive oxygen species play a role in regulating a fungus–perennial ryegrass mutualistic interaction. The Plant Cell Online 18:1052-1066.

24. Tang, X., Xie, M., Kim, Y. J., Zhou, J., Klessig, D. F., and Martin, G. B. 1999. Overexpression of pto activates defense responses and confers broad resistance. The Plant Cell Online 11:15-29.

25. Tenberge, K. B., Beckedorf, M., Hoppe, B., Schouten, A., Solf, M., and von den Driesch, M. 2002. In situ localization of AOS in host-pathogen interactions. Microscopy and Microanalysis 8:250-251.

26. Tiedemann, A. V. 1997. Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. Physiological and Molecular Plant Pathology 50:151-166.

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

28. Torres, M. A. 2010. ROS in biotic interactions. Physiologia Plantarum 138:414-429.

29. Venkatasubbaiah, P., Tisserat, N. A., and Chilton, W. S. 1994. Metabolites of *Ophiosphaerella herpotricha*, a cause of spring dead spot of bermudagrass. Mycopathologia 128:155-159.

30. Wadsworth, D. F., and Young, H. C. 1960. Spring dead spot of bermudagrass. Plant Disease Reporter 44:516-518.

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

32. Wang, C.-F., Huang, L.-L., Buchenauer, H., Han, Q.-M., Zhang, H.-C., and Kang, Z.-S. 2007. Histochemical studies on the accumulation of reactive oxygen species ($O_2$ and $H_2O_2$) in the incompatible and compatible interaction of whea *Puccinia striiformis* f. sp. *tritici*. Physiological and Molecular Plant Pathology 71:230-239.

**TABLES:**

**Table IV-6** Bermudagrass root reaction to fungal culture filtrates of *Ophiosphaerella* spp. grown in Fries, ALM or ATM media.

| Media | Fungus | Concentration method | Organic Solvent | Cultivar | Discoloration |
|---|---|---|---|---|---|
| Fries | *O. herpotricha* | Lyophilization[y] | - | Tifway | + |
| Fries | *O. korrae* | Lyophilization | - | Tifway | - |
| ALM[w] | *O. herpotricha* | Lyophilization | - | Tifway | + |
| ALM | *O. korrae* | Lyophilization | - | Tifway | - |
| ATM[x] | *O. herpotricha* | Lyophilization | - | Tifway | - |
| ATM | *O. korrae* | Lyophilization | - | Tifway | + |
| Fries | *O. herpotricha* | Liquid extraction[z] | Ethyl acetate | Tifway | + |
| Fries | *O. herpotricha* | Liquid extraction | Ethyl acetate | Midlawn | ++ |
| Fries | *O. herpotricha* | Liquid extraction | Ethyl acetate | U3 | + |
| Fries | *O. herpotricha* | Liquid extraction | Ethyl acetate | Bentgrass | - |
| ALM | *O. herpotricha* | Liquid extraction | Ethyl acetate | Midlawn | - |
| Fries 50 % | *O. herpotricha* | Liquid extraction | Ethyl acetate | Midlawn | - |
| Fries 17 % | *O. herpotricha* | Liquid extraction | Ethyl acetate | Midlawn | - |
| Fries | *O. herpotricha* | Sequential extraction | Ethyl acetate | Midlawn | +/- |
| Fries | *O. herpotricha* | Sequential extraction | Hexane | Midlawn | - |
| Fries | *O. herpotricha* | Sequential extraction | Chloroform | Midlawn | - |

[w] *Alternaria alternata* Asparagine L-malic acid medium.

[x] *Alternaria alternata* toxin production medium.

[y] 50 ml of culture filtrate was lyophilized and later resuspended on 2 ml of water

[z] 500 ml of culture filtrate was reduced on a rotary evaporator and later the organic phase was extracted using ethyl acetate 1:3 twice. The ethyl acetate fraction was evaporated to dryness and the residue resuspended on 2 ml ethyl acetate.

[*] Represents the strongest reaction observed.

**Table IV-7**. Likelihood ratio statistics for effects of cultivar, hours post inoculation (HPI), and fungus on the formation of reactive oxygen species (ROS), expressed as green fluorescence, produced in roots of bermudagrass [z] and in *Ophiosphaerella* spp. hyphae in response to fungal colonization [y]

| Source | df | Chi-square | Pr > ChiSq |
|---|---|---|---|
| Root-ROS | | | |
| Cultivar | 2 | 14.73 | 0.0006 |
| HPI | 3 | 10.02 | 0.0184 |
| Fungus | 1 | 0.21 | 0.6463 |
| Fungal-ROS | | | |
| Cultivar | 2 | 2.50 | 0.2870 |
| HPI | 3 | 5.24 | 0.1547 |
| Fungus | 1 | 25.05 | <.0001 |

[z] Rated visually based in a scale of 0 to 2 as follows: 0 = no green fluorescence present in the root, 1 = weak green fluorescence present, 2 = strong green fluorescence present.
[y] Rated visually as follows: 0 = green fluorescence present in hyphal tips only, 1 = green fluorescence extensively present in the hyphae

**Table IV-8** Odds ratio estimates, Wald confidence limits at 95%, and *P* values for comparisons of cultivars[u], hours post infection (hpi)[v], and fungus[w] on the formation of reactive oxygen species produced in roots of bermudagrass in response to *Ophiosphaerella* spp. colonization[x].

| Comparison | Odds ratio Estimate[y] | Wald 95% Confidence Limits | | Pr > ChiSq |
|---|---|---|---|---|
| Cultivar | | | | |
| Midlawn vs. U3 | 0.387 | 0.195 | 0.768 *[z] | 0.0067 |
| Tifway vs. U3 | 0.239 | 0.111 | 0.512 * | 0.0002 |
| HPI | | | | |
| 4 vs. 24 | 1.449 | 0.558 | 3.765 | 0.4463 |
| 48 vs. 24 | 2.308 | 1.046 | 5.093 * | 0.0383 |
| 72 vs. 24 | 3.454 | 1.542 | 7.736 * | 0.0026 |
| Fungus | | | | |
| *O. herpotricha* vs. *O. korrae* | 1.148 | 0.638 | 2.065 | 0.6463 |

[u] Coded with U3 as reference.

[v] Coded with 24 hpi as reference.

[w] Coded with *O. korrae* as reference.

[x] Rated visually based in a scale of 0 to 2 as follows: 0 = no green fluorescence present in the root, 1 = weak green fluorescence present, 2 = strong green fluorescence present.

[y] Odds ratio: 1 is equivalent to no difference in the comparison; values >1 indicate increased probability of generating ROS when compared to the reference; values <1 indicate decreased probability of generating ROS when compared to the reference

[z] "*" significant difference ($P \geq 0.005$) when 1 is not within the confidence interval.

**Table IV-9** Odds ratio estimates, Wald confidence limits at 95%, and *P* values for comparisons of cultivars[w] and hours post infection (hpi)[x] on the formation of reactive oxygen species produced in hyphae of *Ophiosphaerella herpotricha* and *O. korrae* colonizing bermudagrass roots[y].

| Comparison | Odds ratio Estimate[z] | Wald 95% Confidence Limits | | Pr > ChiSq |
|---|---|---|---|---|
| *O. herpotricha* | | | | |
| Cultivar | | | | |
| Midlawn vs. U3 | 1.119 | 0.779 | 1.608 | 0.5422 |
| Tifway vs. U3 | 1.158 | 0.791 | 1.697 | 0.4506 |
| HPI | | | | |
| 4 vs. 72 | 0.441 | 0.126 | 1.539 | 0.1992 |
| 24 vs. 72 | 0.605 | 0.278 | 1.314 | 0.204 |
| 48 vs. 72 | 1.443 | 0.777 | 2.682 | 0.2455 |
| *O. korrae* | | | | |
| Cultivar | | | | |
| Midlawn vs. U3 | 1.448 | 0.767 | 2.733 | 0.2535 |
| Tifway vs. U3 | 1.745 | 0.894 | 3.405 | 0.1027 |
| HPI | | | | |
| 4 vs. 72 | 1.361 | 2.334 | 7.934 | 0.7319 |
| 24 vs. 72 | 3.643 | 0.865 | 15.342 | 0.0780 |
| 48 vs. 72 | 3.000 | 0.714 | 12.614 | 0.1338 |

[w] Coded with U3 as reference.

[x] Coded with 72 hpi as reference.

[y] Rated visually based as follows: 0 = ROS present only on hyphal tips, 1 = extensive ROS formation.

[z] Odds ratio: 1 is equivalent to no difference in the comparison; values >1 indicate increased probability of generating ROS when compared to the reference; values <1 indicate decreased probability of generating ROS when compared to the reference

**FIGURES:**

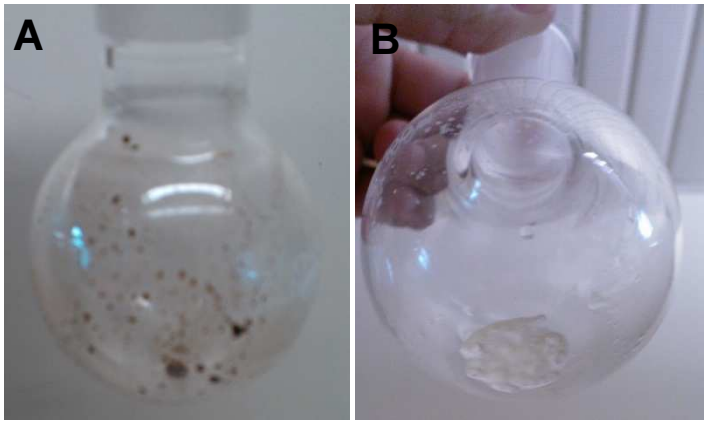


**Figure IV-1** Products of a liquid-liquid extraction from filtrates of *Ophiosphaerella herpotricha* grown on A) Fries medium and B) *Alternaria alternata* asparagine L-malic acid medium

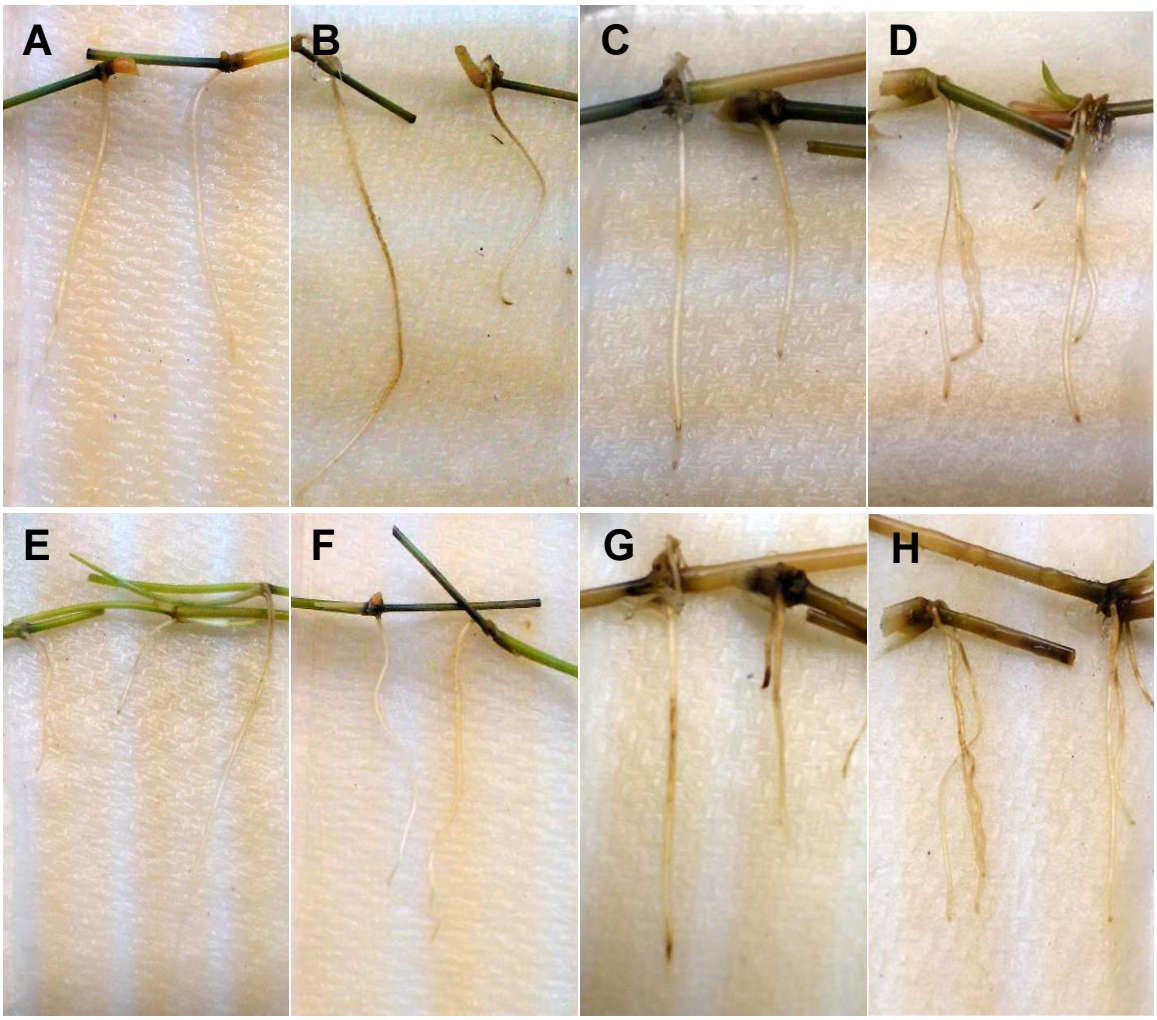


**Figure IV-2** Reaction of turfgrass roots three days after treatment with ethyl acetate extracts of A) non-inoculated Fries medium on Midlawn. B-E) *O. herpotricha* inoculated Fries medium on B) Midlawn, C) Tifway 419, D) U3, and E) creeping bentgrass. F) *O. herpotricha* inoculated Fries 50% sucrose medium on Midlawn. G) Tifway, and H) U3 fourteen days after treatment with extracts of *O. herpotricha* inoculated Fries medium.

