CONFIRMATION OF *SPHAEROBOLUS STELLATUS* AS A CAUSAL ORGANISM OF THATCH COLLAPSE OF TURFGRASSES

A Thesis in
Agronomy
by
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ABSTRACT

In 2011, previously unreported disease symptoms were observed on golf course putting greens in the United States and New Zealand. Symptoms of this disease, referred to as thatch collapse, include dark green depressed patches approximately 8 to 46 cm in diameter. The patches of degraded organic matter result in a disruption of playability of golf course tees, fairways and greens. A single fungal species was commonly isolated from turfgrass grown in California, Michigan, Montana, South Dakota, and New Zealand. Sequences of the internal transcribed spacer region (ITS) revealed 695 to 724-bp sequences that were a 98% to 99% match to Sphaerobolus stellatus in the NCBI database. S. stellatus is a basidiomycete commonly found within wood mulch. Little is known about S. stellatus within turfgrass systems. When mature turf was inoculated with S. stellatus and incubated for 6 weeks, thatch layer depth and total organic matter was reduced 28% and 21%, respectively. Studies were conducted to assess the morphology, growth rates and gleba production of S. stellatus isolated from turf. The general morphology of gleba were closer to S. iowensis, but basidiospores were similar to S. stellatus. Optimal temperatures for growth of S. stellatus ranged from 21 to 30°C. Higher quantities of gleba were produce at 10, 15 and 20°C than at 25°C with the highest quantities occurring within 11 weeks of incubation at 10°C. To understand the mechanism by which organic matter was degraded by S. stellatus, the structure of lignin and lignin modifying enzymes were assessed. Utilizing tetramethylammonium hydroxide thermochemolysis, a significant depolymerization of lignin was found when S. stellatus colonized organic matter for 6 weeks. The presence of laccase in an isolate of S. stellatus from turf, manganese independent peroxidase in S. stellatus isolates and xylanase in all fungal extracts illustrates the mechanism of organic matter reduction in turf. Overall, S. stellatus isolated from turf effectively degrades lignin within organic matter through
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Chapter 1

Literature Review

Introduction

Numerous turfgrass maladies have been associated with various fungal species within the phylum Basidiomycota. Many species in the order *Agaricales* have been shown to produce a wide range of symptoms on maintained turfgrasses. Although several fungi have been found within the thatch and soil interface of turfgrasses, links to pathogenicity are not definitive for all symptoms and their associated “diseases” (Miller, 2010). Regardless, a number of fungal species have been identified and are commonly associated with numerous diseases of turf.

The most common disease associated with basidiomycete fungi in turf is fairy ring. Fairy ring symptoms are highly variable and have been associated with more than 60 different fungal species (Miller, 2010). Several species of *Agaricus* L. Fr., *Calvatia* Fr., *Chlorophyllum* Massee, *Clitocybe* (Fr.) Staude, *Lepiota* (Pers.) Gray, *Lycoperdon* Pers., *Marasmius* Fr., *Scleroderma* Pers. and *Tricholoma* Fries are commonly isolated within fairy ring in turf swards (Smiley et al., 2005).

Fairy ring fungi decompose organic matter as the mycelia grow radially outward through the thatch and soil. The various symptoms are believed to be the result of nutrient excess or deficiency, development of hydrophobic soil conditions, direct pathogenicity to the plant, and other means not well understood (Miller, 2010; Smiley et al., 2005). These symptoms are used to classify fairy ring into three distinct types based on symptoms developing within individual swards. Type I fairy ring is characterized by a necrotic arc of dead or declining turf (Shantz et al., 1917; Smiley et al., 2005). Type II fairy rings generally are characterized by a dark green ring of lush turf (Fidanza, 2007; Fisher, 1977; Shantz et al., 1917; York et al., 2000). Type III fairy rings include those producing no visible change to the turfgrass, but instead are identified by a ring of basidiocarps (Shantz et al., 1917; Smiley et al., 2005). For all three types of fairy ring, arcs and
rings may range in size from a few centimeters to several meters or larger in diameter (Smiley et al., 2005). When at least two symptoms are present in the same area, it is referred to as a disease complex.

Classification of the fairy ring fungi can also be based on the location of fungal activity within the turfgrass sward. Lectophilic fungi colonize the leaf litter and upper 2.5 cm of thatch. Symptoms produced by basidiomycetes colonizing the soils are generally referred to edaphic. This plant-fungi interaction does not depend on host plant or mowing height of turf, as the fungi persist in the soil and thatch (Miller, 2010).

Superficial fairy ring, also known as white patch, is another distinct turf disease characterized by rings or patches of white mycelia on the foliage or within infested thatch of Agrostis, Festuca and Poa annua putting greens (Smiley et al., 2005). Mycelia typically develop during cool and moist conditions and may persist within the thatch, or in select cases induce senescence of older tissues (Jackson, 1972). Patches typically range from 5 to 35 cm in diameter, but have been reported to expand radially up to 1 m (Smiley et al., 2005). Symptoms of superficial fairy ring have been associated with Trechispora cohaerens (Schwein) Julich & Stalpers, T. farinacea (Pers.:Fr.) Liberta, and Coprinus kubickae Pilat & Svrcek (Jackson, 1972, and Smiley et al., 2005).

Yellow ring, caused by the basidiomycete T. alnicola (Bourd. & Galzin) Liberta, is a disease of Kentucky bluegrass (Poa pratensis L.). Symptoms may be present from spring to autumn and include a distinct yellowing of the leaves (Wilkinson, 1987). The saprophytic fungus colonizing the thatch penetrates epidermal root cells during cool and moist weather inducing leaf chlorosis. Stands of Kentucky bluegrass less than 2 years old are more susceptible to yellow ring than established stands of turf and symptoms are often transient with leaves regaining their green color over time.
White blight, caused by *Melanotus phillipsii* (Berk. & Broome) Singer, is primarily a disease of *Festuca*, but has been found on *Agrostis*, *Lolium* and *Poa* spp. (Smiley et al., 2005). Symptoms within infested turf are characterized by blighted circular patches ranging from 8 to 12 cm in diameter, but patches may coalesce to form large irregular areas of blighted turf (Smiley et al., 2005). Signs of the pathogen include grayish white mycelium matted on the leaf surfaces and stalkless basidiocarps emerging from blighted grass blades. The disease is most severe under hot and humid conditions, but due to the pathogen’s inability to infect stems, the affected areas usually recover once cooler, less humid conditions return (Smiley et al., 2005.)

**Thatch Collapse**

In 2010, a new disease believed to be caused by a basidiomycete was reported on *Festuca* spp. in Scotland (Dernoede et al., 2011). Based on the symptoms expressed on finely mown turf, the disease was referred to as thatch collapse. Since the initial report of the disease, similar symptoms have been reported in stands of creeping bentgrass (*Agrostis stolonifera* L.) in New Zealand and Australia, as well as on creeping bentgrass and annual bluegrass (*Poa annua* L.) in California, South Dakota, Michigan and Pennsylvania.

Thatch degradation from this unknown basidiomycete on golf course putting greens and fairways creates sunken, depressed patches. Symptoms consist of circular patches of dark green turf ranging from 8 to 46 cm in diameter (Figure 1). Decomposing thatch within symptomatic areas has a characteristic fawn color and a mushroom odor (Dernoeden et al., 2011). Observed morphological characteristics of mycelia include clamp connections, supporting classification of this fungus as a basidiomycete.

Thatch collapse symptoms are distinct from other symptoms produced by mushroom or puffball-forming basidiomycetes commonly associated with turfgrass. The distinct symptoms may represent a previously unrecognized symptomology caused by one of the aforementioned
species of basidiomycetes or caused by a basidiomycete not previously associated with turf diseases.

Early in 2012, the basidiomycete *Sphaerobolus stellatus* (Tode) Persoon was found in association with thatch collapse in New Zealand, California, South Dakota and Michigan. Consistent development of unique fruiting bodies and mycelium characteristics upon incubation of symptomatic samples has lead to the identification of *S. stellatus* within samples of turf expressing thatch collapse symptoms.

**Biology and ecology of *Sphaerobolus stellatus***

*Sphaerobolus* Tode was first acknowledged almost 300 years ago as *Carpobolus* by Micheli (1729). The passive spore discharge placed *Sphaerobolus* within the formerly known artificial morphological group Gasteromycetes (Ulloa and Hanlin, 2000). Utilization of more recent molecular data has positioned *Sphaerobolus* in the gomphoid phalloid clade (Hibbett et al., 1997).

*Sphaerobolus stellatus* colonizes woody lignocellulose substrates for growth and reproduction. This fungus is commonly found within the U.S., especially the Northeast and even Alaska, and numerous other locations including Africa, Australia, Canada, Europe from Greece to Iceland, Latin America and New Zealand. *Sphaerobolus stellatus* has been observed inhabiting woody substrates, different types of dung and forest floors (Geml, 2004). Lately, the large expansion of landscaping mulch has provided an optimal substrate for *S. stellatus*, making this fungus a common entity in homeowners’ mulch beds (Brantley et al., 2001a; Brantley et al., 2001b; Davis et al., 2011).

The unusual fruiting structures and spore dispersal has interested mycologists for hundreds of years (Buller, 1958). *Sphaerobolus stellatus* forms creamy orange (1 to 2 mm diameter) basidiocarps containing a developing single spore mass (glebae) (Buller, 1958; Dykstra, 1982). Each glebae contains uniucleated, thick walled basidiospores and thin walled
asexual dikaryotic gemmae. The two spore types germinate under different ecological circumstances. Basidiospores germinate when exposed to high temperatures and animal digestion. However, if the spore mass is not consumed the gemmae will germinate on wood or plant debris (Dykstra, 1982). *Sphaerobolus stellatus* mycelia colonizes wood substrates optimally at temperatures of 20 to 25°C. The diploid mycelia cord is derived into a phototrophic peridium. Optimum temperatures for basidiocarp development range from 10 to 20°C, but peridia will not open to properly discharge nor will spores germinate without moisture (Alasoadura, 1963; Buller, 1958).

