Turfgrass and Environmental Research Online

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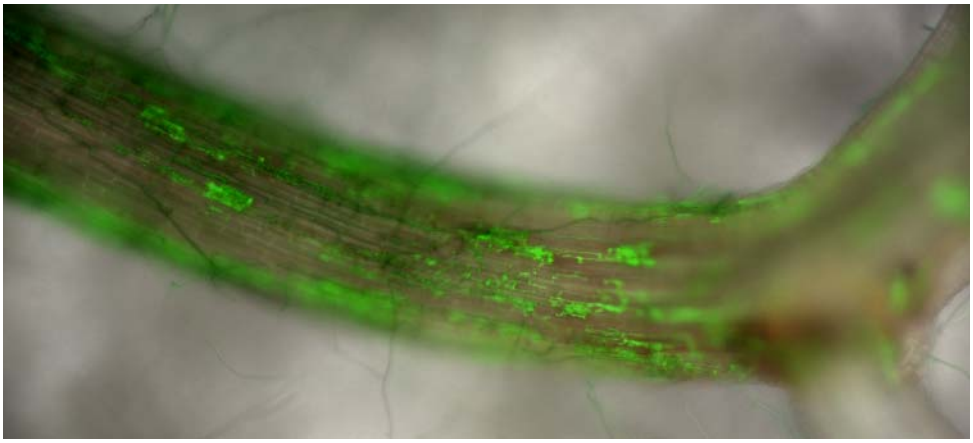

Researchers at Oklahoma State University used an *Agrobacterium tumefaciens* (the bacterium that causes crown gall on broadleaf plants)-mediated transformation method to transform *Ophiosphaerella herpotricha* to express either the Green Florescent Protein (GFP, shown above) or red florescent protein in this fungal pathogen that causes spring dead spot in bermudagrass. The study was conducted to advance the understanding of the interaction between *O. herpotricha* and its bermudagrass hosts (e.g. how different host tissues and organs react to infection) in order to provide a rational basis for the development of strategies for more effective disease control of this important disease of bermudagrass.

Volume 8, Number 3
February 1, 2009

PURPOSE

The purpose of *USGA Turfgrass and Environmental Research Online* is to effectively communicate the results of research projects funded under USGA's Turfgrass and Environmental Research Program to all who can benefit from such knowledge. Since 1983, the USGA has funded more than 350 projects at a cost of $29 million. The private, non-profit research program provides funding opportunities to university faculty interested in working on environmental and turf management problems affecting golf courses. The outstanding playing conditions of today's golf courses are a direct result of ***using science to benefit golf***.

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Host Infection and Colonization by a Causal Agent of Spring Dead Spot of Bermudagrass

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SUMMARY

Researchers at Oklahoma State University used an *Agrobacterium tumefaciens* (the bacterium that causes crown gall on broadleaf plants)-mediated transformation method to transform the *Ophiosphaerella herpotricha* to express either the Green Florescent Protein or red florescent protein in this fungal pathogen that causes spring dead spot in bermudagrass. The study was conducted to advance the understanding of the interaction between *O. herpotricha* and its bermudagrass hosts (e.g. how different host tissues and organs react to infection) in order to provide a rational basis for the development of strategies for more effective disease control of this important disease of bermudagrass. The study's accomplishments included:

- Two fluorescent transgenic fungi were generated.
- These fluorescent fungi were used to study the progression of disease in bermudagrass varieties that differ in susceptibility to the disease.
- These studies were expanded to include an accession of *Cynodon transvaalensis*, one of the parents used to generate hybrid bermudagrass
- A susceptible variety displayed more extensive cell necrosis associated with fungal invasion than that observed for more resistant or tolerant varieties.
- This information will be used to enhance traditional breeding efforts at Oklahoma State University to improve host-plant resistance.