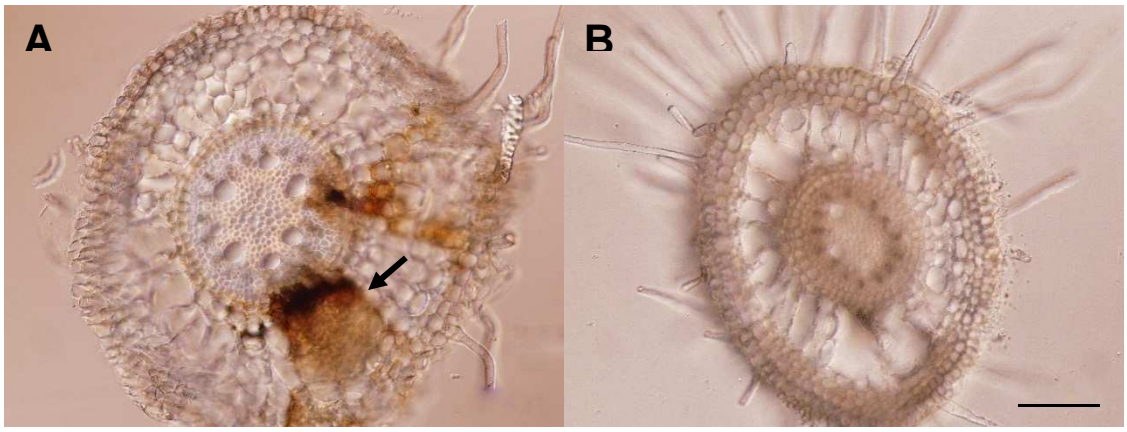


**Figure IV-3** Midlawn roots 5 days after treatment with A) extract of *O. herpotricha* grown on Fries medium culture filtrate, and B) extract of non-inoculated Fries medium. Arrow indicates necrotic emerging lateral root. Bar represents 100 µm

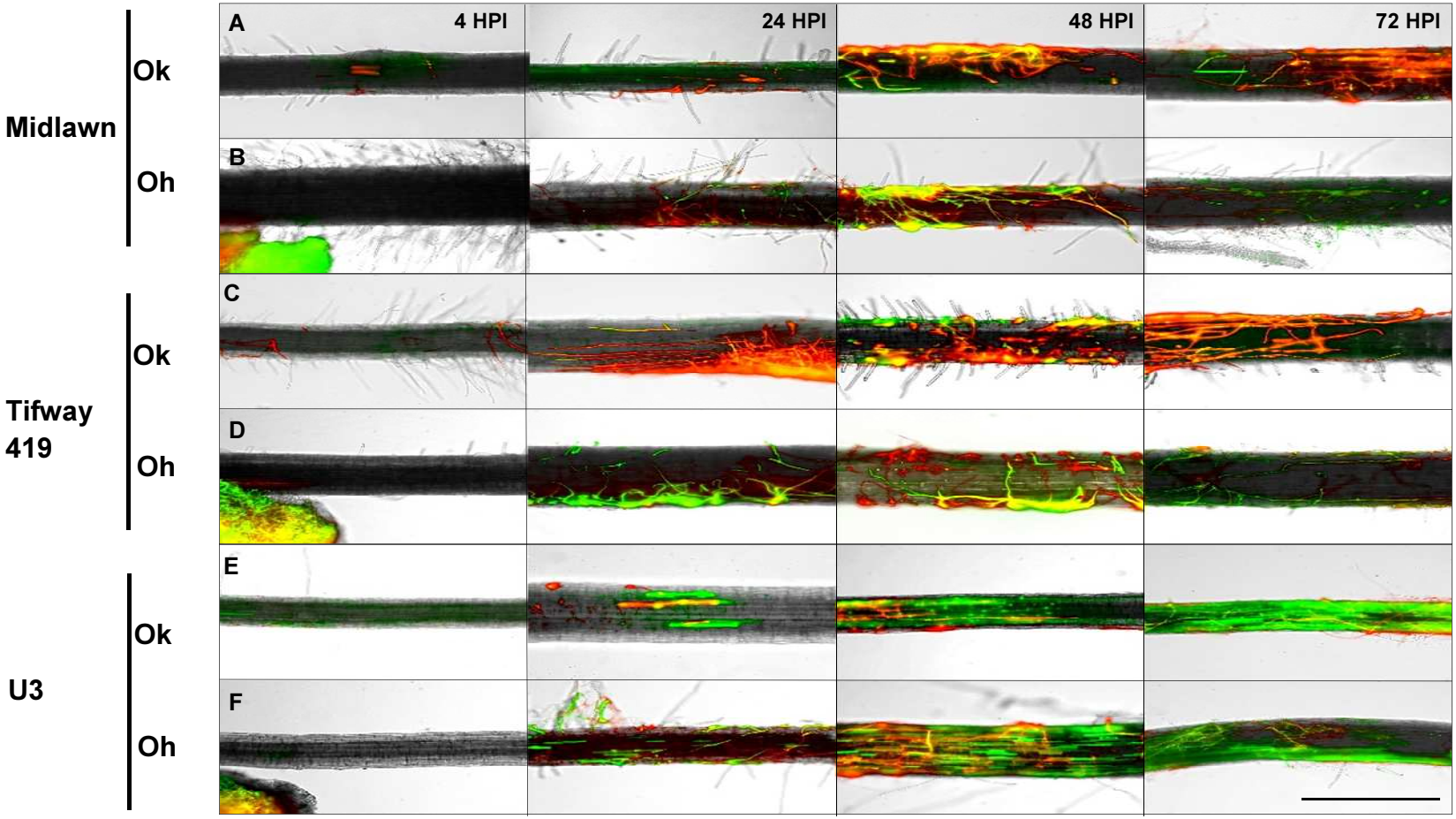


**Figure IV-4** Bermudagrass roots inoculated with *Ophiosphaerella* spp. fungi after 2', 7'-dichlorofluorescein diacetate staining to reveal the formation of reactive oxygen species (green) 4, 24, 48, and 72 hours post inoculation. Interactions pictured: A) Midlawn with *O. korrae* (Ok), B) Midlawn with *O. herpotricha* (Oh), C) Tifway 419 with *O. korrae*, D) Tifway 419 with *O. herpotricha*, E) U3 with *O. korrae*, F) U3 with *O. herpotricha*. Bar represents 1 mm.

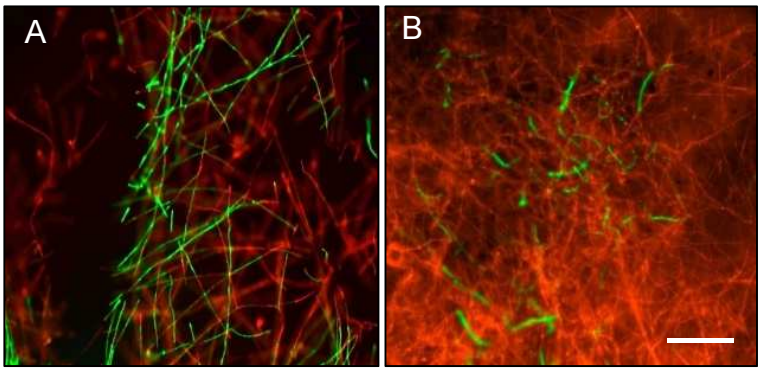


**Figure IV-5** Fluorescent images of cultures of tdTom expressing *Ophiosphaerella herpotricha* isolate B6 (A) and *O. korrae* isolate 26bt2 (B) stained with 2', 7'-dichlorofluorescein diacetate. Green fluorescence indicates reactive oxygen present. Bar represents 50 µm.

# CHAPTER V

## ALTERNATIVE HOSTS OF SPRING DEAD SPOT-CAUSING FUNGI

**Abstract**

Three fungi in the genus *Ophiosphaerella*, *O. herpotricha*, *O. korrae* and *O. narmari*, are the causal agents of spring dead spot of bermudagrass. These fungi can cause disease in grasses other than bermudagrass including zoysiagrass and buffalograss. However, the extent at which *Ophiosphaerella* spp. can colonize and infect other alternative hosts is unknown. To determine the potential host range of *Ophiosphaerella* spp., several warm-season and cool-season grasses and two dicots were evaluated for colonization by *O. korrae* and *O. herpotricha*. Plants were grown from seed under greenhouse conditions. Roots were washed with water, inoculated with tdTom expressing transformants of either *O. herpotricha* or *O. korrae*, and incubated at 17˚C. Intact and transverse sectioned roots were examined using epifluorescence microscopy 4-14 days after inoculation. Most plant species reacted similarly to either species of fungi. In creeping bentgrass, blue panicgrass, and wheat roots the fungi were able to colonize the root vasculature without causing necrosis. In zoysiagrass and buffalograss, the fungi occasionally produced symptoms of necrosis in the root cortex, while vasculature colonization was very rare. Vascular colonization and necrosis was once observed in

Kentucky bluegrass and proso millet. Big bluestem and broadleaf panicum exhibited cortical colonization only, without vascular colonization or necrosis. In tall fescue, the root surface was sparsely colonized and cortical colonization was rare. Tomato and alfalfa were non-hosts, with minimum root surface colonization. *Ophiosphaerella* spp. has a broad monocotyledonous host range showing diverse plant-fungus interactions, in which necrosis is not common at up to 14 days post infection.

**Introduction**

Spring dead spot (SDS) of common bermudagrass (*Cynodon dactylon* (L) Pers.), (*C. dactylon* × *C. transvaalensis* Burtt-Davy) is caused by the ectotrophic, root-infecting fungi *Ophiosphaerella herpotricha* (Fr.) Walker, *O. korrae* (J. Walker and A. M. Smith) R. A. Shoemaker and C. E. Babcock, or *O. narmari* (J. Walker and A. M. Smith) Wetzel, Hubert and Tisserat (2; 4; 14; 15; 17; 21; 22; 24), which tend to colonize the surface of below-ground organs prior to infection. When SDS is present, bermudagrass turf exhibits well defined, circular, dead patches visible in spring as the grass resumes growth after dormancy (21). The fungi infect bermudagrass roots directly while stolons are likely colonized through wounds (1; 15). In susceptible cultivars, the fungi colonizes the root cortex where it can cause extensive necrosis within days (1; 6) In contrast, on roots of partially resistant cultivars SDS-causing fungi seldom produce necrosis even though vascular colonization is often observed (1; 6). While *O. korrae* and *O. narmari* will produce pseudothecia that can be frequently found on infected leaf sheaths and stolons in Australia, in the United States the fruiting structures are not commonly observed (9; 22). Inoculum dispersal is likely through movement of infested root, crown, or stem tissues and soils during establishment or maintenance of the stand (10).

Spring dead spot-causing fungi are a group of closely related species each with a distinctive geographical distribution. All three *Ophiosphaerella* species causing SDS have been found in United States, while in Australia only *O. narmari* and *O. korrae* are present. *Ophiosphaerella korrae* has also been reported in Italy (8; 24). The presence of SDS was also confirmed in New Zealand and Argentina but the specific species causing the disease were not identified (9; 12). A study of *Ophiosphaerella* spp. in North Carolina by Tredway *et al.* (20) concluded that the distribution of these fungi is not random, with certain species typically dominating specific regions.

The first bermudagrass varieties that were reported to be susceptible to SDS were the *C. dactylon* cultivars 'U-3' and 'common', African (*C. transvaalensis*) cultivars, and hybrid cultivars 'Tiffine' and 'Tifgreen'. Fungi that cause SDS can colonize several hosts other than bermudagrasses, and cause disease under conducive conditions. These include zoysiagrass (*Zoysia japonica* Steud.) (7; 19) and buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.) (16). *Ophiosphaerella korrae* is also the causal agent of necrotic ring spot of cool-season grasses including Kentucky bluegrass (*Poa pratensis* L.) (25), creeping red fescue (*Festuca rubra* L. subsp. *rubra* Smith) (3), and annual bluegrass (*Poa annua* L.) (11). Upon identification of *O. korrae* as the cause of SDS of bermudagrass, Smith (15) reported also isolating it from diseased blanket grass (*Axonopus compressus* Beauv.), St. Augustine grass (*Stenotaphrum secundatum* Kuntze), and kikuyu grass (*Pennisetum clandestinum* Hochst). Inoculation with *O. korrae* and *O. narmari* of wheat, oats, barley and rice have resulted in infections (18; 22).