The peridium of *S. stellatus* contains 6 unique cellular layers. Initially, hyphae surround the exterior of the sporocarp and a gelatinous layer is present next to a pseudoparenchyma composing the exoperidium. A fibrous layer consisting of interwoven hyphae and a palisade layer surround the gleba. Another thin layer of pseudoparenchyma coats the gleba completing the endoperidium. The peridia phototrophically position towards a bright light source and eject gleba into the air (Buller, 1958). The active dispersal mechanism in the morphological group of Gasteromycetes has interested mycologists, while the end result of gleba sticking to structures has disturbed homeowners.

Active/passive basidiospores propulsion is derived from breakdown of sugars within the palisade layer of the peridia. Glucose breaks down to maltose, which increases the concentration of the total sugars causing swelling of palisade cells from altered osmolarity (Buller, 1958). As the palisade cells swell, the peridia open stellately and the developed gleba becomes exposed. Eventually the palisade and the fiberous membrane yield to the increasing pressure and evert, causing the gleba to exit the peridia. The stellate points hold the everted membrane forming diamond shaped openings, which allows air to rush into the newly formed cavity and preventing an internal vacuum (Buller, 1958).
Prior to glebae ejection from the peridium, the surrounding pseudoparenchyma layer dissolves, coating the glebae in a slimy coating. The eversion of the palisade layers and fiberous layers causes the glebae to be propelled at high speeds measured up to 9.1 m sec\(^{-1}\) (Buller, 1958). These high speeds flatten the round spore mass upon impact to a solid surface and the slimy coating enables adherence of the spore mass to flat surfaces.

**Lignin degrading abilities of *Sphaerobolus stellatus***

The ability of *S. stellatus* to colonize organic matter and degrade lignocellulose categorizes this fungus as white rot fungi. White rot fungi (WRF) have been studied for thatch degradation within turfgrass stands to decrease the accumulation of a compact layer of stolons, rhizomes and roots between the soil and vegetation (Beard, 1973). Sartain and Volk found *Coriolus versicolor* (L.) Lloyd reduced lignin content of warm and cool season grasses under laboratory conditions (1984). In laboratory studies, Marin and Dale (1980) also found *C. versicolor, Fomes fomentarius* (L.) Fr., *Goeophyllum trabeum* (Pers.) Murrill and *Ganoderma applanatum* (Pers.) Pat. reduced overall mass of organic matter from St. Augustine grass (*Stenotaphrum secundatum* (Walt.) Kuntze), bermudagrass (*Cynodon dactylon* (L.) Pers.) and zoysiagrass (*Zoysia japonica* Steud). However, none of these studies produced significant reductions in organic matter under field evaluations.

In a recent greenhouse study, fungal laccase applications to creeping bentgrass significantly decreased organic content and thatch layer thickness (Sidhu et al., 2012). Similar field studies have found comparable results of decreased organic content and thatch layer thickness after 6 months of laccase applications (Sidhu et al., 2012).

The use of lignocellulose-degrading enzymes has aided in thatch layer reduction. Lignin peroxidase has been widely studied in *Phanerochaete chrysosporium* (Fr.) P. Karst and was found as an extracellular enzyme with peroxide requiring activity (Kirk and Farrell, 1987). The lignin degrading mechanism within ligninase is a one-electron oxidation with lignin model
compounds to produce phenoxy radicals. Through this process, susceptible aromatic nuclei on the lignin molecule are oxidized by one-electron to produce unstable cation radicals which undergo a variety of non-enzymatic reactions (Kirk and Farrell, 1987). Lignin peroxidase degrades lignin extracellularly.

Manganese peroxidase is also essential to reduce lignin. The overall reaction requires manganese (Mn II), but also peroxide and lactate from the surrounding environment. Mechanisms proceed as Mn II is oxidized by peroxidase to Mn III, which then oxidizes organic substrates (Kirk and Farrell, 1987). Manganese chelates oxidize the more reactive phenolic structures, which compose at least 10 percent of the lignin structure. Manganese functions as a phenol-oxidizing enzyme and is thought to participate in peroxide production (Hammel, 1997).

Laccase is another component of lignin degradation and is comprised of a blue copper oxidase. This enzyme catalyzes one electron oxidation of phenols to phenoxy radicals, eventually transferring 4 electrons to oxygen from the aromatic components of lignin (Kirk and Farrell, 1987). Following these processes, the components of lignin become less stable and are more easily broken down (Hammel, 1997).

White rot fungi also contain enzymatic cellulases and xylanases, creating the potential to further degrade organic matter more extensively. However, many microbes are capable of breaking down cellulose or xylose once lignin is degraded (Hammel, 1997).

**Cultural and chemical management of *Sphaerobolus stellatus***

The increasing use of landscaping mulch has expanded the population of *S. stellatus* (Davis et al., 2011). Homeowners are plagued by the spore masses sticking to any bright objects near mulch beds, including house siding, porches and cars. Gleba are difficult to remove and leave a brown residue on surfaces, causing homeowners to file lawsuits and insurance claims from property damage (Brantley et al., 2001a; Brantley et al., 2001b; Davis et al., 2011).
Studies regarding cultural control methods were investigated to reduce growth and sporulation of *Sphaerobolus* spp. on mulch. Davis et al. (2004) assessed 27 mulch types to determine vegetative growth and sporulation impacts. Four years after inoculation with *Sphaerobolus* spp. the mulch types derived from large piles of shredded blends of bark and wood produced significantly greater levels of sporulation. However, large bark nuggets, cypress mulch and 100% spent mushroom substrate reduced *Sphaerobolus* spp. sporulation. Carbon to nitrogen ratios of the mulch types were not found to affect sporulation unless nitrogen percent was exceptionally low (.02%) or exceptionally high (.42%).

Spent mushroom substrate applications to mulch beds were found to reduce spore production. Brantley et al. (2001a) concluded that spent mushroom substrate contains microorganisms detrimental to *Sphaerobolus* spp. *Bacillus subtilis* Ehrenberg and *Trichoderma harzianum* Rifai were found to be antagonistic to *Sphaerobolus* spp. when tested in vitro and are believed to be present in spent mushroom substrate. However, fungal populations are variable within spent mushroom substrate and recommendations of mixing spent mushroom substrate with mulch are not well known.

Chemical control of *S. stellatus* within maintained turf systems is unknown, but fungicidal management strategies have been researched within mulch. An in vitro study based on a *S. stellatus* mulch isolate found EC50 values were lowest for polyoxin-D (Zinc 5-[[2-amino-5-0-(aminocarbonyl)-2-deoxy-L-xylonoyle]amino]-1-(5-carboxy-3,4-dihydro-2,4-dioxo-1(2H)-pyrimidinyl)-1,5-dideoxy-B-Dallofuranuronate) and azoxystrobin (Methyl (E)-2-{2 [6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate, followed by pyraclostrobin, (Methyl N-[2-[1-(4-chlorophenyl)-1H-pyrazol-3-yl] oxymethyl]phenyl](N methoxy)carbamate, triademifon (1-(4-Chlorophenoxy)-3,3-dimethyl-1-[1,2,4]-triazol-1-ylbutan-2-one), tebuconazole ((RS)-1-p-chlorophenyl)-4,4-dimethyl-3-(1H,1,2,4-triazol-1-ylmethyl)pentan-3-ol), thiophanate-methyl (dimethyl 4,4’(o-phenlene)bis(3-thioallophane) and triticonazole ((±) - (E) -5-(4-

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chlorobenzylidene)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol) (Fidanza et al., 2009). These fungicides are registered for use in ornamental and turfgrass systems but need to be evaluated in the field before conclusions can be drawn regarding fungicide recommendations.

Unlike mulch, turfgrass systems require repeated application of fertilizers (Carrow et al., 2001). The severity of select turfgrass diseases may be reduced by altering soil pH through fertility regimes. Acidifying fertilizers (e.g., ammonium sulfate) have been shown to reduce soil pH and disease severity of some ectotrophic root-infecting turfgrass pathogens (Landschoot et al., 1991). Take-all patch caused by Gaeumannomyces graminis (Sacc.) Arx and D. Olivier var avenae (E. M. Turner) Dennis and summer patch caused by Magnaporthe poae Landschoot and Jackson symptoms have also been shown to be suppressed by applications of elemental sulfur or acidifying nitrogen sources (Landschoot et al., 1991). Monthly applications of ammonium sulfate or ammonium chloride have also been shown to reduce spring dead spot caused by Ophiophaerella herpotricha J. C. Walker and O. korrae Walker and Smith by 41% (Dernoeden et al., 1991). Fungal development within the organic matter layer may be impacted by these repeated applications of soil pH-altering fertilizers and therefore influence thatch collapse severity.

The unusual fruiting structures and mechanisms have interested mycologists since the discovery of Sphaerobolus. Although the biology, ecology and management of S. stellatus are understood on mulch, there is a lack of information of S. stellatus in turfgrass systems. Thatch collapse represents a new disease that may be caused by one or more previously unidentified organisms. Little to no information regarding the causal agent(s) of thatch collapse is available and the role of S. stellatus as a causal agent of thatch collapse, despite its consistent association with symptomatic tissues, remains unknown. The objectives of this study are to: 1) identify the causal agent(s) of thatch collapse and document its (their) geographic distribution; 2) elucidate
biological aspects of the fungus; and 3) evaluate the lignocellulose-degrading abilities of selected fungi associated with thatch collapse.
Figure 1. Thatch collapse symptoms include a dark green depressed patch due to the degradation of organic matter.
Chapter 2

Geographic Distribution, Identification of Thatch Collapse Fungi, and Biology of *Sphaerobolus stellatus*