Spring dead spot is the most devastating and important disease of bermudagrass grown in locations where it undergoes winter dormancy (4). The disease is caused by one or more of three fungal species in the genus *Ophiosphaerella* (*spp. herpotricha, korrae, or narmari*). The disease causes unsightly dead patches on fairways, tee boxes and greens, resulting in increased management inputs to eliminate weeds and encourage regrowth of bermudagrass into the dead areas. Dead grass in diseased patches is often sunken and can interfere in ball roll or lie. Play interference and poor turf appearance reduce the quality of golf during the spring when weather conditions are most desirable for play. Often an entire growing season is required for the reestablishment and maturation of bermudagrass in the affected areas.

Despite the identification of the causal agents in the United States in the 1980's, the biology and ecology of these fungi has been poorly understood (1, 2, 5). A critical limitation to the study of turfgrass root diseases is the inability of researchers to rapidly and easily study the plant-fungus interactions because they occur below ground and often inside of roots. Our understanding of spring dead spot has been based on its perceived similarity to diseases of agronomic crops, such as take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici*.

Figure 1. Spring dead spot is caused by one or more of three fungal species (*Ophiosphaerella herpotricha*, *O. korrae*, or *O. narmari*) and is the most devastating disease of bermudagrass. Note weed encroachment in some of the patches.

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USGA Turfgrass and Environmental Research Online 8(3):1-6.
TGIF Record Number: 145695

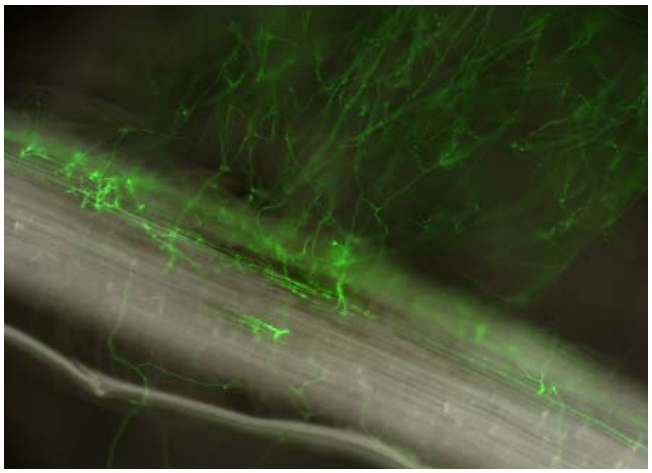

Figure 2. Initial infection and colonization of 'Tifway 419' three days after inoculation by an isolate of *Ophiosphaerella herpotricha* expressing green fluorescent protein (200x).

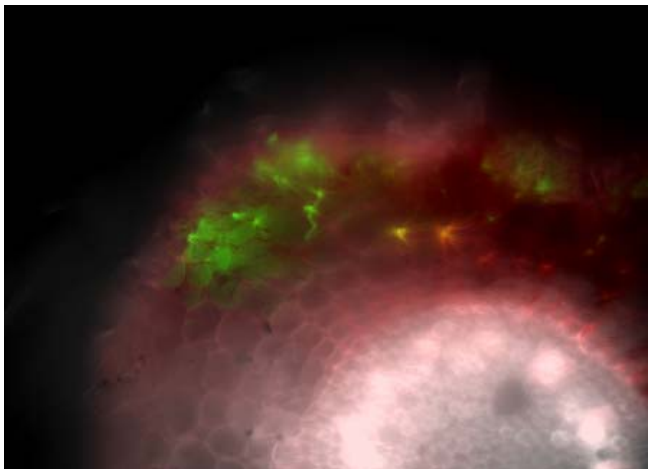

Figure 4. Colonization and collapse of the outer root cells for 'Midlawn' 12 days after inoculation (400x).

Currently, it is thought that as bermudagrass enters dormancy, the fungus uses specialized structures to colonize the exterior of roots with dark mycelium, while finer hyphae infect root tissues (4). Infection eventually causes a characteristic dark brown or black discoloration of the root system of infected plants. It has been believed that the extensive colonization and subsequent necrosis of bermudagrass root systems impairs root function severely enough to eventually lead to the death of the whole plant.