The host range of SDS-causing *Ophiosphaerella* spp. is reported to be wide but the extent of root colonization by the fungi for plant species other that bermudagrass is

not known. Hence, the objectives of this study were to 1) test various plant species, including warm-season grasses, cool-season grasses, and dicotyledonous plants for their reaction to *O. korrae* and *O. herpotricha*, and 2) characterize the colonization process of these two fungal species on roots of the plants evaluated.

## Materials and Methods

The plant species selected for this study included the cool-season grass species including creeping bentgrass (*Agrostis stolonifera* L.) 'SR1020', tall fescue (*Festuca arundinacea* Schreb.) 'Rebel IV', hard red winter wheat (*Triticum aestivum* L.) 'PBW-343', and Kentucky bluegrass (*Poa pratensis* L.) 'Touchdown'; and the warm-season grasses buffalograss (*Buchloe dactyloides*) 'Cody', zoysiagrass (*Zoysia japonica* (L.) Merr.) 'Meyer', broadleaf panicum (*Panicum deustum* Thunb.), proso millet (*Panicum miliaceum* L.), blue panicgrass (*Panicum coloratum* L.), and big bluestem (*Andropogon gerardii* Vitman.); and the dicots alfalfa (*Medicago sativa* L.) 'Vernal' and tomato (*Solanum lycopersicum* L.) 'Glamour'. Seeds of all plants were planted in a commercial potting mix (Miracle-Gro potting mix, Scotts, Marysville, OH) and grown for one to three months under greenhouse conditions or in a growth chamber at 27ºC and a 12h photoperiod. Single plants were selected and roots cleaned with deionized water until no soil debris were visible. Clean plants were transferred individually to sterile petri plates (100 x 15 mm, VWR, Sugar Land, TX) lined with a double layer of wet white paper towel and placed on a microscope slide (75 x 25 x 1 mm, Fisher Scientific Inc., Pittsburgh, PA). The paper towel was saturated with sterile water. A single root from

each plant was inoculated with a potato dextrose agar plug (approximately 0.25 mm in diameter) containing mycelium from the edge of a 10 to 14-day old culture of the tdTom expressing transformants, *O. herpotricha* isolate B6 (1) or *O. korrae* isolate 26bt2 (6) One non-inoculated control root was included for every three inoculated root replicates. Aluminum foil was placed over the paper towel-wrapped roots to prevent root exposure to light. Plates were sealed with parafilm and incubated upright (roots-down) in a growth chamber at 17°C with 12 h light cycle. A minimum of three infected roots were examined per plant species and the experiment was repeated twice.

Roots adjacent to the points of inoculation were observed with epifluorescence microscopy and stereomicroscopy at 4 and 14 days post-inoculation (DPI). Surface colonization was observed directly by mounting excised roots on a slide. Internal colonization observed using transverse sections made by hand-sectioning the plant organs with a razor blade prior to mounting in water. Whole and sectioned roots were mounted in deionized water and observed with a Nikon Eclipse E800 microscope (Nikon Inc., Tokyo, Japan) equipped with mercury-arc lamp using the UV-2E/C DAPI and G-2E/C TRITC epifluorescence filter sets and bright field observations. Digital images were captured using a monochrome QImaging Retiga 2000R charge-coupled device (CCD) camera (Quantitative Imaging Corp., Surrey, BC, Canada). Monochromatic grayscale images taken with each epifluorescent filter were pseudo colored appropriately and combined as layers over a bright field image of the same field using QCapture Pro version 5.1.1.14 (Quantitative Imaging Corp., Surrey, BC, Canada). Color images corresponding to the composites were captured using a Nikon SMZ-2T stereomicroscope

(Nikon Inc., Tokyo, Japan) equipped with an Olympus DP71 digital camera (Olympus Imaging America Inc., Center Valley, PA).

**Results**

For most plant species, there were no differences observed between *O. herpotricha* and *O. korrae* for infection and colonization of plant organs (Table V-1). In blue panicgrass and wheat, *O. herpotricha* and *O. korrae* colonized the root surface and then the root cortex by 4 DPI with vascular colonization common by 14 DPI, both without symptoms of necrosis (Figs. V-1 and V-2). In creeping bentgrass, there was a difference in colonization between the two fungi. *Ophiosphaerella korrae* colonized bentgrass roots at a more rapid rate than *O. herpotricha.* By 4 DPI, extensive colonization of the root surface and cortex was observed for *O. korrae* (Fig. V-3A), while for *O. herpotricha,* root surface colonization was minimal (Fig. V-3B). By 14 DPI, *O. korrae* had penetrated and colonized the stele in creeping bentgrass roots and spread broadly through the vasculature (Fig. V-3C). In contrast to *O. herpotricha* had only just begun to colonize the vasculature at 14 DPI (Fig. V-3E). Neither fungi caused necrosis in creeping bentgrass roots at 14 DPI (Fig V-3D and V-3F).

The cortex of zoysiagrass and buffalograss roots were colonized by *O. korrae* and *O. herpotricha* by 4 DPI (Figs. V-4A-D). No symptoms of necrosis were observed in zoysiagrass colonized by *O. korrae* at 14 DPI (Fig. V-4E and V-4F), while necrotic epidermal and cortical cells were observed occasionally for plants colonized by *O. herpotricha* as early as 4 DPI (Fig. V-4G and V-4H). In buffalograss roots, necrotic cortical cells near the stele were observed at 14 DPI when colonized by *O. korrae* (Fig. V-4I and V-4J), while no necrotic symptoms were produced by cortical colonization of

*O. herpotricha* (Fig. V-4K and V-4L). No vascular colonization was observed at 14 DPI in buffalograss roots (Fig. V-4I and V-4L), while zoysiagrass showed vascular colonization in a single root, which was completely colonized by *O. herpotricha* (Fig. V-5).

Colonization of proso millet by *Ophiosphaerella* spp. was slower compared to Kentucky bluegrass (Fig V-6A-D), but the reaction by plants after 14 days were similar. Even though most proso millet and Kentucky bluegrass roots displayed vascular colonization by *O. korrae*, all were asymptomatic by 14 DPI (Figs. V-6E-F and V-6I-J). However, symptoms of necrosis were observed, once in each of these two plants species, when infected by *O. herpotricha* (Fig. V-6G-H and V-6K-L).

On big bluestem and broadleaf panicum, colonization of the root surface by both *Ophiosphaerella* spp. was extensive by 4 DPI (Fig. V-7A-D), but appeared to slow and stop by 14 DPI. Limited cortical colonization was observed by 14 DPI and the hyphae did not penetrate into the vasculature (Fig. V-7E, V-7G, V-7I, and V-7K). No symptoms of necrosis related to fungal colonization were observed on either big bluestem or broadleaf panicum (Fig. V-7F, V-7H, V-7I, and V-7L).

Minimal surface colonization by both *Ophiosphaerella* spp. was observed on tall fescue by 4 DPI (data not shown) and hyphae did not spread on the root surface or penetrated into the cortex by 14 DPI (Fig. V-8A and B). In alfalfa, both fungi exhibited limited colonization of the surface of primary and secondary roots; however, both were able to penetrate thin tertiary roots by 14 DPI (Fig V-8D). By 14 DPI, hyphae were not present where surface colonization was previously observed but it persisted on senescing

roots (Fig V-8C). In tomato, the fungi were not found beyond the epidermis of any roots examined and hyphae were only sparsely present near the point of inoculation (Fig V-8E and F). No symptoms of necrosis related to fungal colonization were observed in either of the two dicotyledonous plants examined.

Both *O. herpotricha* and *O. korrae* had identical host ranges and few differences were detected between the two fungi. *Ophiosphaerella korrae* colonized creeping bentgrass roots more rapidly than *O. herpotricha*. In contrast, *O. herpotricha* induced occasional symptoms of necrosis in Kentucky bluegrass and proso millet while *O. korrae* did not.

**Discussion**

The identic host range of *O. herpotricha* and *O. korrae* may be attributed to their close relatedness (5). Furthermore, these two species have been shown to be similar when infecting bermudagrass cultivars (1; 6).

*Ophiosphaerella korrae* is reported to cause necrotic ringspot of Kentucky bluegrass (26), but in this study it did not cause symptoms of necrosis at 14 DPI. This is likely due to several factors including the cool temperature used during the incubation period since necrotic ring spot is associated with warm soil temperatures.

Root infecting necrotrophic fungi generally have a wide host ranges (13) and SDS-causing fungi are not an exception. Although the three *Ophiosphaerella* spp. are known for causing a devastating disease of bermudagrass, many monocotyledonous plants appear to be colonized without symptoms of disease. With the exception of tall fescue, all turf-type grasses evaluated were readily colonized by *O. herpotricha* and *O.*

*korrae*. A correspondence between vascular colonization and tolerance to colonization by *Ophiosphaerella* spp. (i.e. the absence of necrosis) has been previously observed for bermudagrasses (1; 6). In this study, when vascular colonization occurred plants typically did not exhibit symptoms of necrosis at 14 DPI. This suggests that *Ophiosphaerella* spp. may have asymptomatic relationships with certain plant species where the fungi can survive in the root system as it was observed in plant species native to the Unites States Great Plains (23). Most plants were asymptomatic at 14 DPI, and these associations may represent commensalism or latent infections.

Since spring dead spot has been reported on zoysiagrass and buffalograss, it is likely that the fungi colonized these turf-type grasses and upon environmentally or maintenance-induced stresses, the fungi caused symptoms of the disease (7; 16; 19). Even though there was colonization of the turf-type grasses evaluated in this study, symptoms of necrosis were minimal when compared to susceptible bermudagrass cultivars which generally exhibit necrosis quickly, often within 4 DPI (1; 6). Annual grass hosts may serve as a reservoir of inoculum and a possible additional means for dispersal of the fungi. Dicotyledonous plants are not likely hosts for SDS-causing fungi and survival of these fungi on decaying roots is probably an artifact of experimental design that exclude normal soil microflora.

**Acknowledgements**

Published with the approval of the Director, Oklahoma Agricultural Experiment Station, Stillwater, OK. This project was supported in part by the United States Golf Association and Oklahoma Agricultural Experiment Station project 2833.

**Literature cited**


1. Caasi, O. C., Walker, N. R., Marek, S. M., Enis, J. N., and Mitchell, T. K. 2010. Infection and colonization of turf-type bermudagrass by *Ophiosphaerella herpotricha* expressing green or red fluorescent proteins. Phytopathology 100:415-423.
2. Dale, J. L., and Diaz, C. 1963. A new disease of bermudagrass turf. Arkansas Farm Research 12:6.
3. Dernoeden, P. H., Zhang, M., and Wetzel, H. C. 1995. First report of necrotic ring spot (*Leptosphaeria korrae*) in creeping red fescue in Maryland. Plant Disease 79:966.
4. Endo, R. M., Ohr, H. D., and Krausman, E. M. 1985. *Leptosphaeria korrae*, a cause of the spring dead spot disease of bermudagrass in California. Plant Disease 69:235-237.
5. Flores, F., Marek, S., and Walker, N. 2014. Multilocus phylogeny of *Ophiosphaerella* species causing spring dead spot of bermudagrass. (submitted).
6. Flores, F. J., Marek, S. M., Anderson, J. A., Mitchell, T. K., and Walker, N. R. 2014. Infection and colonization of several bermudagrasses species by *Ophiosphaerella korrae* (submitted).
7. Green, D. E. 1993. Pathogenicity of *Rhizoctonia solani* AG-2-2 and *Ophiosphaerella herpotricha* on zoysiagrass. Plant Disease 77:1040-1044.
8. Gullino, M. L., Mocioni, M., and Titone, P. 2007. First report of *Ophiosphaerella korrae* causing spring dead spot of bermudagrass in Italy. Plant Disease 91:1200.

9. Jackson, N. 1993. Geographic distribution, host range, and symptomatology of patch diseases caused by ectotrophic fungi. Pages 161. in: Turfgrass patch diseases caused by ectotrophic root-infecting fungi B. B. Clarke, and A. B. Gould, eds. APS Press, Saint Paul, MN.

10. Landschoot, P. J. 1993. Ecology and epidemiology of ectotrophic root-infecting fungi associated with patch diseases of turfgrasses. Pages 161. in: Turfgrass patch diseases caused by ectotrophic root-infecting fungi B. B. Clarke, and A. B. Gould, eds. APS Press, Saint Paul, MN.

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

12. Luc, J. E., Canegallo, A., and Martin, B. 2005. Spring dead spot in bermudagrass greens in Argentina and South Carolina. Golf Course Management 73:92-95.

13. Okubara, P., and Paulitz, T. 2005. Root defense responses to fungal pathogens: A molecular perspective. Pages 215-226. in: Root Physiology: from Gene to Function H. Lambers, and T. Colmer, eds. Springer Netherlands.

14. Smiley, R. W., Dernoeden, P. H., and Clarke, B. B. 2005. Compendium of Turfgrass Diseases. 3ed ed. The American Phytopatological Society, St. Paul, MN.

15. Smith, A. M. 1965. *Ophiobolus herpotrichus* a cause of spring dead spot in couch turf. Agricultural Gazette of New South Wales 76:753-758.

16. Tisserat, N., Wetzel, H., Fry, J., and Martin, D. L. 1999. Spring dead spot of buffalograss caused by *Ophiosphaerella herpotricha* in Kansas and Oklahoma. Plant Disease 83:199.

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

18. Titone, P., Mocioni, M., Landschoot, P. J., and Gullino, M. L. 2004. Survey of ectotrophic root infecting fungi associated with turfgrass patch diseases in Italy. Acta Horticulturae 661:491-498.