Abstract

In 2010, previously unreported disease symptoms were observed on golf course putting greens located in the United Kingdom and Pennsylvania. Since this initial discovery, similar symptoms were subsequently reported in California, Michigan, South Dakota, and New Zealand on golf putting greens and fairways. Symptoms of this disease include circular patches of degraded organic matter ranging from 8 to 46 cm in diameter. Thatch degradation results in an indentation of the turf surface and disruption in playability. Commonly found in association with the thatch degradation is a basidiomycete not previously reported within stands of any turfgrass. Fungal signs include profuse mycelium with clamp connections within the upper 2.5 cm of the soil/thatch profile and peridia similar to those produced by *Sphaerobolus stellatus* within the thatch and canopy of golf course putting greens. Little is known about *S. stellatus* within turfgrass systems or the association of this fungus to thatch collapse. Therefore, the objectives of this study were to: 1) identify the causal agent(s) of thatch collapse and document its (their) geographic distribution; 2) document the morphology of collected isolates; 3) determine the cardinal temperatures for growth of thatch collapse isolates; and 4) assess optimal temperatures for gleba production. Peridia of *S. stellatus* were surface disinfested and grown on antibiotic water agar prior to growth on antibiotic oatmeal agar (AOA). After 2 to 3 weeks of growth on AOA, plates were covered with compressed white mycelium and new peridia with mature gleba were produced. Mycelial plugs were incubated in nutrient broth and the DNA of isolates (n = 11) from California, Michigan, Montana, South Dakota, and New Zealand were extracted using a DNEasy DNA extraction kit. Amplification of the ribosomal internal transcribed spacer region (ITS) with primer set ITS4/ITS5 resulted in 695 to 724-bp sequences from all isolates with a 98% to 99%
similarity to known isolates of *Sphaerobolus stellatus*. To confirm the thatch degrading ability of *S. stellatus*, 12 samples (15 cm diameter x 5 cm deep) of mature Penn ‘A-1’ creeping bentgrass were inoculated with 0.6 g of *S. stellatus*-infested thatch. After 6 weeks of incubation at 12°C in continuous light, *S. stellatus* was successfully re-isolated from colonized thatch. Total degradation of organic matter was determined using the loss on ignition method and by direct measurements of thatch depth. When compared to the uninoculated control, plugs inoculated with *S. stellatus* exhibited a 21% and 28% decrease of organic matter and thatch depth, respectively.

Morphological characteristics of *S. stellatus* isolates from turf were determined from measurement of gleba and basidiospores. The general morphology of gleba and basidiospores were within the range reported for *S. iowensis* and *S. stellatus*, respectively. The growth rate of three *S. stellatus* isolates in culture was compared at 6 temperatures (5, 10, 15, 21, 25, and 30°C). Differences were found among temperatures, but optimal growth rate ranged from 21 to 30°C. Gleba production was assessed for three *S. stellatus* isolates over 4 temperatures (10, 15, 21, and 25 °C) for 11 weeks. Gleba were produce at 10, 15, 21°C and 25°C by one isolate. Based on these results, *S. stellatus* is a casual agent for rapid organic matter degradation resulting in thatch collapse on golf course turfgrass species within the United States and New Zealand. Based on biological, morphological and molecular information obtained from this study, the fungus isolated from turf is similar to known mulch isolates of *S. stellatus*. 
Introduction

In 2010, a new disease believed to be caused by a basidiomycete was reported on *Festuca* spp. on a golf course in Scotland (Dernoeden et al., 2011). Based on the symptoms expressed on finely mown turf, the disease has been referred to as thatch collapse. Thatch degradation on golf course putting greens and fairways creates sunken, depressed patches. Symptoms of thatch collapse consist of circular patches of dark green turf ranging from 8 to 46 cm in diameter. Decomposing thatch within symptomatic areas contains a characteristic fawn color and a mushroom odor (Dernoeden et al., 2011). Morphological characteristics of mycelia include clamp connections, supporting classification of this fungus as a basidiomycete.

Thatch collapse symptoms are distinct from those caused by other mushroom or puffball-forming basidiomycetes commonly associated with turfgrass. The symptoms may represent a previously unrecognized symptomology caused by one or more species of basidiomycetes or caused by a basidiomycete not previously associated with turf diseases. In late 2011 and 2012, the basidiomycete *Sphaerobolus stellatus* (Tode) Persoon was found in association with thatch collapse symptoms in New Zealand, California, Michigan, Montana and South Dakota. This consistent association has led to the connotation of *S. stellatus* as one possible causal agent of thatch collapse.

The genus *Sphaerobolus* was first acknowledged almost 300 years ago as *Carpobolus* by Micheli (1729). *Sphaerobolus stellatus* colonizes woody lignocellulose substrates for growth and reproduction. This fungus is commonly found on numerous continents inhabiting woody substrates, many different types of dung and forest floors (Buller, 1958). Lately, the large expansion of landscaping mulch has provided an optimal substrate for *S. stellatus*, making this fungus a common entity in homeowners’ mulch beds (Brantley et al., 2001a; Brantley et al., 2001b; Davis et al., 2011).
There have been no published reports regarding basic biology of *S. stellatus* colonizing managed turfgrass systems or causing thatch collapse. Linking *S. stellatus* or any basidiomycete to thatch collapse symptoms remains unwarranted until the following objectives are met: 1) identification of the causal agent(s) of thatch collapse and document its (their) geographic distribution; 2) documentation of the morphology of collected isolates; 3) elucidation of cardinal temperatures for growth of thatch collapse isolates; and 4) determination of optimal temperature for gleba production.

**Materials and Methods**

**Sample collection and isolation**

Symptomatic turf samples were collected from 5 golf courses in 4 states across the United States. Upon submission to the laboratory, samples were incubated in a plastic container lined with moist paper towels. When peridia were present, the fruiting bodies were sterilized by completing a 1 min rinse in 10 % sodium hypochlorite (Bleach Clorox, Oakland, CA) followed by three 30 sec washes in sterilized distilled water. Gleba were dried on sterilized filter paper and transferred to oatmeal agar amended with penicillin G and streptomycin (AOA). Growth medium was prepared by autoclaving single grain baby oatmeal 20 g L\(^{-1}\) (Gerber, Fremont, MI) and bacteriological agar 15 g L\(^{-1}\) (Amresco, Solon, OH) for 25 min at 121°C. Media was allowed to cool to 55°C prior to the addition of penicillin G 0.5 g L\(^{-1}\) (TCI, Portland, OR) and streptomycin sulfate 0.5 g L\(^{-1}\) (Sigma Aldrich Corp., St. Louis, MO). Liquid media with antibiotics was poured in 100 x 15 mm dishes. Peridia-seeded petri plates were sealed with parafilm (American National Can, Chicago, IL) and cultures incubated for 2 weeks at 21°C in constant darkness.

When fruiting bodies were not present, samples were incubated to induce sporulation. Samples were placed within a sealed plastic container lined with moist paper towels and incubated under constant light at 12°C for two weeks or until fruiting bodies developed.
Hyphae development was monitored microscopically (Carl Zeiss Microscopy, LLC, Thornwood, NY) on symptomatic samples. The presence of intracellularly hyphae in plant tissues including leaf, roots, crowns and stolons was examined by staining with methyl blue (Sigma Aldrich Corp., St. Louis, MO). Infested organic matter was also microscopically assessed for intracellular colonization using the methyl blue stain.

**DNA sequencing and comparison with known fungal species**

Autoclaved potato dextrose broth (PDB) containing 9 mL of 24 g L\(^{-1}\) (Difco, Franklin Lakes, NJ) was added to 20 x 116 mm test tubes. Cultures were grown in PDB for 3 weeks at 22±1°C. DNA was extracted using a DNEasy isolation kit (QIAGEN, Inc., Valencia, CA) from lyophilized mycelia fragments that had been ground with a mortar and pestle in lysis buffer. Polymerase chain reaction (PCR) was used to amplify the ITS region of the nuclear ribosomal RNA gene repeat. The PCR reaction mixture contained 27.9 μL PCR water, 10 μL 10x PCR buffer (0.5M KCl, 0.1M Tris HCl pH 8.3), 5μL 2 mM dNTP mix, 4 μL 25 mM MgCl\(_2\), 1 μL reverse primer ITS4 and 1 μL forward primer ITS5, 1 μL DNA template and 0.1 μL of Taq DNA polymerase (White et al., 1990). PCR reactions were performed in a thermocycler (PTC-100 Programmable Thermal Controller, MJ Research, Inc.) using the following program: 94°C for 5 min; followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min; and 72°C for 5 min. Amplification products were separated by electrophoresis in a 1.0% agarose gel, stained with ethidium bromide for visualization, and purified using an EXOSAP-IT PCR Purification Kit (USB Corporation, Cleveland, OH). Purified amplification products were sequenced at the Pennsylvania State University Nucleic Acid Facility on an ABI 3730XL automated DNA sequencer.

Consensus sequence data were assembled and edited using Sequencer 3.1 (Gene Codes, Ann Arbor, MI) from both forward and reverse DNA sequences. Consensus 20 sequences were scanned through NCBI BLASTN (National Center for Biotechnology Information, Bethesda, MD) and compared with sequences from known fungal species.
The consensus sequences were imported into Molecular Evolutionary Genetics Analysis (MEGA), version 5 (Center for Evolutionary Medicine and Informatics, The Biodesign Institute, Tempe, AZ). Sequences were aligned using ClustalW (European Bioinformatics Institute, Cambridgeshire, UK). Phylogenetic trees were constructed using the Neighbor-Joining method and a bootstrap consensus tree was inferred by 1000 replicates (Saitou et al., 1987). The evolutionary distances were computed using the P-distance method (Nei et al., 2000). To confirm the Neighbor-Joining reconstructed tree, Maximum Likelihood method analyses was also performed based on the Tamura-Nei model. A bootstrap consensus tree was inferred from 1000 replicates (Tamura et al., 1993; Felsenstein, 1985). The initial tree for the heuristic search was obtained by applying the Neighbor-Join and BIoNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach and then selecting the topology with a superior log likelihood value.

**Confirmation of the causal agent**

To confirm a cause and effect relationship between thatch collapse and *S. stellatus*, Koch’s postulates were performed with *S. stellatus* isolated from turf. A thatch-infested inoculum was developed and used for the procedure. Organic matter was obtained from the Joseph E. Valentine Turfgrass Research Center located in University Park, PA in November of 2011. Living turf from a mature stand of ‘A-4’ creeping bentgrass was removed by mowing to a height of 2.0 mm with a Greensmaster 1000 walk mower (Toro, Bloomington, Minnesota). Organic matter was then obtained by vertically slicing into the canopy with a Ryan Mataway slicer (Ryan, Johnson Creek, WI) and all material collected. Organic matter was dried at 65°C for 7 days and stored in dry plastic containers prior to use.