The overall goal of this study was to advance our understanding of the interaction between *O. herpotricha* and its bermudagrass hosts (e.g. how different host tissues and organs react to infection) in order to provide a rational basis for the development of strategies for more effective disease control.

An *Agrobacterium tumefaciens* (the bacterium that causes crown gall on broadleaf plants)-mediated transformation method was used to transform the *O. herpotricha* genome to express either the Green Florescent Protein (GFP) or red florescent protein (tdTom) (3). This was done by incubating fragments of the fungus with an isolate of *A. tumefaciens* capable of transferring a piece of DNA containing a selectable marker gene and a fluorescent reporter gene into the fungal genome. Thus, a fungus successfully 'transformed' with the DNA grew on selective media containing an antifungal antibiotic and most of these 'transformants' also produced a fluorescent reporter signal.

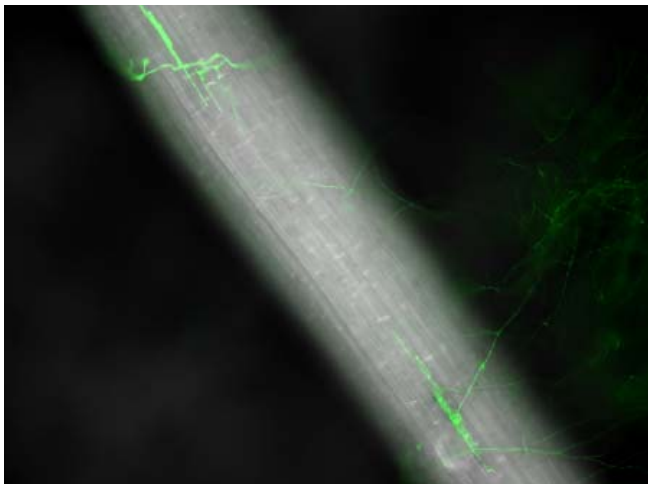

Figure 3. Initial infection and colonization of 'Midlawn' four days after inoculation (100x).

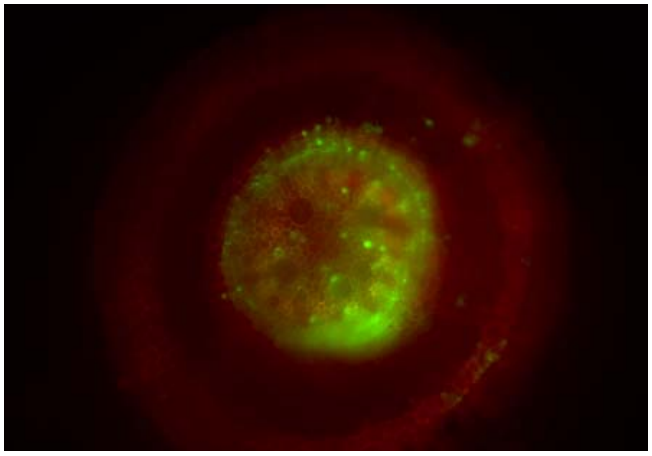

Figure 5. Extensive infection and colonization of the stele and vascular tissues of a *Cynodon transvaalensis* accession 14 days after inoculation (300x).