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

20. Tredway, L. P., Butler, E. L., Soika, M. D., and Bunting, M. L. 2008. Etiology and management of spring dead spot of hybrid bermudagrass in North Carolina, USA. Acta Horticulturae 783:535-546.

21. Wadsworth, D. F., and Young, H. C. 1960. Spring dead spot of bermudagrass. Plant Disease Reporter 44:516-518.

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

23. Walker, N. R. 2007. Root colonization of several species of native grasses by *Ophiosphaerella herpotricha*. Phytopathology 97:S119.

24. Wetzel, H. C., Skinner, D. Z., and 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.

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

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

**TABLE:**

**Table V-10** Summary of colonization (C) and necrosis (N) occurring in root tissues 14 days after *Ophiosphaerella* spp. inoculation of different plant species

| Host | Fungi | Epidermis | | Cortex | | Vasculature | |
|---|---|---|---|---|---|---|---|
| | | C | N | C | N | C | N |
| Creeping | *O. herpotricha* | + | - | + | - | + | - |
| bentgrass | *O. korrae* | ++ | - | ++ | - | ++ | - |
| Wheat | *O. herpotricha* | + | - | + | - | ++ | - |
| | *O. korrae* | + | - | + | - | ++ | - |
| Kentucky | *O. herpotricha* | ++ | - | + | - | + | +/- |
| bluegrass | *O. korrae* | ++ | - | + | - | + | - |
| Tall fescue | *O. herpotricha* | + | - | - | - | - | - |
| | *O. korrae* | + | - | - | - | - | - |
| Buffalograss | *O. herpotricha* | + | - | + | - | - | - |
| | *O. korrae* | + | - | + | +/- | - | - |
| Zoysiagrass | *O. herpotricha* | + | +/- | + | +/- | - | - |
| | *O. korrae* | + | - | + | - | - | - |
| Proso millet | *O. herpotricha* | + | - | + | - | + | +/- |
| | *O. korrae* | + | - | + | - | + | - |
| Broadleaf | *O. herpotricha* | ++ | - | + | - | - | - |
| panicum | *O. korrae* | ++ | - | + | - | - | - |
| Big bluestem | *O. herpotricha* | + | - | + | - | - | - |
| | *O. korrae* | ++ | - | + | - | - | - |
| Blue | *O. herpotricha* | + | - | + | - | ++ | - |
| Panicgrass | *O. korrae* | + | - | + | - | ++ | - |
| Alfalfa | *O. herpotricha* | + | - | - | - | - | - |
| | *O. korrae* | + | - | - | - | - | - |
| Tomato | *O. herpotricha* | + | - | - | - | - | - |
| | *O. korrae* | + | - | - | - | - | - |

**FIGURES:**

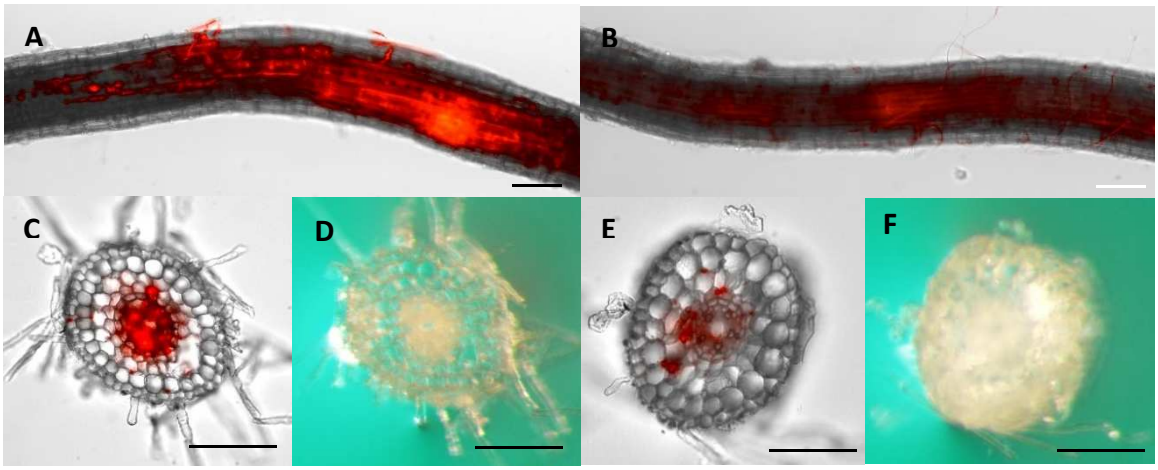


**Figure V-11** Colonization of blue panicgrass at 4 days post inoculation (DPI) by *Ophiosphaerella korrae* (A) and *O. herpotricha* (B), and at 14 DPI by *O. korrae* (C, D) and *O. herpotricha* (E, F). Bars represent 200 µm.

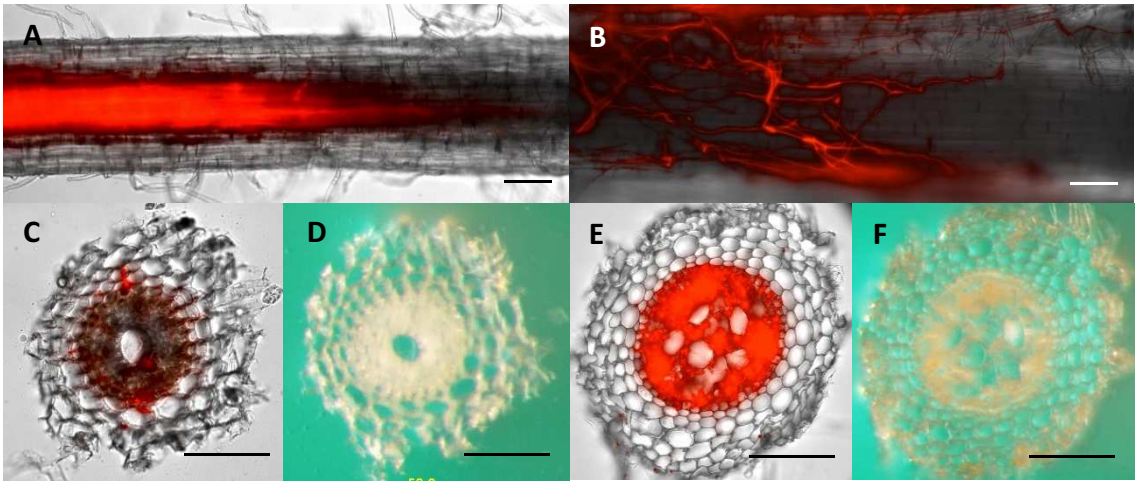


**Figure V-12** Colonization of wheat at 4 days post inoculation (DPI) by *Ophiosphaerella korrae* (A) and *O. herpotricha* (B), and at 14 DPI by *O. korrae* (C, D) and *O. herpotricha* (E, F). Bars represent 200 µm.

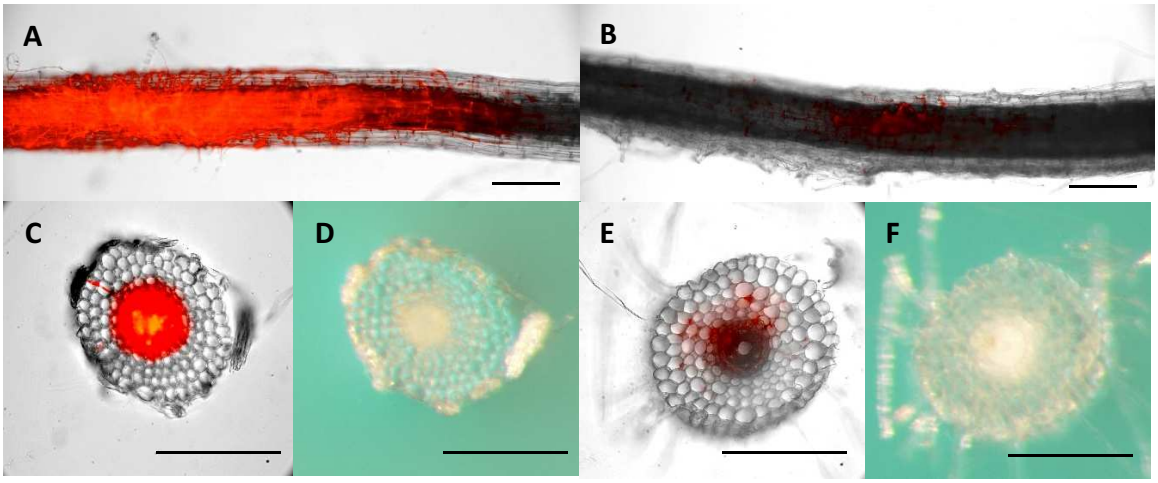


**Figure V-13** Colonization of creeping bentgrass at 4 days post inoculation (DPI) by *Ophiosphaerella korrae* (A) and *O. herpotricha* (B), and at 14 DPI by *O. korrae* (C, D) and *O. herpotricha* (E, F). Bars represent 200 µm.

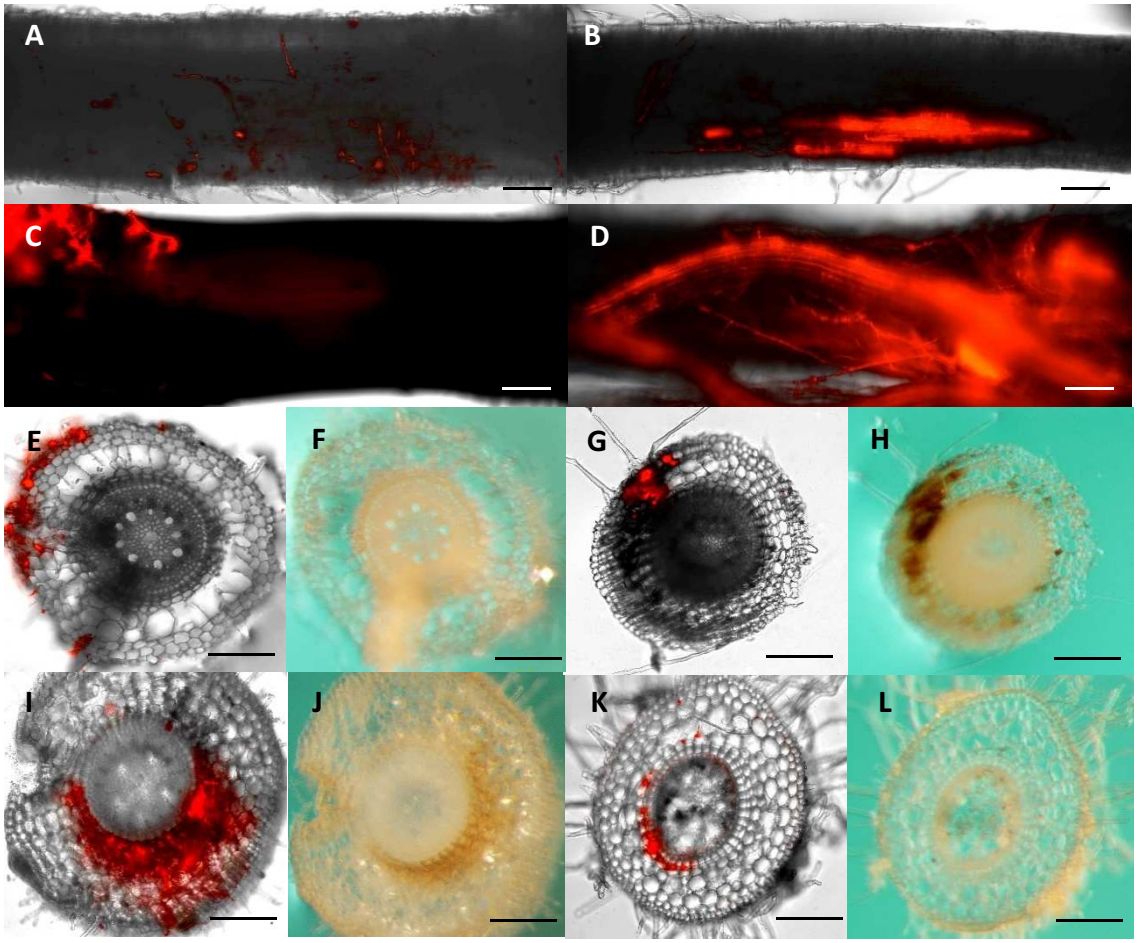


**Figure V-14** Colonization of zoysiagrass by *Ophiosphaerella korrae* (A) and *O. herpotricha* (B), and of buffalograss by *O. korrae* (C) and *O. herpotricha* at 4 days post inoculation (DPI) (D). Colonization of zoysiagrass by *O. korrae* (E, F) and *O. herpotricha* (G, H), and of buffalograss by *O. korrae* (I, J) and *O. herpotricha* at 14 DPI (K, L). Bars represent 200 µm.