Flasks (250 mL) containing 1.0 g of dried organic matter were stopped with cotton and covered with aluminum foil prior to sterilization at 121°C for 90 min on 3 consecutive days. Three equally-spaced gleba obtained from pure cultures of *S. stellatus* isolated from a golf course
in Spearfish, SD (SS-SD) were placed on AOA in a 15-cm plate. One gram of sterile organic matter moistened with 3 mL of sterilized distilled water was placed atop the seeded AOA plates. Plates were sealed with parafilm and incubated under continuous light at 22 ±1°C until peridia formed. Infested organic matter with developed peridia was utilized to inoculate healthy turf.

In May and July of 2012, 12 plugs (15 cm diameter) of mature Penn ‘A-1’ creeping bentgrass were removed from the Joseph E. Valentine Turfgrass Research Facility. Four plugs each were inoculated with 0.6 g of *S. stellatus*-infested organic matter (isolate SS-SD) at the soil/thatch interface (2 cm depth) within the center of the plug. Inoculations with uninfested sterilized organic matter as well as non-inoculated plugs served as untreated controls. A plug from each treatment was placed in plastic containers and incubated at 12°C under 24 hr light and monitored for gleba development in the thatch layer as well as degradation of organic matter. Upon fruiting body development and thatch degradation, *S. stellatus* was re-isolated onto AOA. Thatch layer depth was determined by placing a 1 kg weight on the surface of a vertically bisected plug (Linde et al., 1999). Compressed thatch depth was measured from the crowns of the turf to the beginning of the soil layer.

Total organic matter content across treatments was compared 6 weeks post inoculation by loss-on-ignition (Ben-Dor et al., 1989). One 1.5 cm diameter x 4 cm long core was taken 3 cm from the inoculation point on each plug. Cores were air-dried and ground to < 0.4 mm using a mortar and pestle. Crucibles were heated to 100°C for 2 hours and placed in a desiccator over CaCl₂ to cool. A subsample of 2.5 g of each ground sample was placed in the tared crucible and placed inside a 1330GM VWR drying oven (VWR, Radnor, PA) for 24 hours at 105°C. The sample was reweighed after the crucible had cooled in the desiccator. Crucibles were placed in a Thermolyne ashing oven (Sybron Corp., Dubuque, IA) at 400°C for 16 hours and then cooled in a desiccator over CaCl₂. The subtraction method was used to calculate the weight of the 400°C samples (Ben-Dor et al., 1989). Loss-on-ignition (LOI) content of the sample was calculated using the following formula:
LOI, % = \( \frac{\text{Weight}_{105} - \text{Weight}_{400}}{\text{Weight}_{105}} \times 100 \)

The experiment was arranged as a completely randomized design with 4 replications and repeated twice. Statistical analyses were performed using PROC MIXED of SAS v. 9.3 (SAS institute, Cary, NC). Means were separated at \( P \leq 0.05 \) using Tukey’s least significant different test.

**Morphology of isolates**

Gleba and basidiospore morphology were assessed using three isolates obtained from golf courses in California (SS-CA-1), Michigan (SS-MI) and South Dakota (SS-SD) (Table 1). Isolates of *S. stellatus* were grown on OA and placed in the dark for 3 weeks. Growth medium was made using the aforementioned agar recipe without antibiotics. Cultures were incubated at room temperature (22±1°C) under 24 hr fluorescent lighting to induce fruiting body production. Ejected gleba were randomly collected and characterized. Ten basidiospores were randomly selected within a crushed glebae for measurement and repeated for 5 gleba. The length and width of total 50 basidiospores and 30 gleba were measured using an Axio Cam HRC (Carl Zeiss Microscopy, LLC, Thornwood, NY) at 10x for gleba and 40x for basidiospores using Axio Vision software AxioVs40V4.8.0.0 (Carl Zeiss Microscopy, LLC, Thornwood, NY) (Geml et al., 2005). Mean length and width of *S. stellatus* isolates from turf were compared using PROC MIXED of SAS v. 9.3 (SAS institute, Cary, NC). Means were separated at \( P \leq 0.05 \) using Tukey’s least significant difference test.

**Cardinal temperatures for growth of pathogen in culture**

Growth rates of three *S. stellatus* isolates (SS-CA-1, SS-MI and SS-SD) were assessed at 6 temperatures (5, 10, 15, 21, 25 and 30°C). A 5-mm circular plug from the edge of an actively growing fungal colony was placed in the center of a petri plate containing 15 to 20 mL OA. Plates
were sealed with parafilm and incubated in the dark at one of the 6 aforementioned temperatures. Colony diameters (cm) were measured twice week\(^1\) in 2 perpendicular directions until *S. stellatus* crossed the plate. The experiment within each growth chamber was arranged as a completely randomized design with 4 replications and the experiment was repeated twice (Ingold, 1972). Average daily growth was analyzed using PROC MIXED of SAS v. 9.3 (SAS institute, Cary, NC). Means were separated at \(P \leq 0.05\) using Tukey’s least significant different test.

**Temperatures favoring gleba production in culture**

Gleba production was evaluated at 4 temperatures (10, 15, 21 and 25°C) using three isolates obtained from golf courses in California (SS-CA-1), Michigan (SS-MI) and South Dakota (SS-SD) (Ingold, 1972). One surface-sterilized glebae from each isolate was placed in the center of a petri plate containing 15 to 20 mL OA. Plates were sealed with parafilm and incubated in the dark for 5 weeks at 21°C prior to incubation in 12 hr light at one of 4 temperatures. Gleba production was assessed weekly for 11 weeks by counting the number of gleba discharged (Geml, 2004). The experiment within each incubator was arranged as a completely randomized design with 4 replications and the experiment repeated twice. Average weekly gleba production was analyzed using PROC MIXED of SAS v. 9.3 (SAS institute, Cary, NC). Means were separated at \(P \leq 0.05\) using Tukey’s least significant difference test.

**Results**

**Sample collection, isolation, and description**

Between 2011 and 2012, *S. stellatus* was isolated from mixed stands of creeping bentgrass and annual bluegrass in California (n=2), Michigan (n=1), Montana (n=1), and South Dakota (n=1). Five isolates were also received from two separate locations in New Zealand (n=5) (Table 1).

The SD isolate obtained from L. Miller, was isolated from a modified USGA green in which the organic mixture component was comprised of wood fibers from a local sawmill. This isolate produced large quantities of peridia and gleba when grown on OA. Five isolates provided
by M. Cushnahan were isolated by M. Christensen from symptomatic turfgrass from three
locations in Lake Wanaka (n=1) and Hawkes Bay (n=3), New Zealand. The MI isolate was
acquired from a putting green approach with excessive thatch (~3 cm). A large dense area of
creamy-orange mycelia within the thatch was noted in the MI sample, but no peridia were present
upon submission to the lab. After 3 wks of exposure to 24 hr of light, fruiting bodies developed
within the thatch layer, but few peridia developed in culture.

In January 2012, a symptomatic turf sample from Harding Park G.C. in San Francisco
CA contained peridia and gleba within the turf canopy. The sample was from a creeping
bentgrass/annual bluegrass green with a Californian greens construction (Davis, 1973). Once in
culture, very few fruiting bodies developed on OA. In late July, a symptomatic sample was
submitted from Franklin Canyon G.C. located in Hollister, CA. No peridia were found in the
sample, and dense areas of mycelia were not observed. After incubating for 4 months, however,
peridia developed in the creeping bentgrass turf canopy. In August 2012, an infested turf sample
from Larchmont G.C. in Missoula, MT contained numerous peridia and gleba within the turf
canopy. The sample was from a modified USGA green containing a high percentage of fine sand.
When the sample was exposed to light, no further peridia developed and few were produced in
culture.

*Sphaerobolus stellatus* was isolated from fruiting body development in the turf canopy or
thatch layer from all symptomatic samples. Hyphae containing clamp connections were observed
intertwining with the organic matter and on the outside of leaf tissue. The white mycelia
colonizing the organic matter produced an orange hue in the thatch along the outer edges of
patches (Figure 1). However, microscopic examination of stained tissues within samples revealed
that hyphae were not found inside leaves, roots, crowns or stolons.

Once isolates were in culture, colony morphology varied among isolates. In culture,
South Dakota and California isolates produced creamy white, stringy, aerial mycelia that
extended upwards toward the lid of the petri dish. The Michigan and Montana isolates formed
creamy white appressed mycelia. All isolates produced concentric rings as the mycelia grew across the media.

**Confirmation of the causal agent**

The growth of *S. stellatus* was allowed to naturally progress over 6 weeks on incubated plugs. No differences between thatch depth ($P = 0.0723$) or total organic matter ($P = 0.5390$) were observed between successive experiments and therefore data were combined for statistical analyses (Table 2; Table 3). Thatch layer depth was significant ($P < 0.0001$) across treatments (Table 2). The *S. stellatus*-infested treatment had a thatch layer depth of 1.46 cm and was significantly different when compared to the control (1.95 cm) and sterilized thatch (1.88 cm) treatments (Figure 2). Due to the lack of differences between the uninfested control and sterilized-thatch control, percent organic matter was only determined for *S. stellatus* infested samples and the uninfested control samples. Percent organic matter was significantly lower in the *S. stellatus*-infested treatment (1.13%) when compared to the control (1.42%) (Figure 3). In all experiments, *S. stellatus* was successfully re-isolated from infested plugs.