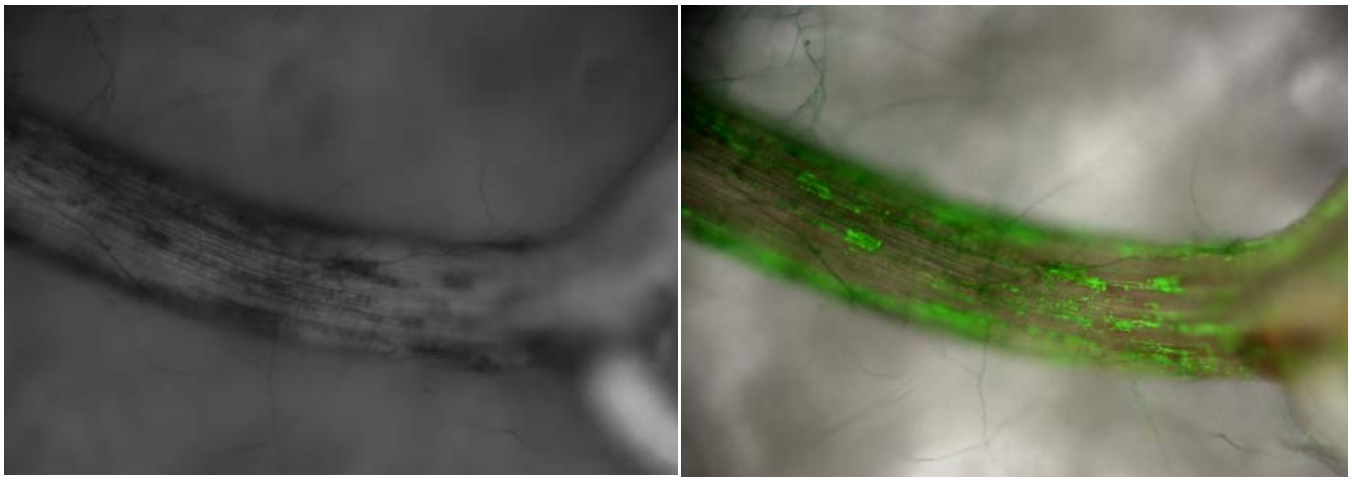

Figure 6. Extensive colonization and areas of necrosis for 'Tifway 419' 10 days after inoculation (100x). Side by side comparison showing underlying necrosis (left) corresponding with colonization (right).

Transformants used in the experiments were selected based on fluorescent signal strength and similarity to the recipient 'wild type' isolate's appearance, growth, and infection of bermudagrass.

Stolons of the interspecific hybrid (*Cynodon dactylon* × *C. transvaalensis*) bermudagrass cultivars, 'Tifway 419' and 'Midlawn', and an African bermudagrass accession (*C. transvaalensis*) were cut into segments containing at least a single node. After 3 to 5 days, stolons with roots were selected and transferred individually to sterile Petri plates lined with double layer of wet white paper towel. A single root from each stolon was inoculated with a plug (approximately 1/32 inch in diameter) of a fluorescent transformant of *O. herpotricha*. Plates were sealed and incubated in a growth chamber at 17° C. This study was repeated four times with one non-inoculated control plant for every five inoculated replicate plants.

Roots were observed from 1 day post-inoculation (DPI) to 14 DPI using a using a Nikon epi-fluorescent microscope. To observe the fungus-plant interaction, roots' surfaces were examined directly or some of the roots were embedded in soft media and hand-sectioned crosswise or lengthwise with a double-edge razor.

In the cultivars Tifway 419 and Midlawn, root cortical cells were rapidly colonized by O. herpotricha (Figures 2 and 3), while the roots' steles remained uncolonized twelve days after inoculation (Figure 4). Conversely, in *C. transvaalensis* roots, the cortical cells were sparsely colo-