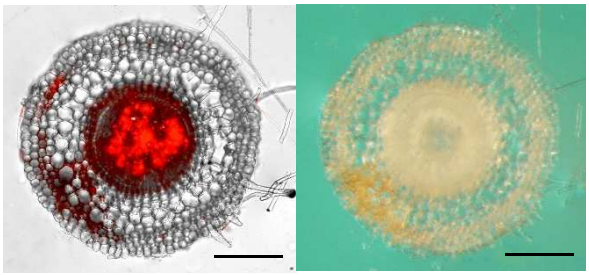

**Figure V-15** Ophiosphaerella herpotricha colonizing zoysiagrass at 14 days post inoculation. Bars represent 200 µm.

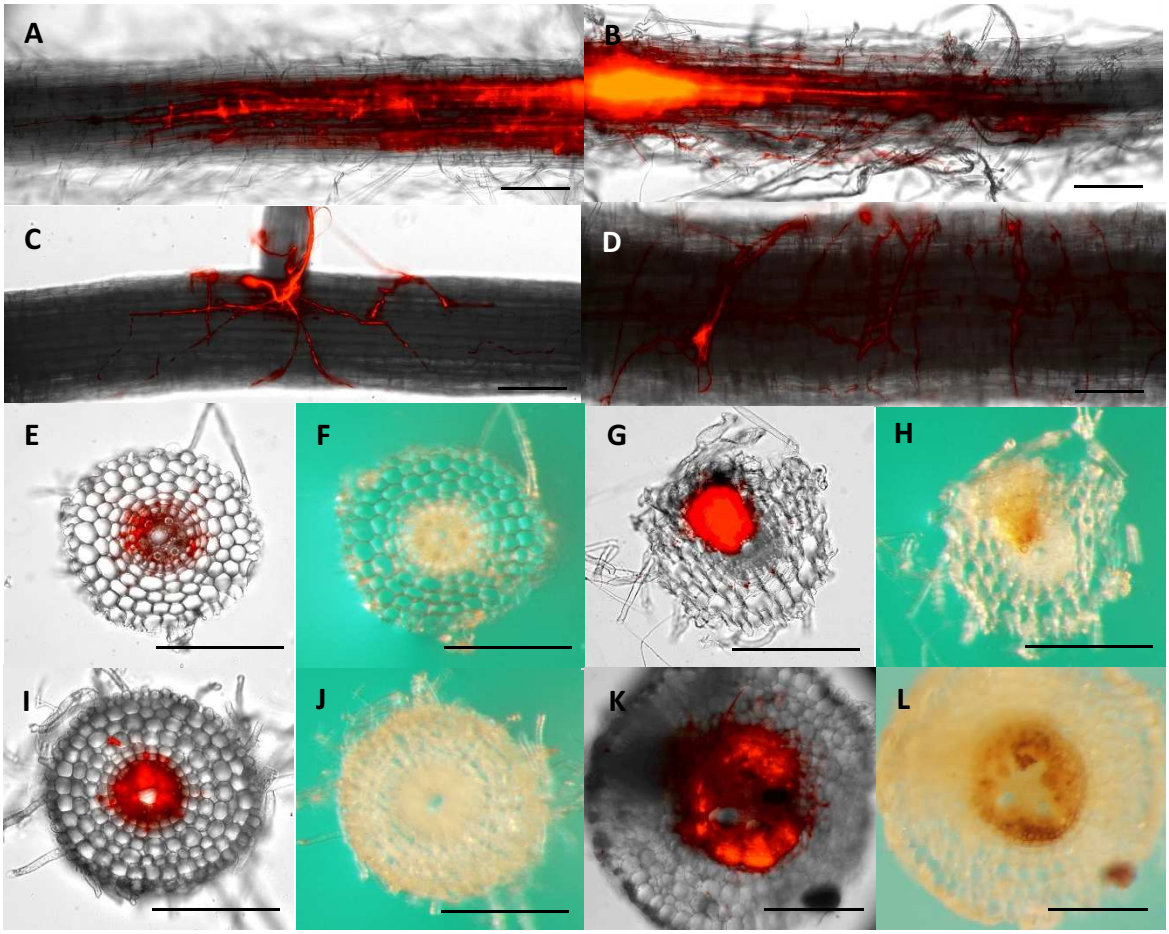


**Figure V-16** Colonization of Kentucky bluegrass by *Ophiosphaerella korrae* (A) and *O. herpotricha* (B), and of proso millet by *O. korrae* (C) and *O. herpotricha* at 4 days post inoculation (DPI) (D). Colonization of Kentucky bluegrass by *O. korrae* (E, F) and *O. herpotricha* (G, H), and proso millet by *O. korrae* (I, J) and *O. herpotricha* at 14 DPI (K, L). Bars represent 200 µm.

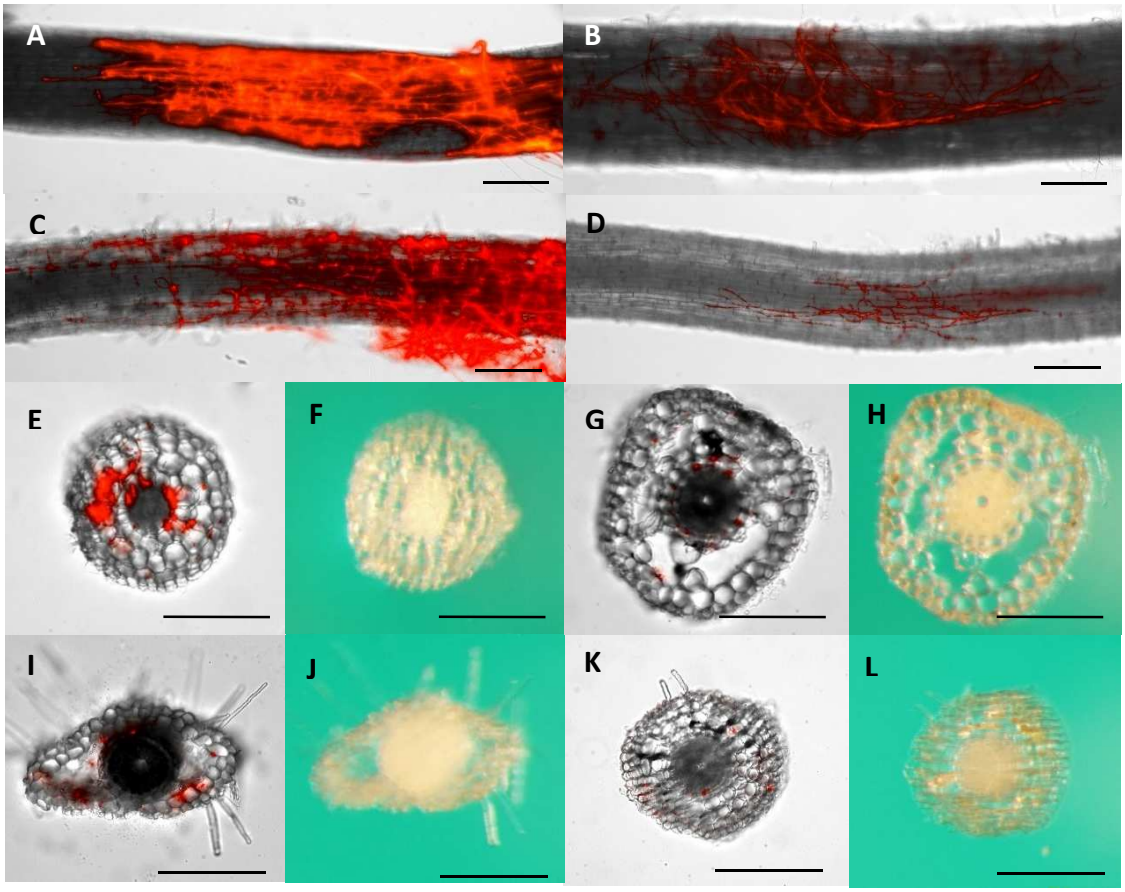


**Figure V-17** Colonization of broadleaf panicum by *Ophiosphaerella korrae* (A) and *O. herpotricha* (B), and of big bluestem by *O. korrae* (C) and *O. herpotricha* at 4 days post inoculation (DPI) (D). Colonization of broadleaf panicum by *O. korrae* (E, F) and *O. herpotricha* (G, H), and of big bluestem by *O. korrae* (I, J) and *O. herpotricha* at 14 DPI (K, L). Bars represent 200 µm.

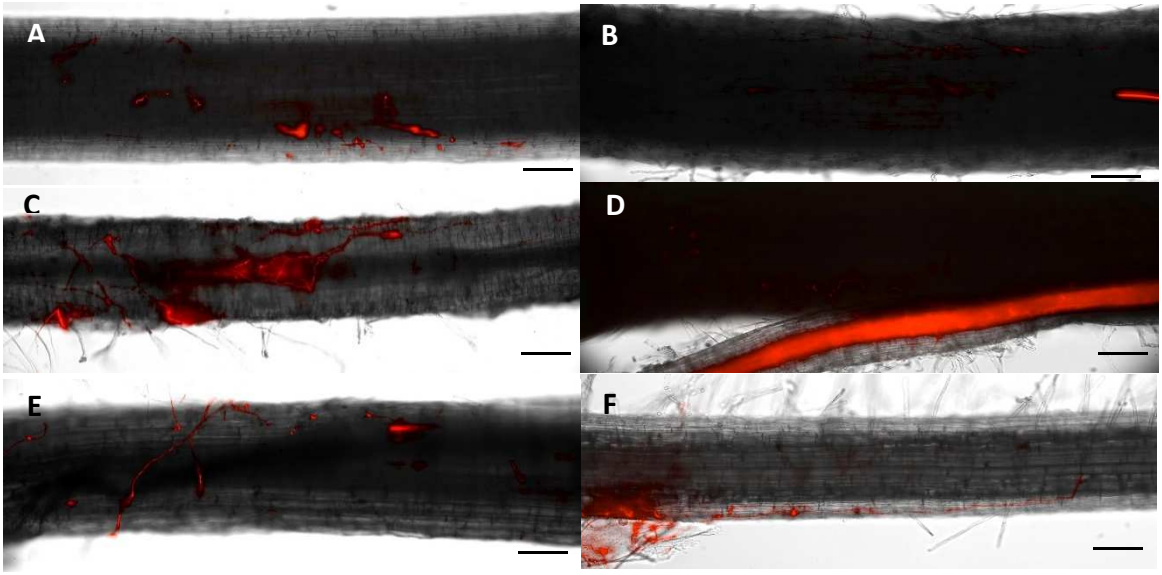


**Figure V-18** Colonization at 14 days post inoculation of tall fescue by A) *Ophiosphaerella korrae* and B) *O. herpotricha*; of alfalfa by C) *O. korrae* and D) *O. herpotricha*; and of tomato by E) *O. korrae* and F) *O. herpotricha*. Bars represent 200 µm.

APPENDICES

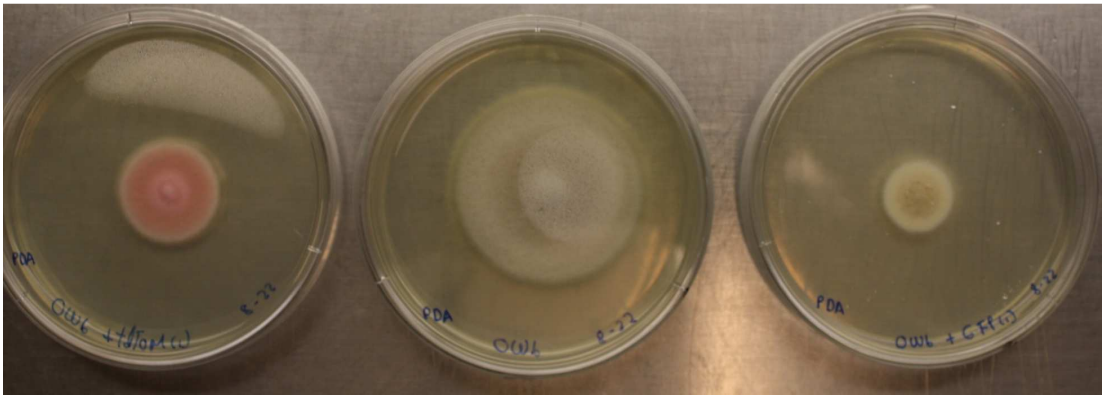


**Figure A-1** *Ophiosphaerella korrae* transformed to express the red fluorescent protein tdTOM (left), and green fluorescent protein GFP (right) along with the wild type (middle) growing on potato dextrose agar for 14 days. Even though the wild type growth in media is faster than the growth of the transformants no significant differences were found on the severity of the disease caused by these fungi *in planta*.

.