**DNA sequencing**

In general, DNA sequencing of the ITS region revealed a 99% sequence similarity to a known *S. stellatus* mulch isolate (Accession number: AY487975.1). One exception was for isolate SS-MT, which was 98% similar to *S. stellatus* accession AY487975.1. The phylogenetic tree created using the Neighbor-Joining method utilized 656 of the 695 to 724 characters in the data sets. The characters deleted were gaps created from sequence alignment. Two distinct clades resulted from the Neighbor-Joining method within the *S. stellatus* clade (Figure 4). The first clade included *S. stellatus* from New Zealand (ICMP 18280), SS-SD, ICMP 18281, SS-CA-1, ICMP 18279, and SS-MI. The second clade included isolates, SS-MT and SS-CA-2. A second phylogenetic analysis created using the Maximum Likelihood method confirmed the tree structure produced by the Neighbor-Joining method (Figure 5). Bootstrap values were greater than 50% for all sub clades of *S. stellatus*. 
Gleba and basidiospore characteristics

Thirty gleba and fifty basidiospores from 5 measured gleba were characterized for isolates SS-CA-1, SS-MI and SS-SD. *Sphaerobolus stellatus* isolate SS-MI produced no gleba in culture and therefore was excluded from the analyses. Average glebae length and width for turf isolates was 833.71 μm and 686.29 μm, respectively (Table 4). Gleba produced by SS-CA-1 were significantly ($P = 0.017$) wider (718.04±22.14 μm) than SS-SD (654.54±11.67 μm), but both isolates had similar glebae lengths of 830.31±20.12 μm and 837.12±20.12 μm for SS-SD and SS-CA-1, respectively.

Average basidiospore length and width was 7.62 μm and 4.77 μm, respectively and lengths were similar for all isolates. Widths of basidiospores were significantly different ($P < 0.0001$) with SS-CA producing wider spores (5.0931±0.06 μm) than SS-SD (4.45±0.07 μm) (Table 4).

Cardinal temperatures for growth of *S. stellatus* in culture

Growth rates of three *S. stellatus* isolates (SS-CA-1, SS-MI and SS-SD) were examined over 6 temperatures. A significant study effect ($P = 0.0057$) was observed, therefore, data from each study were analyzed separately (Table 5).

*Study I.* Differences in average daily growth rate of all isolates were significant at 4 temperatures ($P < 0.0001$) (Table 6). All isolates had the slowest daily growth when incubated at 5°C (Figure 5A). Average daily growth increased with increasing temperatures from 5 to 25°C. Both SS-CA-1 and SS-MI had a significant decrease of daily growth at 30°C (Figure 6A). Generally, at 10, 21, 25 and 30°C SS-SD and SS-CA-1 isolates sustained the greatest and lowest growth rate, respectively of the three isolates (Figure 6A).

*Study II.* Significant differences of growth rates were observed at 5 of the 6 temperatures included in this study ($P < 0.0001$). When incubated at 5°C, all isolates grew at the slowest rate. Daily growth increased for all isolates as the temperature increased from 5 to 21°C (Figure 6B). At 25 and 30°C, growth rates for each isolate varied, but growth generally was sustained or
slightly decreased for all three isolates (Figure 6B). At each temperature evaluated, SS-SD generally exhibited the greatest daily growth, while SS-CA-1 exhibited the lowest growth of the three isolates.

**Temperatures favoring gleba production in culture**

The influence of temperature on *S. stellatus* gleba production of three isolates (SS-CA-1, SS-MI and SS-SD) was examined for 11 weeks. No significant study effect was observed for gleba production, therefore data were combined for statistical analyses (Table 7).

Fruiting bodies started developing 1 week after exposure to light and gleba were discharged during the second week of the study when incubated at 21 and 25°C. Gleba production of isolates incubated at 10 to 15°C was not observed until approximately 3 to 4 weeks after incubation (Figure 7). The length of time gleba were produced also varied by temperature. At 21°C, all gleba production occurred within 4 weeks. Gleba production at 10, 15 and 25°C, however, ceased after 11, 8 and 6 weeks, respectively (Figure 7). Isolate SS-SD produced gleba at all temperatures, whereas isolate SS-CA-1 only produced gleba at 10 and 15°C and isolate SS-MI produced gleba at 15°C (Table 8; Figure 8).

Differences of total gleba production were found at two temperatures for SS-SD. Gleba production was highest at 10°C and the least amount were produced at 25°C. Gleba production was similar at 15 and 21°C and between production levels at 10 or 25°C (Table 8).

**Discussion**

*Sphaerobolus stellatus* was isolated from fruiting bodies within the turf canopy or thatch layer from all symptomatic samples. Peridia developed on organic matter, illustrating the organic matter use as a growing substrate. Although hyphae were observed within the organic matter, no visible signs of *S. stellatus* were found within plant tissues, providing further evidence that the organism is not pathogenic. The white mycelia colonizing the organic matter produced an orange hue where the organic matter was breaking down, associating profuse mycelia development with organic matter degradation.
DNA sequencing of the ITS region revealed that the thatch collapse isolates were S. *stellatus*. The sub clade development within the species of *S. stellatus* revealed slight changes within the ITS region. The amount of base pairs deleted during alignment indicates the ITS region are difficult to align between species (Geiser et al., 2001; Geml and Royse 2002; Geml et al., 2000; O’Donnell et al., 1998; Peinter et al., 2003). This difficulty may be correlated to a large number of unknown taxa that could fill the phylogenetic gaps (Geml, 2005).

*Sphaerobolus stellatus* isolate SS-SD induced a reduction of the thatch layer and developed an orange hue upon colonization of thatch. The reduction of the thatch layer and total organic matter in plugs inoculated with *S. stellatus* indicates that thatch was decreasing and organic matter was degrading. Reisolation of *S. stellatus* from symptomatic samples confirm its association with and role in the development of thatch collapse (Agrios, 1997).

The organic matter breakdown by *S. stellatus* is consistent with prior applications of this fungus for organic matter reduction in contaminated soils. *Sphaerobolus stellatus* was found to reduce soil organic carbon by 20% over 6 months in laboratory experiments (Winquist, 2009). Percent organic matter reduction from a *S. stellatus* isolate from turf occurred over a shorter period of time in this study when compared to the level of reduction found by Winquist over 6 months (2009). The accelerated organic matter reduction in turf plugs from thatch collapse isolate SS-SD exhibits the capabilities of this fungus to cause thatch collapse on golf playing surfaces.

The morphology of the thatch collapse causal agent was similar to a combination of two known *Sphaerobolus* species. Gleba were generally rectangular in shape. This unusual morphology likely was due to compression of gleba when hitting the lids of petri plates. The gleba length of *S. stellatus* isolates examined in this study were more closely related to the diameter of *S. ingoldii* (977 μm) than *S. stellatus* from studies where diameter was recorded because gleba were found to be oval (Geml, 2004). The basidiospores for both isolates were similar to *S. stellatus*, however, isolate SS-CA-1 produced slightly larger basidiospores than SS-SD (Geml, 2004). Gleba were smaller than previously reported for *S. stellatus*, which may
correlate to the placement of thatch collapse isolates into a sub clade of *S. stellatus*. Limited sample populations may account for the slight variation as speciation of *Sphaerobolus* based on morphology alone is difficult due to overlap within the measured structures.

To understand the biology of *S. stellatus* isolated from turf, average daily growth of three turf isolates in culture was determined. Growth of isolates evaluated in this study were similar to those previously reported (Alasuadura, 1963). Although slight differences were observed, *S. stellatus* growth increased with temperatures from 5 to 25°C. Growth then remained constant or slightly decreased daily growth at 30°C. Mean daily growth of isolates SS-CA-1 and SS-MI were similar to known *S. stellatus* at 25°C (Geml, 2004). However, daily growth of isolate SS-SD was faster and more comparable to *S. ingoldii* (Geml, 2004).

The projectile gleba could potentially serve as an important source of inoculum for the spread of *S. Stellatus* and development of thatch collapse. Assessment of gleba production illustrated that gleba were formed over a wide range of temperatures. However, only isolate SS-SD produced copious amounts of gleba in our growth chamber study. The maximum amount of gleba was produced at 10°C, but production did not begin until 4 weeks after exposure to light. This delayed abundant production of gleba at 10°C was also noted by Alasuadura (1963). Isolate SS-SD followed the same trend as reported by Alasuadura with higher amounts of gleba produce at 10, 15 and 20°C than at 25°C. However, the SS-SD isolate produced the highest levels at 10°C, whereas with Alasuadura’s study isolates of *S. stellatus* produced the most gleba at 15°C. The decrease in gleba production at 25°C was also observed in other studies, indicating an optimum smaller temperature range for basidiocarp production, than that observed for vegetative growth (Walker, 1927; Ingold, 1972). While higher temperatures appear to be optimum for mycelia growth, gleba production of *S. stellatus* isolates is maximized at lower temperatures.

The isolation of *S. stellatus* from numerous locations across from the continental United States and New Zealand, and conclusive fulfilling of Koch’s postulates confirms *S. stellatus* as a causal agent of thatch collapse.
Table 1. Isolate designation, location, date received and turfgrass species of 9 golf courses confirmed to be affected by thatch collapse or *Sphaerobolus stellatus*, 2011 to 2012.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Golf Course</th>
<th>City, State</th>
<th>Date Received</th>
<th>Species</th>
<th>Collector</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-SD</td>
<td>Spearfish Canyon G.C.</td>
<td>Spearfish, SD</td>
<td>10 Oct 2011</td>
<td>CBG&lt;sup&gt;a&lt;/sup&gt;, ABG</td>
<td>Miller</td>
</tr>
<tr>
<td>SS-CA-1</td>
<td>Harding Park G.C.</td>
<td>San Francisco, CA</td>
<td>16 Sept 2011</td>
<td>CBG, ABG</td>
<td>Baetsen</td>
</tr>
<tr>
<td>SS-MI</td>
<td>Little Traverse Bay G.C.</td>
<td>Harbor Springs, MI</td>
<td>20 Nov 2011</td>
<td>CBG, ABG</td>
<td>Baetsen</td>
</tr>
<tr>
<td>SS-CA 2</td>
<td>Franklin Canyon G.C.</td>
<td>Hercules, CA</td>
<td>27 Jul 2012</td>
<td>CBG</td>
<td>Baetsen</td>
</tr>
<tr>
<td>SS-MT</td>
<td>Larchmont G.C.</td>
<td>Missoula, MT</td>
<td>13 Aug 2012</td>
<td>CBG, ABG</td>
<td>Baetsen</td>
</tr>
<tr>
<td>ICMP 18282</td>
<td>Jacks Point G.C.</td>
<td>Lake Wanaka, NZ</td>
<td>13 Nov 2009</td>
<td>BTBG</td>
<td>Christensen</td>
</tr>
<tr>
<td>ICMP 18279</td>
<td>Cape Kidnappers G.C.</td>
<td>Hawkes Bay, NZ</td>
<td>13 Nov 2009</td>
<td>BTBG</td>
<td>Christensen</td>
</tr>
<tr>
<td>ICMP 18280</td>
<td>Cape Kidnappers G.C.</td>
<td>Hawkes Bay, NZ</td>
<td>13 Nov 2009</td>
<td>BTBG</td>
<td>Christensen</td>
</tr>
<tr>
<td>ICMP 18281</td>
<td>Cape Kidnappers G.C.</td>
<td>Hawkes Bay, NZ</td>
<td>13 Nov 2009</td>
<td>BTBG</td>
<td>Christensen</td>
</tr>
</tbody>
</table>

<sup>a</sup> CBG = creeping bentgrass (*Agrostis stolonifera* L.), ABG = annual bluegrass (*Poa annua* L.) and BTBG = browntop bentgrass (*Agrostis capillaris* L.).
Table 2. Influence of *Sphaerobolus stellatus* on thatch degradation of Penn ‘A-1’ creeping bentgrass as measured by thatch depth in a controlled environment chamber.