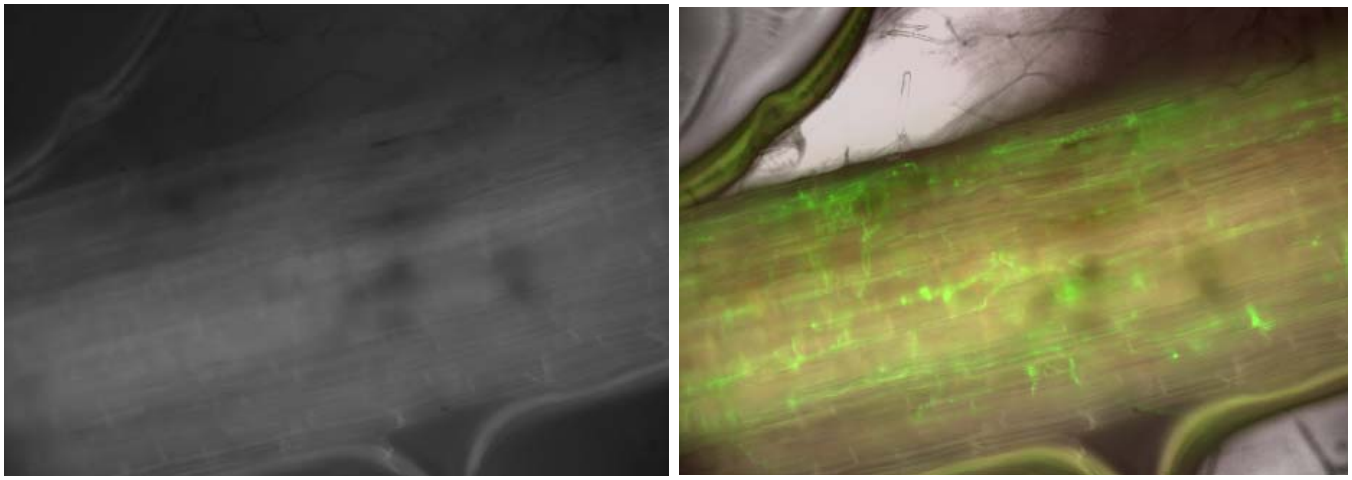

Figure 7. Extensive infection and colonization of *Cynodon transvaalensis* 10 days after inoculation (100x). Side by side comparison not showing extensive underlying necrosis (left) corresponding with colonization (right).

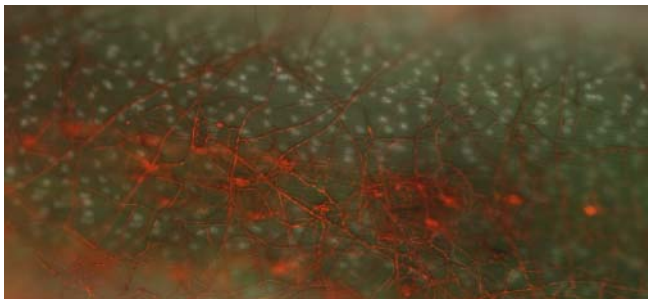

Figure 8. Surface colonization and aggregates of hyphae on 'Tifway 419' stolon one month after inoculation by an isolate of *Ophiosphaerella herpotricha* expressing red fluorescent protein (100x).

nized, while the roots' stele was extensively colonized, fourteen days after inoculation (Figure 5). In general, 'Tifway 419' roots exhibited greater colonization and necrosis (Figure 6) than the more tolerant cultivar 'Midlawn' and *C. transvaalensis*; the latter which, though its roots were heavily colonized, exhibited very little necrosis (Figure 7).

Similar studies were also conducted on the stolons of these same bermudagrasses. Stolons were observed at 3, 14 and 28 days post-inoculation (DPI) with fluorescent transformants of *O. herpotricha*. Fungal colonization of stolon surfaces and internal tissues (crosswise and lengthwise hand-sections) were observed. When stolons were inoculated at unwounded internodes, epidermal colonization varied after 28 days. 'Tifway 419' stolon surfaces appeared to be colonized more greatly than the stolons of either 'Midlawn' or *C. transvaalensis* (Figure 8). In addition, the

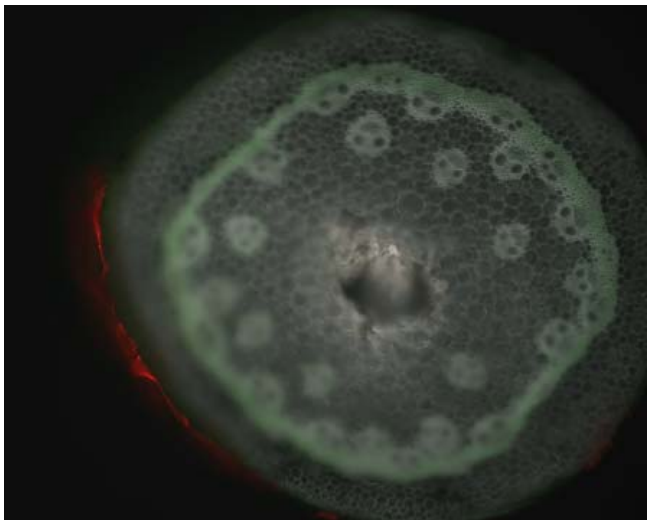

Figure 9. Cross section of 'Tifway 419' stolon with surface and no internal colonization one month after inoculation (100x).