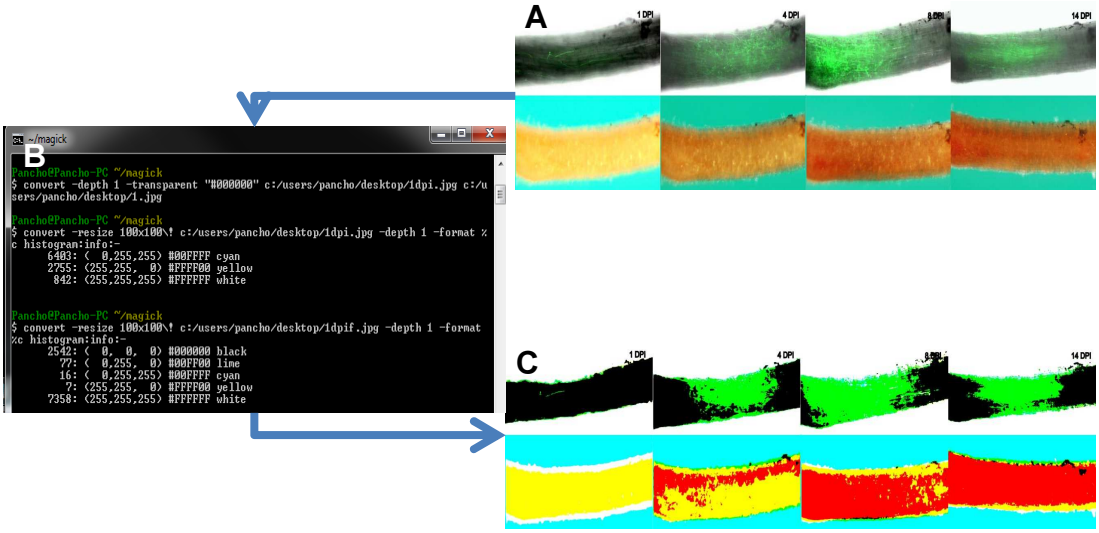


**Figure A-2** Image processing for the quantification of fungal colonization and disease severity. A) Fluorescence and bright field microscopies of bermudagrass roots infected by a green fluorescent *O. korrae* isolate. B) Commands used on Imagemagick to transform pictures into 8 color images and to quantify pixels with different colors. C) Resulting eight-color images.

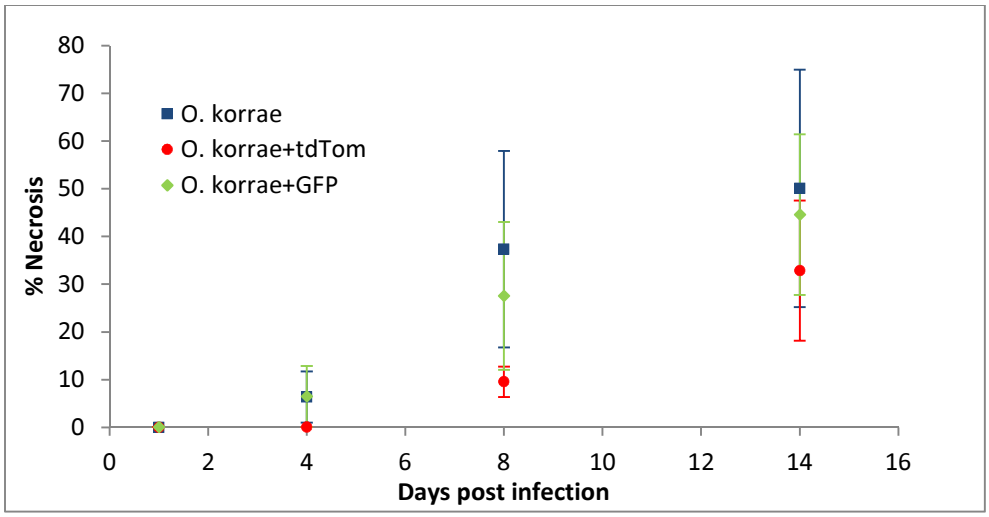


**Figure A-3** Necrosis produced by *O. korrae* wild type, and GFP and TdTom transformants on Midlawn roots. Four roots were infected with each isolate and tracked through 14 days. Percentage of necrosis was measured on digital images using Imagemagick.

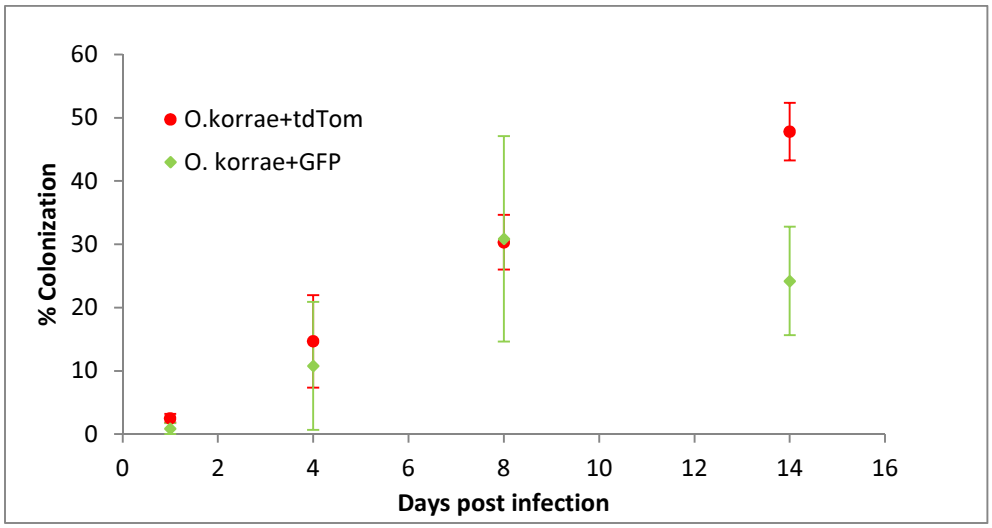


**Figure A-4** Colonization of *O. korrae* GFP and TdTom transformants on Midlawn roots. Four roots were infected with each isolate and tracked through 14 days. Percentage of colonization was measured on digital images using Imagemagick.

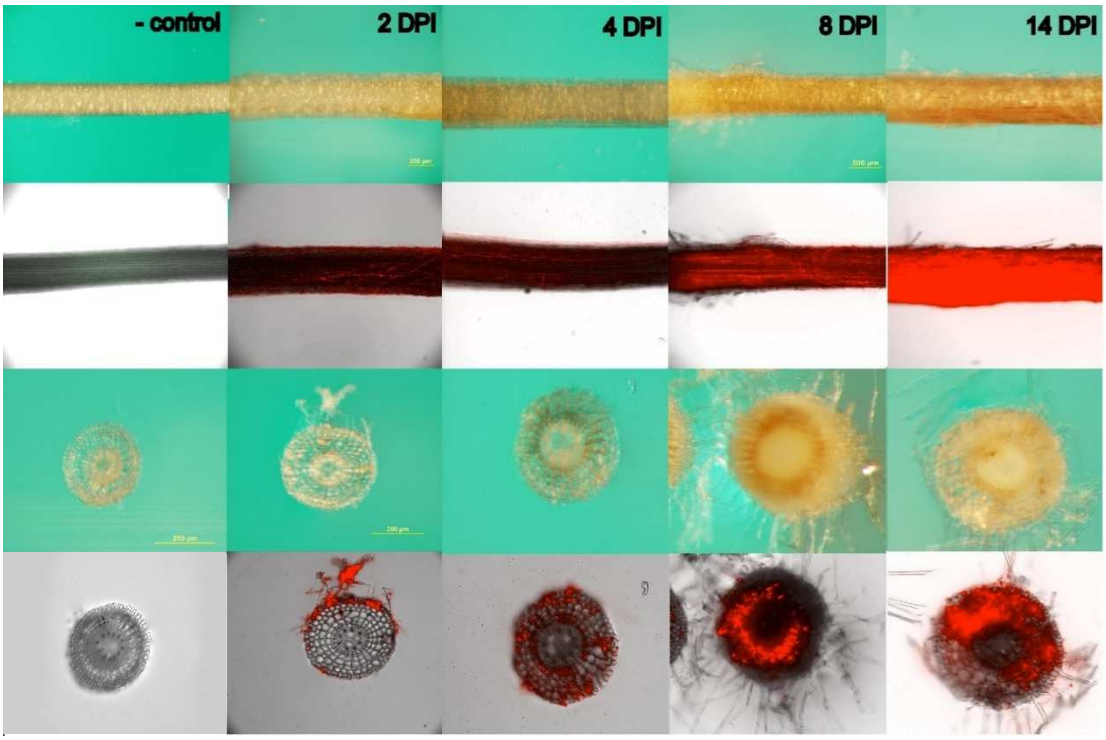


**Figure A-5** Colonization and infection of roots of hybrid bermudagrass cultivar Midlawn by *Ophiosphaerella korrae* expressing tdTom from 2 to 14 days post inoculation (dpi).

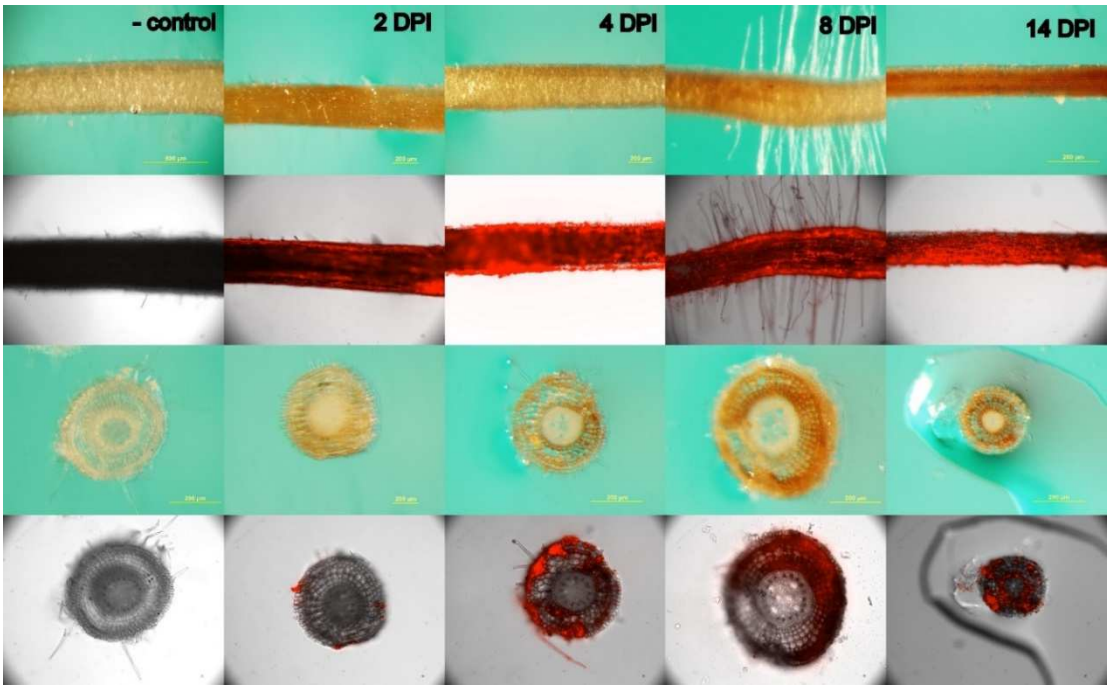


**Figure A-6** Colonization and infection of roots of hybrid bermudagrass cultivar Tifway 419 by *Ophiosphaerella korrae* expressing tdTom from 2 to 14 days post inoculation (dpi).

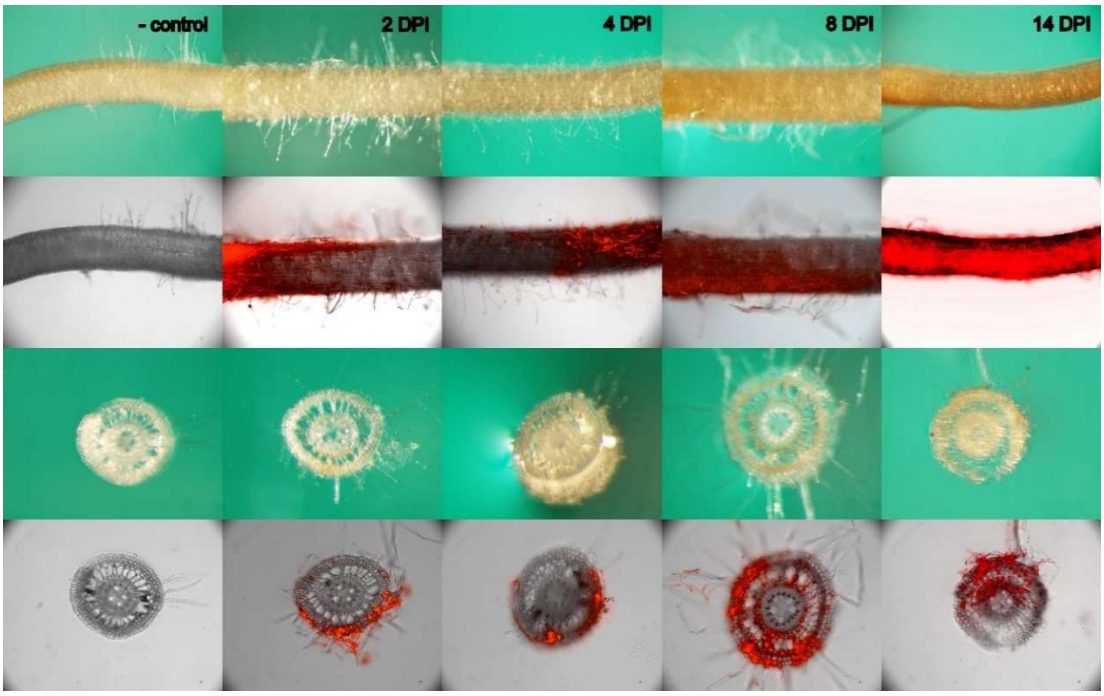


**Figure A-7** Colonization and infection of roots of *Cynodon transvaalensis* cultivar Uganda by *Ophiosphaerella korrae* expressing tdTom from 2 to 14 days post inoculation (dpi).