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>Num DF(^a)</th>
<th>Den DF(^b)</th>
<th>F Value(^c)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum</td>
<td>2</td>
<td>15</td>
<td>0.64</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time</td>
<td>1</td>
<td>15</td>
<td>22.32</td>
<td>1.0000</td>
</tr>
<tr>
<td>Inoculum*time</td>
<td>2</td>
<td>15</td>
<td>0.00</td>
<td>0.5390</td>
</tr>
</tbody>
</table>

\(^a\) Numerator degrees of freedom.

\(^b\) Denominator degrees of freedom.

\(^c\) Calculated F value according to the equation F = MS\(_{between}\)/MS\(_{within}\).
Table 3. Influence of *Sphaerobolus stellatus* on thatch degradation of Penn A-1 creeping bentgrass as measured by percent total organic matter in a controlled environment chamber.

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>Num DF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Den DF&lt;sup&gt;b&lt;/sup&gt;</th>
<th>F Value&lt;sup&gt;c&lt;/sup&gt;</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum</td>
<td>1</td>
<td>9</td>
<td>12.49</td>
<td>0.0064</td>
</tr>
<tr>
<td>Time</td>
<td>1</td>
<td>9</td>
<td>0.22</td>
<td>0.6513</td>
</tr>
<tr>
<td>Inoculum*time</td>
<td>1</td>
<td>9</td>
<td>4.14</td>
<td>0.0723</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numerator degrees of freedom.

<sup>b</sup> Denominator degrees of freedom.

<sup>c</sup> Calculated F value according to the equation $F = \frac{MS_{between}}{MS_{within}}$. 
Table 4. Gleba and basidiospore dimensions of two *Sphaerobolus stellatus* isolates from California (SS-CA-1) and South Dakota (SS-SD) in culture.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gleba</th>
<th></th>
<th>Basidiospore</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (μm)</td>
<td>Width (μm)</td>
<td>Length (μm)</td>
<td>Width (μm)</td>
</tr>
<tr>
<td>SS-SD</td>
<td>830.31±20.12</td>
<td>654.54±11.67</td>
<td>7.83±1.02</td>
<td>4.45±0.06</td>
</tr>
<tr>
<td>SS-CA</td>
<td>837.12±20.12</td>
<td>718.04±22.41</td>
<td>7.42±0.11</td>
<td>5.09±0.07</td>
</tr>
<tr>
<td><em>P</em>-value</td>
<td>0.817</td>
<td>0.017</td>
<td>0.701</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Length and width of 30 gleba and 50 basidiospores measured with a Zeiss Axio Cam HRC at 10x for gleba and 40x for basidiospores in program AxioVs40V4.8.0.0.

*y* Mean ± standard error.
Table 5. Influence of temperature on average daily growth in culture of *Sphaerobolus stellatus* isolates in a controlled environment chamber.

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>Num DF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Den DF&lt;sup&gt;b&lt;/sup&gt;</th>
<th>F Value&lt;sup&gt;c&lt;/sup&gt;</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2</td>
<td>101</td>
<td>5.86</td>
<td>0.0039</td>
</tr>
<tr>
<td>Isolate&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5</td>
<td>101</td>
<td>4.43</td>
<td>0.0011</td>
</tr>
<tr>
<td>Time</td>
<td>10</td>
<td>101</td>
<td>2.42</td>
<td>0.0126</td>
</tr>
<tr>
<td>Temperature*isolate</td>
<td>10</td>
<td>101</td>
<td>1.24</td>
<td>0.2744</td>
</tr>
<tr>
<td>Temperature*time</td>
<td>1</td>
<td>101</td>
<td>36.90</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Isolate*time</td>
<td>2</td>
<td>101</td>
<td>49.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Temperature<em>isolate</em>time</td>
<td>5</td>
<td>101</td>
<td>560.21</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numerator degrees of freedom.

<sup>b</sup> Denominator degrees of freedom.

<sup>c</sup> Calculated F value according to the equation $F = \frac{MS_{between}}{MS_{within}}$.

<sup>d</sup> Temperatures ranged from 5 to 30°C.

<sup>e</sup> *Sphaerobolus stellatus* isolates from California (SS-CA-1), Michigan (SS-MI) and South Dakota (SS-SD).
Table 6. Average daily growth of three *Sphaerobolus stellatus* isolates in culture from California (SS-CA-1), Michigan (SS-MI) and South Dakota (SS-SD) in a controlled environment chamber.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>SS-CA-1</th>
<th>SS-MI</th>
<th>SS-SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature °C</td>
<td>Daily growth rate (mm)(^z)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.2 (^d)</td>
<td>0.2 (^e)</td>
<td>0.5 (^d)</td>
</tr>
<tr>
<td>10</td>
<td>0.4 (^c)</td>
<td>0.5 (^d)</td>
<td>0.6 (^c)</td>
</tr>
<tr>
<td>15</td>
<td>1.1 (^b)</td>
<td>1.1 (^c)</td>
<td>1.2 (^b)</td>
</tr>
<tr>
<td>21</td>
<td>1.5 (^a)</td>
<td>1.6 (^a)</td>
<td>1.7 (^a)</td>
</tr>
<tr>
<td>25</td>
<td>1.6 (^a)</td>
<td>1.6 (^a)</td>
<td>1.8 (^a)</td>
</tr>
<tr>
<td>30</td>
<td>1.1 (^b)</td>
<td>1.4 (^b)</td>
<td>1.6 (^a)</td>
</tr>
<tr>
<td><em>P</em>-value</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.4 (^e)</td>
<td>0.3 (^d)</td>
<td>0.5 (^e)</td>
</tr>
<tr>
<td>10</td>
<td>0.5 (^d)</td>
<td>0.6 (^c)</td>
<td>0.8 (^d)</td>
</tr>
<tr>
<td>15</td>
<td>1.0 (^c)</td>
<td>1.1 (^b)</td>
<td>1.2 (^c)</td>
</tr>
<tr>
<td>21</td>
<td>1.7 (^a)</td>
<td>1.5 (^a)</td>
<td>2.0 (^a)</td>
</tr>
<tr>
<td>25</td>
<td>1.7 (^a)</td>
<td>1.5 (^a)</td>
<td>1.9 (^ab)</td>
</tr>
<tr>
<td>30</td>
<td>1.5 (^b)</td>
<td>1.5 (^a)</td>
<td>1.7 (^b)</td>
</tr>
<tr>
<td><em>P</em>-value</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\(^z\) Daily growth was determined by measuring colony diameter in two perpendicular directions bi-weekly divided by total growing days.

\(^y\) Means followed by the same letter are not significantly different at *P* ≤ 0.05 according to the Tukey’s least significance difference test.
Table 7. Influence of temperature on gleba production in culture of three *Sphaerobolus stellatus* isolates by week in a controlled environment chamber.

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>0.0144</td>
<td>0.0132</td>
<td>0.0546</td>
<td>0.0165</td>
<td>0.2364</td>
<td>0.0987</td>
<td>0.0005</td>
<td>0.0009</td>
<td>0.0673</td>
<td>0.0117</td>
<td>0.1304</td>
</tr>
<tr>
<td>Isolate</td>
<td>0.0047</td>
<td>&lt;0.0001</td>
<td>0.0003</td>
<td>0.0013</td>
<td>0.0015</td>
<td>0.0056</td>
<td>0.0592</td>
<td>0.1027</td>
<td>0.0902</td>
<td>0.4113</td>
<td>0.0001</td>
</tr>
<tr>
<td>Temperature*isolate</td>
<td>0.0026</td>
<td>0.0023</td>
<td>0.0220</td>
<td>0.0031</td>
<td>0.2075</td>
<td>0.0669</td>
<td>0.0612</td>
<td>0.0047</td>
<td>0.0306</td>
<td>0.5001</td>
<td>0.1553</td>
</tr>
<tr>
<td>Temperature*Time</td>
<td>0.8335</td>
<td>0.2048</td>
<td>0.8047</td>
<td>0.6469</td>
<td>0.1870</td>
<td>0.3361</td>
<td>0.9595</td>
<td>0.9573</td>
<td>0.0673</td>
<td>0.0177</td>
<td>0.5904</td>
</tr>
<tr>
<td>Isolate*Time</td>
<td>0.9918</td>
<td>0.4432</td>
<td>0.2891</td>
<td>0.3288</td>
<td>0.6315</td>
<td>0.6312</td>
<td>0.1289</td>
<td>0.0183</td>
<td>0.0902</td>
<td>0.4113</td>
<td>0.7701</td>
</tr>
<tr>
<td>Temperature<em>isolate</em>Time</td>
<td>0.9405</td>
<td>0.1694</td>
<td>0.9198</td>
<td>0.7761</td>
<td>0.1425</td>
<td>0.2614</td>
<td>0.0915</td>
<td>0.0047</td>
<td>0.0306</td>
<td>0.5001</td>
<td>0.4885</td>
</tr>
</tbody>
</table>

*a* P-values ≥ 0.05 indicate no significant difference between data groups.