fungus formed small hyphal aggregates on the surface of 'Tifway' stolons and not on the other grasses. Also, fungal colonization of inoculated stolons of all three bermudagrasses appeared limited to the surface (cuticle) of the stolon, with no fungal ingress into stolon cortical tissues observed (Figure 9).

However, internal colonization of stolons was observed when root-inoculated bermudagrasses were observed 42 DPI. In these cases, root-colonizing *O. herpotricha* had grown up the root to the stolon and the fungus penetrated the stolon through the cut internode of the stolon. In Tifway 419, stolon infection resulted in internal necrosis of stolon cortical tissues (Figure 10), while in 'Midlawn' stolons, similar internal infec-

Figure 10. Lengthwise section of 'Tifway 419' stolon with internal colonization and necrosis six weeks after inoculation (100x).

tion resulted in less severe necrosis. However, in *C. transvaalensis* stolon tissues were internally colonized without any apparent necrosis (Figure 11).

A confocal scanning laser microscope, a type of microscope that uses a set of lasers to optically section images from inside a specimen, was used to image infected roots and stolons. Each optical slice can be viewed individually (Fig. 12) or combined to generate three dimensional images of the fungal plant interaction. These images suggest that the fungus grows covertly in between host cells and prior to inducing necrosis of host cells.

Conclusions

We observed that the fungus was able to vigorously colonize roots without causing necrosis in early stages of infection of 'Midlawn' and all stages of infection of *C. transvaalensis*. However, infection of 'Tifway 419' roots almost always resulted in necrosis. This necrotic response of 'Tifway 419' to *O. herpotricha* infection may be a misdirected hypersensitive defense designed to restrict colonization and growth of the fungus by depriving it of living host cells. However, the rapid necrosis of cells to infection could provide a nutritionally rich environment for fungal growth. Thus, the cell death response of bermudagrass cultivars, ironically, may be the cause of their susceptibility to *O. herpotricha*.

On the other hand, the ability of the *C. transvaalensis* accession to tolerate colonization could be attributed to its lack of recognition of the fungus or necrotic response to the sparse presence of the fungus. Alternatively, *Ophiosphaerella* species belong to a group of plant pathogenic fungi known to produce host specific toxins that disarm and kill susceptible host cells, facilitating a necrotrophic life style. Thus, the susceptible bermudagrass cultivars could possess a vulnerable target (e.g. a genetic "Achilles heel") for a necrosis-inducing toxin secreted by *O. herpotricha*, while *C. transvaalensis* lacks this target or possesses a resistant form of the target. Elucidation of the mechanisms underlying the observations reported here will required further research involving genetic crosses of susceptible and tolerant bermudagrasses.