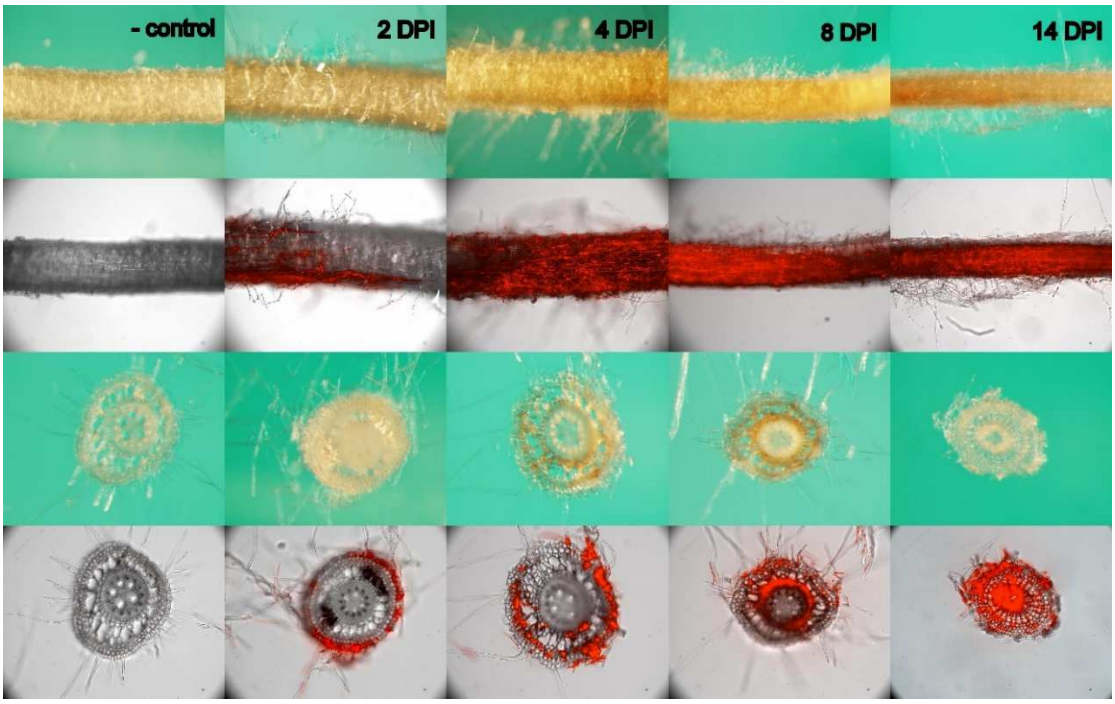


**Figure A-8** Colonization and infection of roots of *Cynodon transvaalensis* cultivar 3200 by *Ophiosphaerella korrae* expressing tdTom from 2 to 14 days post inoculation (dpi).

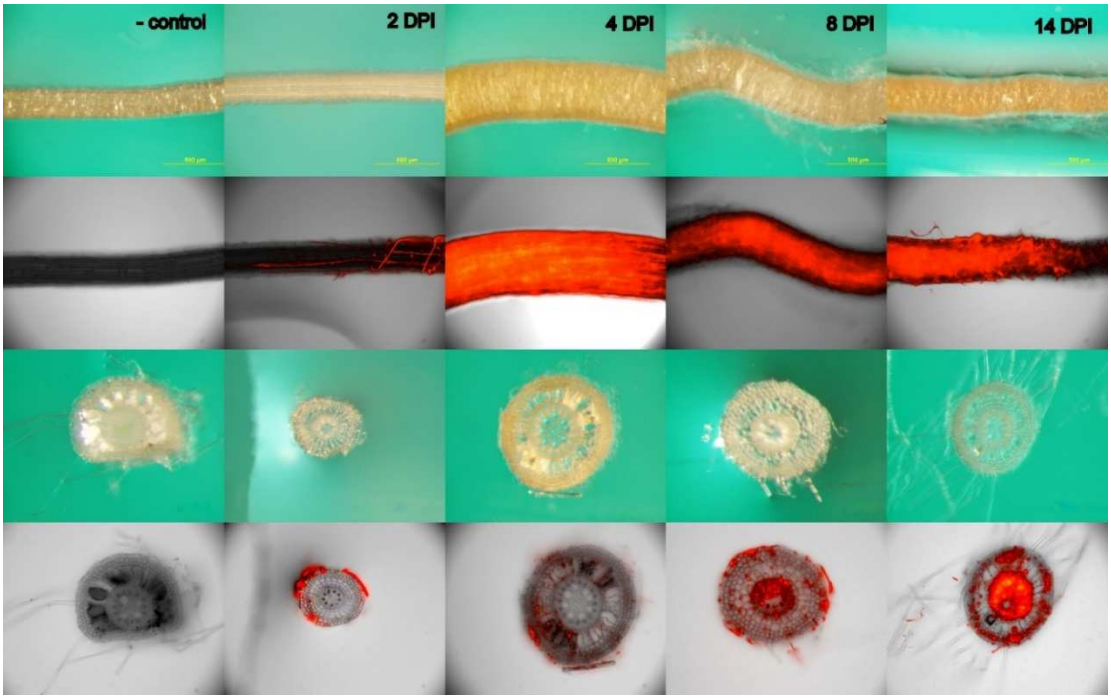


**Figure A-9** Colonization and infection of roots of *Cynodon dactylon* bermudagrass cultivar U3 by *Ophiosphaerella korrae* expressing tdTom from 2 to 14 days post inoculation (dpi).

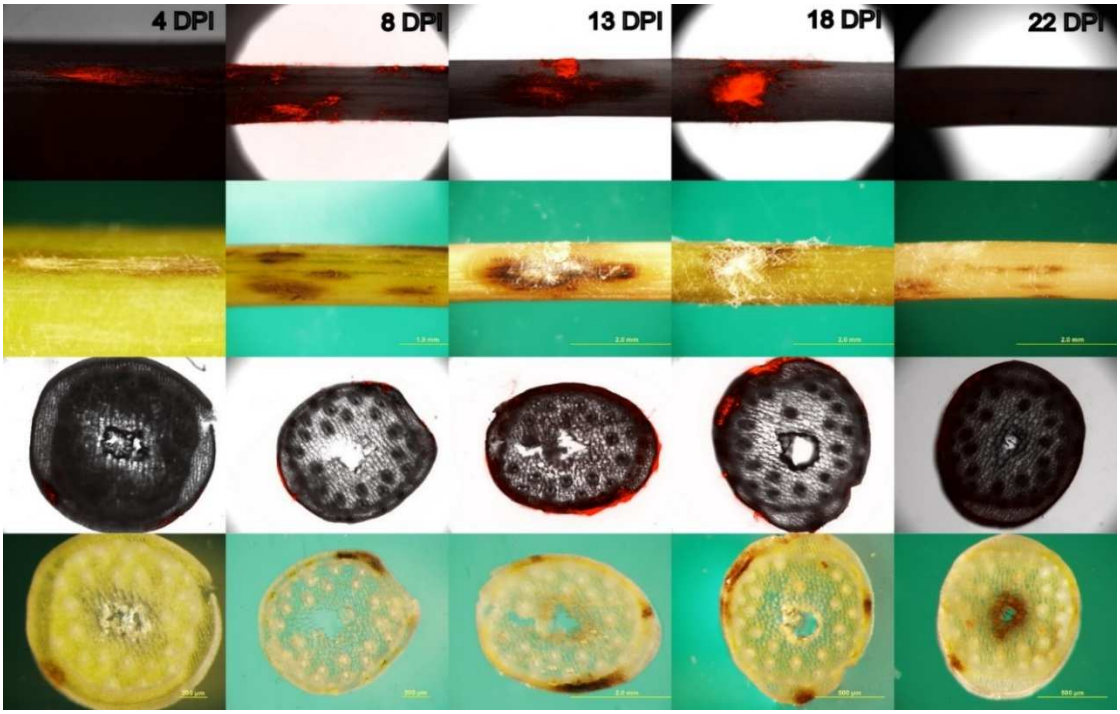


**Figure A-10** Colonization and infection of stolons of hybrid bermudagrass cultivar Midlawn by *Ophiosphaerella korrae* expressing tdTom from 4 to 22 days post inoculation (dpi).

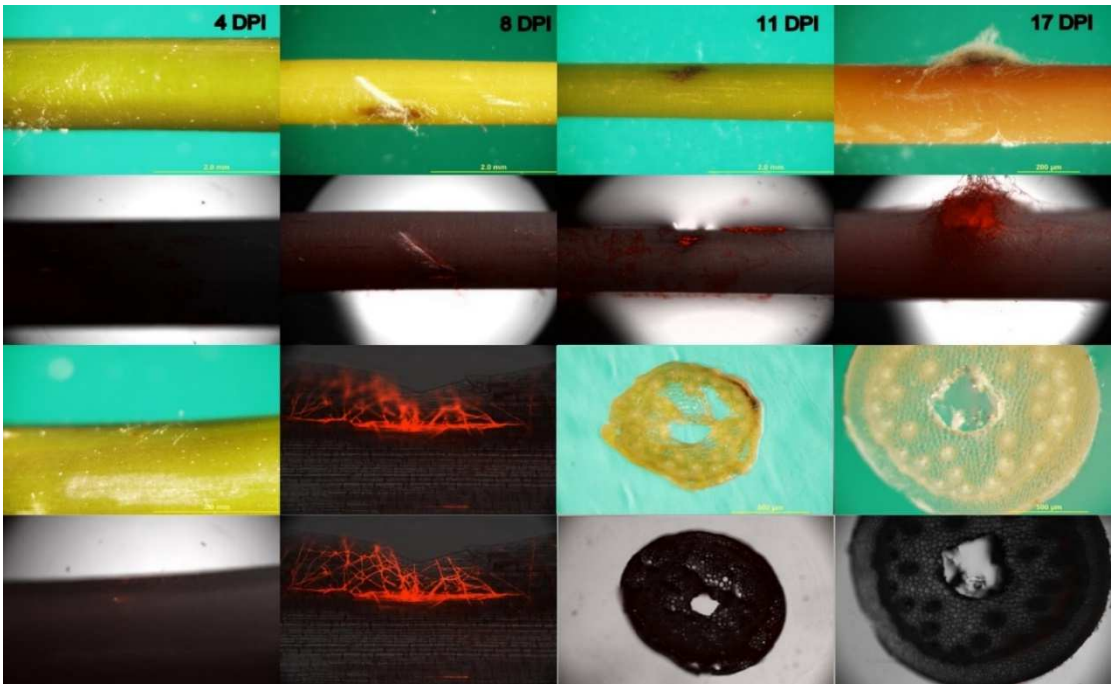


**Figure A-11** Colonization and infection of roots of hybrid bermudagrass cultivar Tifway by *Ophiosphaerella korrae* expressing tdTom from 4 to 17 days post inoculation (dpi).

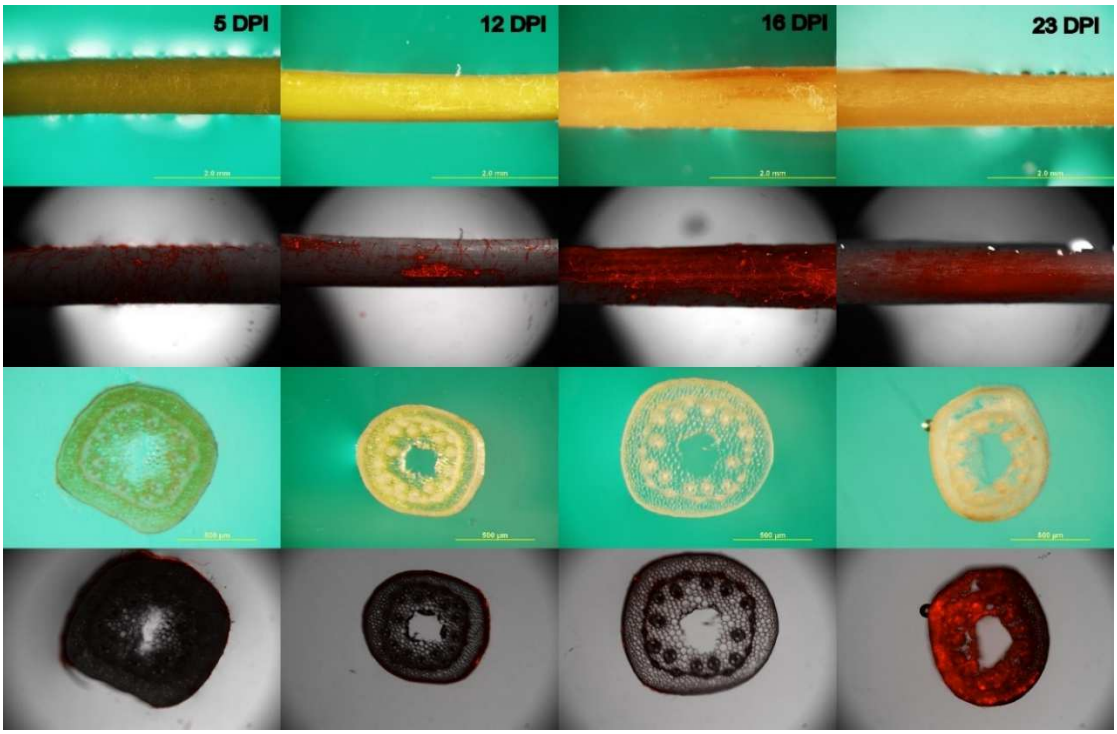


**Figure A-12** Colonization and infection of stolons of *Cynodon transvaalensis* bermudagrass cultivar Uganda by *Ophiosphaerella korrae* expressing tdTom from 5 to 23 days post inoculation (dpi).