*b* *Sphaerobolus stellatus* isolates from California (SS-CA-1), Michigan (SS-MI) and South Dakota (SS-SD).
Table 8. Average gleba production in culture of three *Sphaerobolus stellatus* isolates from California (SS-CA-1), Michigan (SS-MI) and South Dakota (SS-SD) at temperatures ranging from 10 to 25°C after 11 weeks in a controlled environment chamber.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Temperature °C</th>
<th>Temperature °C</th>
<th>Temperature °C</th>
<th>Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>15</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>SS-CA-1</td>
<td>3 b(^y)</td>
<td>14 b</td>
<td>0 b</td>
<td>0 b</td>
</tr>
<tr>
<td>SS-MI</td>
<td>0 b</td>
<td>4 b</td>
<td>0 b</td>
<td>0 b</td>
</tr>
<tr>
<td>SS-SD</td>
<td>53 a</td>
<td>21 a</td>
<td>38 a</td>
<td>13 a</td>
</tr>
<tr>
<td>(P)-value</td>
<td>0.0063</td>
<td>0.0031</td>
<td>0.0064</td>
<td>0.0091</td>
</tr>
</tbody>
</table>

\(^z\) Gleba production was assessed by counting new gleba produced on a weekly basis in a colonized plate exposed to 24 hr. light.

\(^y\) Means followed by the same letter are not significantly different at \(P \leq 0.05\) according to the Tukey’s least significance difference test.
Figure 1. Symptoms of thatch collapse are circular patches of dark green turf ranging from 15 to 46 cm (A), degraded organic matter creates sunken depressed areas (B), decomposing thatch contains a fawn color and a mushroom odor (C), and signs contain profuse mycelia within the upper 2.5 cm of the soil/thatch interface (D), clamp connections within mycelia (E) and peridia development within thatch and the turfgrass canopy (F).
Figure 2. Thatch depth of Penn ‘A-1’ creeping bentgrass plugs after 6 weeks of incubation at 12°C. Inoculum included an untreated control, sterilized thatch and *Sphaerobolus stellatus*-infested thatch. Treatment means followed by the same letter are not significantly different according to Tukey’s least significant difference test at $P \leq 0.05$. 
Figure 3. Total percent organic matter of Penn ‘A-1’ creeping bentgrass samples as determined by the loss on ignition method following incubation at 12°C for 6 weeks. Inoculation treatments included and untreated control and *Sphaerobolus stellatus*-infested thatch. Means followed by the same letter are not significantly different according to Tukey’s least significant difference test at $P \leq 0.05$. 
Figure 4. Evolutionary relationships between ITS sequences from *S. stellatus* isolates, *S. iowensis* and *S. ingoldii* conducted in MEGA5. The evolutionary history was inferred using the Neighbor-Joining method. The percentages of the replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) and are show next to the branches. The tree is drawn to scale, with branch lengths in the same unit as those of the evolutionary distances to infer the phylogenetic tree. The evolutionary distances were computed using the P-distance method and are the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. All gaps and missing data were eliminated. A total of 656 positions were included in the final data set.
Figure 5. Evolutionary relationships between ITS sequences from *S. stellatus* isolates, *S. iowensis* and *S. ingoldii* conducted in MEGA5. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus trees were inferred from 1000 replicates and are taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees are clustered together in the bootstrap test (1000 replicates) and are shown next to the branches. The initial trees for the heuristic search were obtained automatically and applied to the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. The analysis involved 14 nucleotide sequences. All gaps and missing data were eliminated. A total of 656 positions were included in the final data set.
Figure 6. Average daily growth in culture of three *Sphaerobolus stellatus* isolates (SS-CA-1, SS-MI and SS-SD) at 6 temperatures. Duplicate experiments (A and B) were analyzed separately. Means followed by the same letter are not significantly different according to Tukey’s least significant difference test at $P \leq 0.05$. 

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Figure 7. Average gleba production in culture over 11 weeks of *Sphaerobolus stellatus* isolate from South Dakota (SS-SD) at 4 temperatures. *S. stellatus* isolates from California (SS-CA-1) and Michigan (SS-MI) not included (< 5 gleba produced).
Figure 8. Mean gleba production of three *Sphaerobolus stellatus* isolates in culture on oatmeal agar from California (SS-CA-1), Michigan (SS-MI) and South Dakota (SS-SD) by temperatures ranging from 10 to 25°C after 11 weeks. Combined means followed by the same letter are not significantly different according to Tukey’s least significant difference test at $P \leq 0.05$. 

---

```
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Total gleba produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-SD</td>
<td>a</td>
</tr>
<tr>
<td>SS-CA-1</td>
<td>b</td>
</tr>
<tr>
<td>SS-MI</td>
<td>b</td>
</tr>
</tbody>
</table>
```
Chapter 3

Lignocellulose Degrading Capabilities of *Sphaerobolus stellatus* in Creeping Bentgrass

Abstract

Thatch collapse is a new disease of finely managed turfgrasses caused by *Sphaerobolus stellatus*. Symptoms include dark green, circular patches of turf atop degraded organic matter (e.g., thatch) that results in an indentation of the playing surface. In as short at 6 weeks, *S. stellatus* has been shown to reduce organic matter and thatch depth by 21% and 28%, respectively. To elucidate the enzymes responsible for degradation of organic matter by *S. stellatus* the following objectives were investigated: 1) quantify lignin degradation by *S. stellatus*; and 2) detect manganese independent and dependent peroxidase, laccase, xylanase and cellulase production from *S. stellatus* and *Panaeolina* spp. Utilizing tetramethylammonium hydroxide thermochemolysis, a significant depolymerization of lignin was found when *S. stellatus* colonized sterilized organic matter for 6 weeks. To understand the mechanism responsible for lignin degradation within *S. stellatus*, crude enzymes were extracted from *S. stellatus* turf and mulch isolates and *Panaeolina* spp. at various times over three months. Extracts were assayed for manganese independent and dependent peroxidases, laccase, xylanase and cellulase activities. An elevated level of laccase was found within *S. stellatus* isolates from turf and both isolates (e.g., from mulch and turf) contained low levels of manganese independent peroxidases. All fungal species contained xylanase activity. Cellulase was not verifiably detected. The presence of laccase in *S. stellatus* from turfgrass, manganese independent peroxidase in *S. stellatus* isolates and xylanase in all fungal extracts illustrates the mechanism of organic matter reduction in thatch collapse. Overall, *S. stellatus* isolated from turf effectively degrades lignin within organic matter through laccase and peroxidase production.
Introduction

In 2010, a new disease believed to be caused by a basidiomycete was reported on *Festuca* spp. in Scotland and given the name thatch collapse (Dernoeden et al., 2011). Symptoms of thatch collapse on golf course putting greens and fairways include sunken, depressed patches of dark green turf ranging from 8 to 46 cm in diameter. Decomposing thatch within symptomatic areas has a characteristic fawn color and a mushroom odor (Dernoeden et al., 2011). Morphological characteristics of mycelia include clamp connections, supporting classification of this fungus as a basidiomycete.

Thatch collapse symptoms are distinct from other symptoms produced by mushroom or puffball-forming basidiomycetes commonly associated with turfgrass. The basidiomycete *Sphaerobolus stellatus* (Tode) Persoon was found as a casual agent of thatch collapse in turfgrass swards from New Zealand, California, South Dakota and Michigan. The fungus has also been identified from symptomatic turfgrass in Montana and Australia.

*Sphaerobolus stellatus* is a common white rot fungus on mulch, but only recently was associated with rapid organic matter degradation of golf course turf. Organic matter quickly accumulates on golf courses from the higher concentration of lignin within dead stolons and sheaths of grasses. The average lignin content for velvet bentgrass (*Agrostis canina* L.) was 27% for thatch, where as living tissues contained 15%. Also, Velvet bentgrass has been shown to contain 10% more lignin than cereal straws (Ledeboer et al., 1967). The inability of the microbial populations to break down lignin leads to the accumulation of organic matter and development of a thatch layer (Ledeboer et al., 1967). *S. stellatus* may be modifying the lignin for energy utilization through expression of lignin degrading enzymes.

White rot fungi within the basidiomycota phylum have been extensively researched for enzymatic degradation of lignocellulose (Mandels and Sternberg, 1976). Enzymes involved in the
modification of lignin include ligninases, peroxidases and laccases (Martinez, 2005). These enzymes modify the aromatic structure through the production of unstable radicals, which oxidatively depolymerize the lignin.

Little information is available regarding the breakdown of organic matter on turfgrasses by *S. stellatus*. Therefore, the objectives of this research are to: 1) quantify lignin degradation by *S. stellatus*; and 2) detect manganese independent and dependent peroxidase, laccase, xylanase and cellulase production from isolates of *S. stellatus* and *Panaeolina* spp. to determine the mechanism of lignin degradation.

**Materials and Methods**

**Organic matter collection and sterilization**

In November of 2011, organic matter was obtained from the Joseph E. Valentine Turfgrass Research Center located in University Park, PA. Living turf from a mature stand of ‘Penn A-4’ creeping bentgrass was removed by mowing to a height of 2.0 mm with a Greensmaster 1000 walk mower (Toro, Bloomington, Minnesota). Organic matter was then obtained by vertically slicing into the canopy with a Ryan Mataway slicer (Ryan, Johnson Creek, WI) and all material collected. Organic matter was dried at 65°C for 7 days and stored in dry plastic containers prior to use.