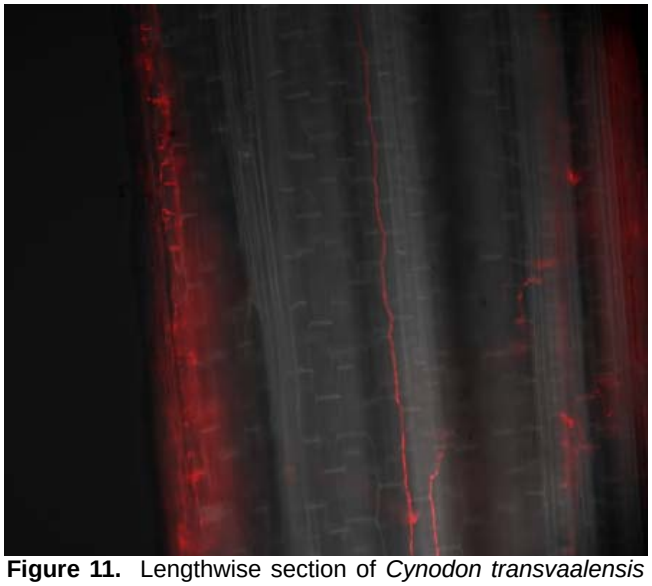

Figure 11. Lengthwise section of *Cynodon transvaalensis* stolon with internal colonization and no observable necrosis six weeks after inoculation (200x).

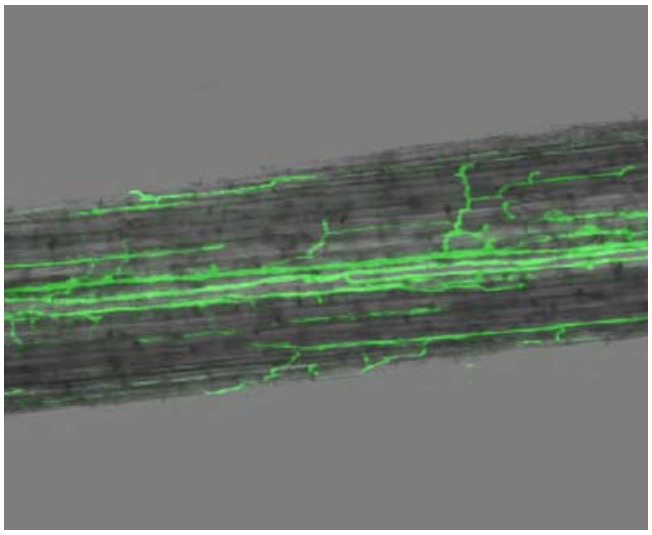

Figure 12. A confocal scanning laser microscope generated image of a root internally infected by an isolate of *Ophiosphaerella herpotricha* expressing green fluorescent protein 13 days after inoculation (150x).

Epiphytic colonization of stolons by *O. herpotricha* differed across the three hosts. The most extensive colonization, including hyphal aggregates, was observed on 'Tifway 419' stolons. The hyphal aggregates may have originally been perceived to be infection mats (penetration structures) by previous investigators. Necrotic epidermal cells were observed in the colonized stolons of both 'Tifway 419' and 'Midlawn'. *Ophiosphaerella herpotricha* can slowly penetrate the stolon epidermis, though less efficiently than roots, and may eventually colonize stolons through this process.

The additional observation of *O. herpotricha* entering stolons through even healed wounds is significant. Turf bermudagrass stolons are often injured by chewing insects, mowing, aerification, and recreational activities and thus, may provide a readily available infection court for *O. herpotricha*. The importance of stolon infection to disease progress of SDS is not yet understood.

This study suggests that direct root infection by *O. herpotricha* is rapid and varies across

cultivars. In susceptible bermudagrasses, infection and colonization occurs within 10 to 14 days resulting in lesions and root necrosis. The observed tolerance of *C. transvaalensis* to colonization by *O. herpotricha* implies that breeding efforts to incorporate cold tolerance from this species into interspecific hybrids may have unintentionally incorporated greater tolerance to *O. herpotricha*.

This study also demonstrated the utility of using *O. herpotricha* transformants expressing fluorescent protein for preliminary screening of bermudagrass germplasm for spring dead spot resistance (or tolerance). Current field screening methods usually require 5 to 7 years, whereas this rapid screening technique would only require a few weeks. This approach should reduce the time and cost of screening bermudagrass genotypes and permit detection of SDS susceptibility earlier in cultivar development.

Acknowledgements

The authors wish to acknowledge Oliver Cassi, a former graduate student who performed almost all of the experiments in this project. We also wish to thank the USGA's Turfgrass and Environmental Research Program and Oklahoma State University Agricultural Experiment Station for support of this research.

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