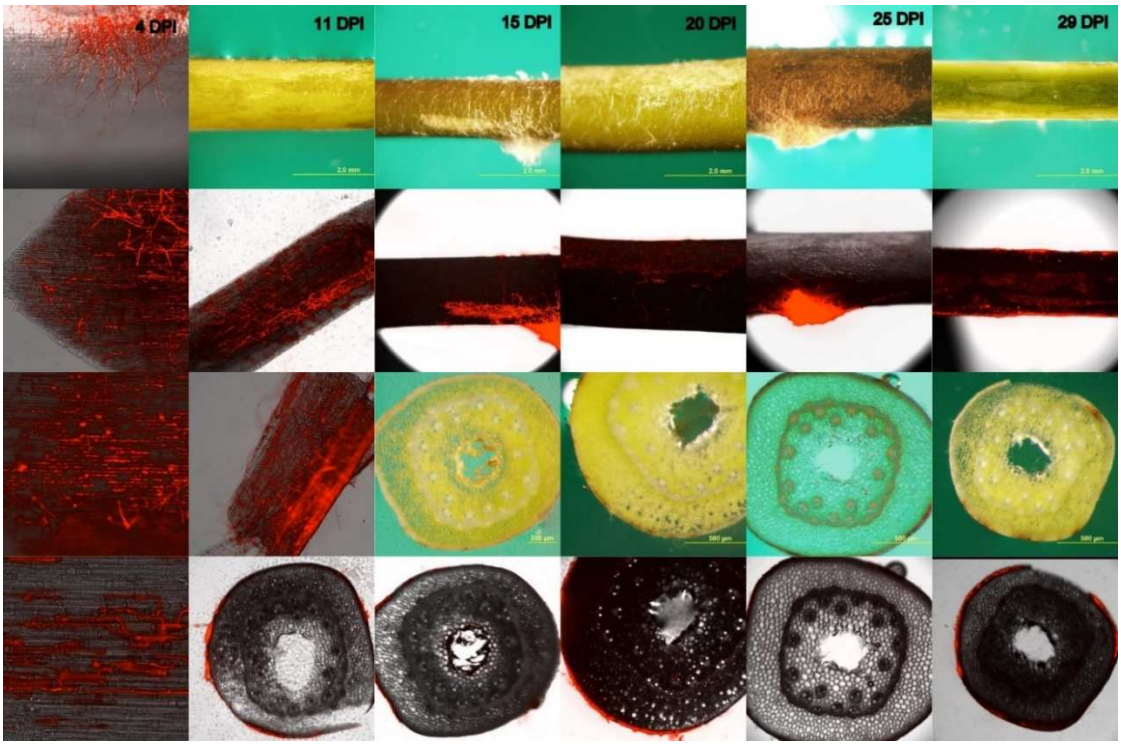


**Figure A-13** Colonization and infection of stolons of *Cynodon transvaalensis* bermudagrass cultivar 3200 by *Ophiosphaerella korrae* expressing tdTom from 4 to 29 days post inoculation (dpi).

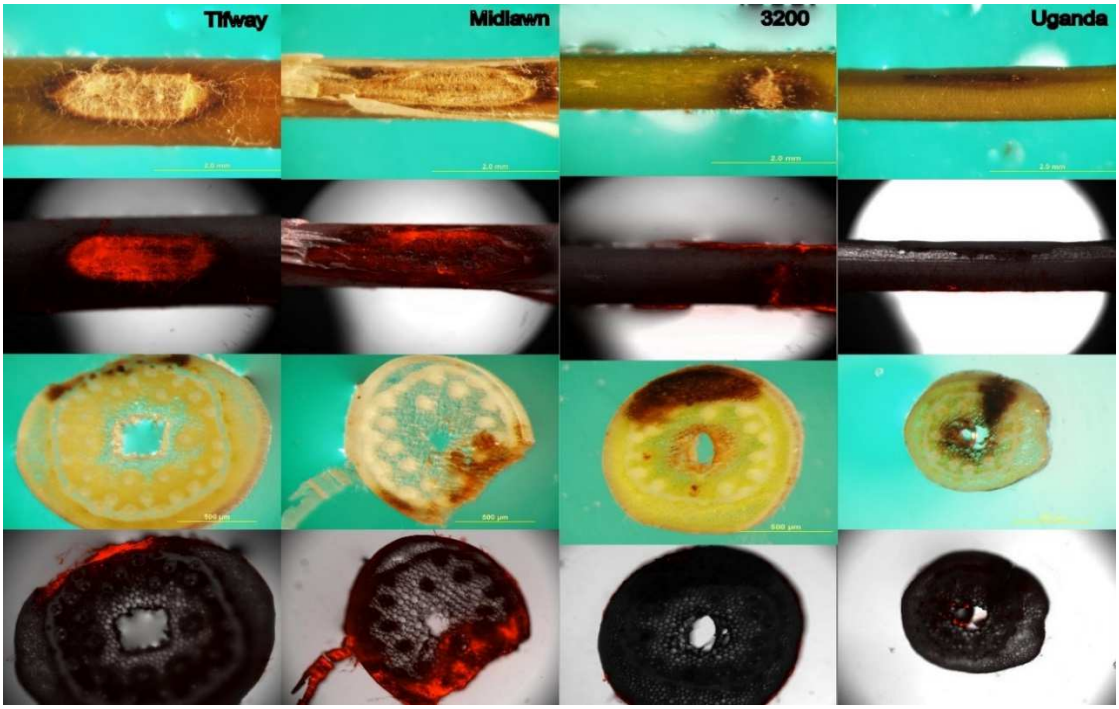


**Figure A-14** Colonization and infection of wounded bermudagrass stolons by *Ophiosphaerella korrae* expressing tdTom at 15 days post inoculation.

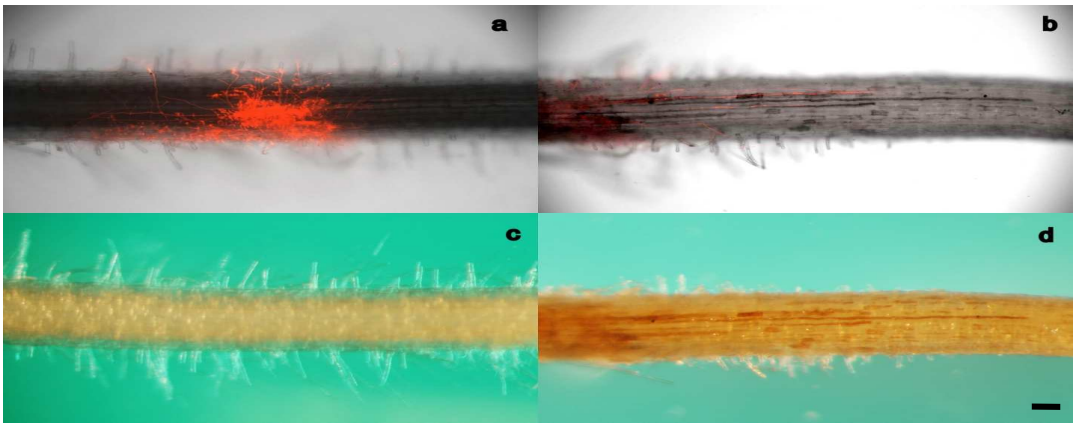


**Figure A-15** Midlawn bermudagrass roots colonized with *Ophiosphaerella korrae* expressing tdTom at one day post infection, A, C) before, and B, D) and staining with 3, 3'-diaminobenzidine (DAB). Staining with DAB proved to be inconsistent so results obtained with this technique were not used for the final analysis. Bar represents 100 µm**.**

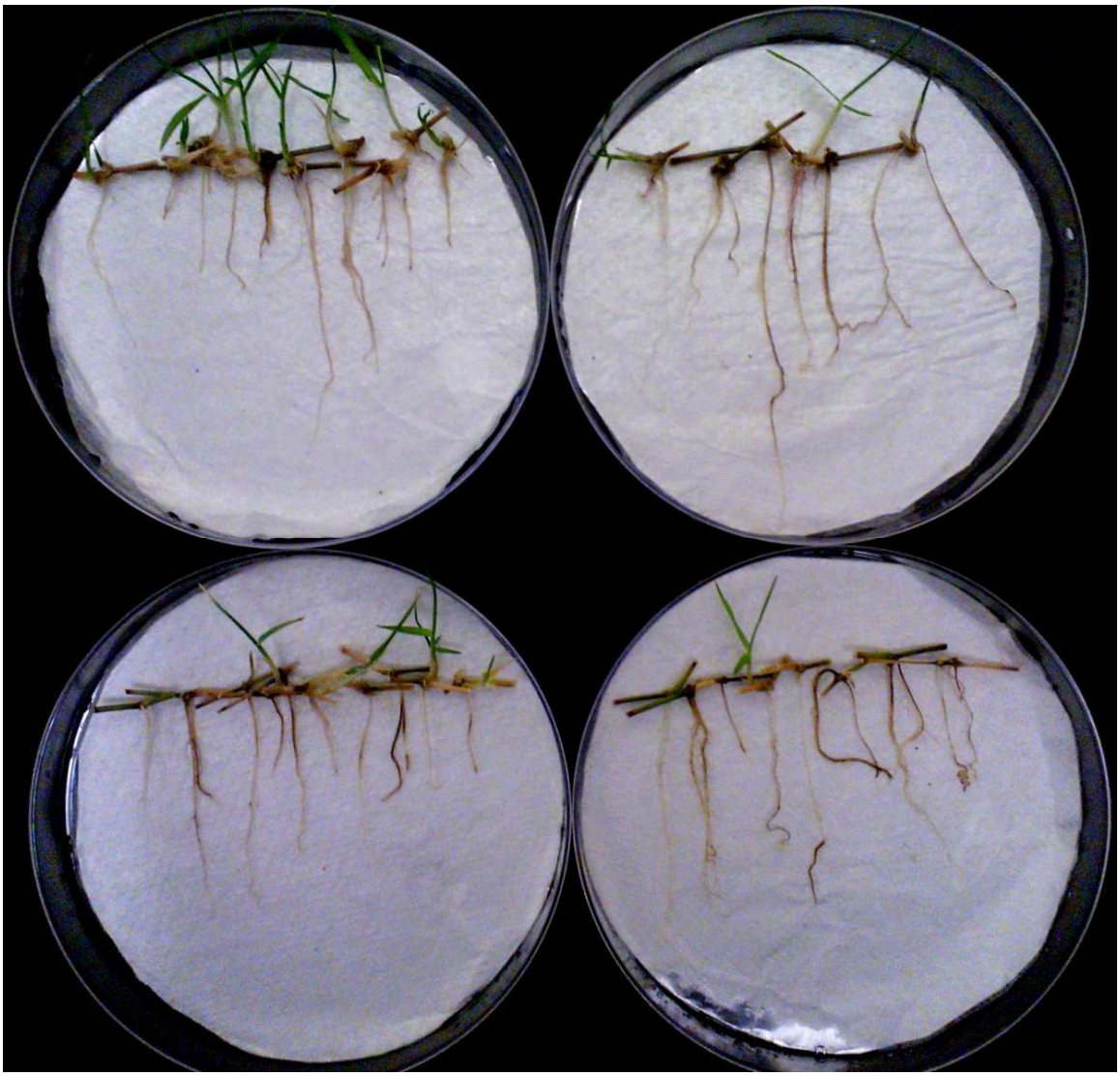

**Figure A-16** *Cynodon dactylon* cultivar U3 infected with *Ophiosphaerella korrae* (top left) and *O. herpotricha* (top right), and hybrid bermudagrass cultivar Midlawn infected with *O. korrae* (bottom left) and *O. herpotricha* (bottom right) 21 days post inoculation (dpi). Roots were stained with 2', 7'-dichlorofluorescein diacetate to detect ROS production at 2 and 3 dpi. A non-infected control root was placed in the far left of each plate for contrast.

VITA

Francisco Javier Flores

Candidate for the Degree of

Doctor of Philosophy

Thesis: ETIOLOGY OF SPRING DEAD SPOT OF BERMUDAGRASS

Major Field: Plant Pathology

Biographical:

Education:
Completed the requirements for the Doctor of Philosophy in Plant Pathology at Oklahoma State University, Stillwater, Oklahoma in December, 2014.
Completed the requirements for the Master of Science in Plant Pathology at Oklahoma State University, Stillwater, Oklahoma in December, 2010
Completed the requirements for the Bachelor of Science in Biotechnology at Escuela Politécnica del Ejército, Sangolquí, Ecuador in 2008

Experience:
Graduate Research Assistant and Teaching Assistant, Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater OK, January 2009 to August 2014
Research Assistant, Microbiology Laboratory, Escuela Politécnica del Ejército, Sangolquí, Ecuador, August 2007 to August 2008
Intern, International Potato Center, Cutuglagua, Ecuador, August to September 2006

Professional Memberships:
American Phytopathological Society (APS)
Oklahoma Academy of Science (OAS)
Gamma Sigma Delta Honor Society of Agriculture
Phi Kappa Phi Honor Society
Golden Key Honor Society
American Association for the Advancement of Science (AAAS)