**Inoculation of *Sphaerobolus stellatus* into turf**

Flasks (250 mL) containing 1.0 g of dried organic matter were stopped with cotton and covered with aluminum foil, and sterilized by exposure to 121°C for 90 min on 3 consecutive days. Three gleba equally spaced from each other derived from pure cultures of *S. stellatus* isolated from a golf course in Spearfish, SD (SS-SD) were placed on AOA. Growth medium was prepared by autoclaving single grain baby oatmeal 20 g L⁻¹ (Gerber, Fremont, MI) and bacteriological agar 15 g L⁻¹ (Amresco, Solon, OH) for 25 min at 121°C. Media was allowed to
cool to 55°C prior to the addition of penicillin G 0.5 g L⁻¹ (TCI, Portland, OR) and streptomycin sulfate 0.5 g L⁻¹ (Sigma Aldrich Corp., St. Louis, MO). Liquid media with antibiotics was poured in 150 x 15 mm dishes. One gram of sterile organic matter moistened with 3 mL of sterilized distilled water was placed atop of the seeded AOA plates. Plates were sealed with parafilm and incubated under continuous light at 22 ±1°C until peridia formed. Infested organic matter with developed peridia was utilized to inoculate healthy turf.

In May and July of 2012, 12 plugs (15 cm diameter) of mature Penn ‘A-1’ creeping bentgrass were removed from the Joseph E. Valentine Turfgrass Research Facility. Four plugs each were inoculated with 0.6 g of *S. stellatus*-infested organic matter (isolate SS-SD) at the soil/thatch interface (2 cm) within the center of the plug. Inoculations with uninfested sterilized organic matter as well as non-inoculated plugs served as untreated controls. A plug from each treatment was placed in plastic containers and incubated at 12°C under 24 hr of light and monitored for gleba development in the thatch layer as well as degradation of thatch. Upon fruiting body development and thatch degradation, *S. stellatus* was re-isolated onto AOA.

**Tetramethylammonium hydroxide thermochemolysis**

Organic matter was removed 3 cm from the point of inoculation within the uninfested control (n=8) and SS-SD infested plugs (n= 8) from each replicate of the inoculation experiments. Thatch was air-dried and ground to a fine powder with a mortar and pestle. Samples were put in Eppendorf tubes and sent to the Purdue Stable Isotope Facility within the Earth and Atmospheric Sciences Department. Samples were further ground and 0.5 mg was mixed with 150 μL of tetramethylammonium hydroxide (TMAH) (25 wt % TMAH in methanol; Thermo Fisher Scientific Inc. Waltham, MA) in borosilicate glass tubes by vortexting. Samples were dried under a stream of nitrogen and vacuumed sealed on a manifold before baking at 250°C for 30 min. Cooled tubes were opened and an internal standard of linolenic acid methyl ester (Sigma Aldrich Corp., St. Louis, MO) was added at 54.2 ng μL⁻¹ in ethyl acetate. Sample extraction was
completed by washing the sides of tubes with ethyl acetate (Thermo Fisher Scientific Inc. Waltham, MA). All washings (1 mL per sample) were filtered through glass wool, combined and concentrated to 200 μL under a stream of nitrogen. Products were analyzed by gas chromatography (GC) and mass spectroscopy (MS) on a Shimadzu PYR-4A pyrolyzer connected to a Shimadzu 17A GC and a QP-5000 mass spectrometer (Shimadzu Corp. Kyoto, Japan). The column used for GC separation was 15 m x 0.25 μm (5% penylpolysiloxane – 95% methylpolysiloxane, Restek Rtx-5). Samples (1 μL) were injected onto a split/splitless injector operating in splitless mode at a temperature of 280°C. The column, using helium as the carrier gas, was run with a constant flow (1.0 ml min⁻¹), and the oven temperature program was: 50°C for 2 min, increased from 50°C to 300 at 15°C per minute, and held at 300°C for 6 min 20 sec. The ionization mode on the mass spectrometer was electron ionization at 70 eV, the ion source temperature was 200°C, and the transfer line temperature was 280°C. Mass spectra were collected at a rate of 20 spectra per second after the 120-sec solvent delay. Masses were obtained between m/z 45 and 500. All analyses were preformed using LECO Pegasus III software (version 3.22) (LECO, St. Joseph, MI).

The normal structure for undegraded depolymerized lignin contains 3,4-dimethoxybenzaldehyde (G4) from guaiacyl (G) lignin. The syringyl (S) lignin components produce 3,4,5-trimethoxybenzaldehyde (S4) when depolymerized with TMAH. When fungal enzymes degrade lignin the G4 structure becomes altered to 3,4-dimethoxybenzoic acid (G6) and S4 becomes 3,4,5-trimethoxybenzoic acid (S6) (Figure 1).

Chromatograms obtained from high-throughput gas chromatography with time-of-flight mass spectrometer (GC/TOF-MS) were integrated using masses 166, 196, 196 and 226, specific to G4, G6, S4 and S6, respectively. This was done to remove small peaks that coelute with G6 from the total ion count. Upon integration, the peak area of each of the 4 compounds was
determined and G6:G4 and S6:S4 ratios calculated. Ratios of degraded and undegraded forms of 
G and S were separated with a one-way anova using PROC MIXED of SAS v. 9.3 (SAS institute, 
Cary, NC). Means were separated at $P \leq 0.05$ using Tukey’s least significance difference test.

**Detection of Enzymes**

**Inoculation and incubation**

Inoculation procedures were dependent upon the fungi being evaluated. For *S. stellatus*, 
three gleba were placed 8 cm apart on 15-cm dishes containing 50 mL of oatmeal agar (OA). 
Oatmeal agar was made with the aforementioned AOA recipe, without antibiotics. One gram of 
sterilized organic matter moistened with 3 mL of sterilized distilled water was set on the seeded 
agar. Three 5-mm diameter agar plugs obtained from the edge of an actively growing *Panaeolina* 
spp. isolate were placed 8 cm apart on the media, and 1 g of sterilized organic matter moistened 
with 3 mL sterilized distilled water was set on the seeded agar. *Panaeolina* spp. was isolated from 
basidiocarps found within or in close proximity to active thatch collapse symptoms (Figure 2). A 
control treatment consisted of OA and sterilized organic matter.

Enzyme production for each isolate (*S. stellatus* from turf (SS-SD) and mulch (SS-8), 
*Panaeolina* spp. and control) was measured at 0, 30, 60 and 90 days. The study was performed in 
triplicate (Chamberlain and Crawford, 2000).

**Enzyme preparations and quantification**

Unless otherwise noted, enzyme preparations and all standards were purchased from 
Sigma Aldrich (Sigma Aldrich Corp., St. Louis, MO). A Pharmacia LKB-Ultrospec II (Pfizer, 
New York, NY) was used to measure absorbency of all enzyme reactions.

At each time interval, three plates of each isolate and the uninfested control were treated 
with 100 mL of 50 mmol L$^{-1}$ sodium chloride buffer (pH 6.0) for 24 hr at 4°C. The extract with 
colonized thatch were ground with a mortar and pestle for 2 min and incubated at 4°C for 24 hr. 
Resulting extracts were filtered through miracloth (MiraCloth, Huntsertown, IN) and centrifuged
in a Sorvall RC 3B Plus (Thermo Fisher Scientific Inc. Waltham, MA) at 2800 g for 30 min. Supernatants were stored in 150 mL Erlenmeyer flasks at 4°C prior to analyses (Valentin et al., 2009).

**Manganese Peroxidase**

Manganese independent and dependent peroxidase activity were assayed with 2,6-dinitrophenol (2,6-DNP) as the substrate. The initial reaction mixture in a 1.5 mL cuvette contained 620 μL of enzyme supernatant, 200 μL of 0.25 M sodium tartrate buffer at pH 4.5 and 50 μL of 20 mM 2,6-dimethoxyphenol. The reference of the spectrophotometer was set at 470 nm to this solution and activity was measured over 5 min. After measuring this initial activity, 80 μL of 5 mM hydrogen peroxide was added to the cuvette and absorbance measured over an additional 5 min. Lastly, 50 μL of 20 mM manganese sulfate was added and absorbance measured for 5 min. Manganese peroxidase activity was determined from subtracting the peroxidase increase in absorbance from the manganese peroxidase increase in absorbance over their respective 5 min periods (Field et al., 1996). Calculation of activity was completed with Beer’s law with the extinction coefficient of 49,800 L mol⁻¹ cm⁻¹ (Beer, 1852). All reactions were completed in duplicate.

**Laccase**

Laccase activity was determined by the increase in the absorbance due to the formation of tetramethoxy-azo-bis-methylenequinone, resulting from the reaction of laccase with syringaldazine. Laccase was assayed using 0.185 mL of supernatant, 0.81 mL of 50 mM sodium citrate buffer (pH 4.8) and 0.1 mL of 0.1 mM syringaldazine in 50% ethanol. One unit was defined as the amount of enzyme producing one unit change in absorbance min⁻¹ at 530 nm (Kumaran et al., 1997). Calculations of enzymatic activity were computed following Beer’s law with the syringaldazine extinction coefficient of 65,000 L mol⁻¹ cm⁻¹ (Beer, 1852). All reactions were completed in duplicate.
**Protein Determination**

Protein content was determined through the Bradford assay (Bradford, 1976). Crude extract containing 10 to 100 μg of protein in a volume of 0.1 mL was pipetted into 1.5 mL cuvettes. One milliliter of Bradford determination reagent was added to the test tube and the contents mixed by inversion. Absorbance at 595 nm was measured after 10 min. A standard curve used to determine the concentration of proteins within unknown samples was developed using the weight of bovine serum albumin plotted against the corresponding absorbance (Bradford, 1976). All reactions were completed in duplicate.

**Cellulase**

Measurement of cellulase activity was determined by incubating 0.6 mL of culture supernatant with 850 mg of qualitative filter paper 494, (VWR, Radnor, PA) in 3.0 mL of 0.1 M sodium citrate buffer (pH 5.0) in a 13 mm x 100 mm tube. Incubated cultures were vertically shaken at 37°C for 4 hours. Every 30 min, 0.5 mL of reaction solution was removed and added to a 1.7 mL Eppendorf tube with 1.0 mL dinitrosalicylic acid reagent (1416 mL distilled water, 10.6 g 3, 5-dinitrosalicylic acid, 19.8 g sodium hydroxide, 306 g sodium potassium tartrate, 7.6 mL phenol and 8.3 g sodium metabisulfite) for determination of reducing sugars. Reactions were halted by setting tubes in a boiling water bath for 5 min. Absorbance was read at 575 nm with glucose standards in a 1.5 mL cuvette with the dilution of 0.1 mL of reaction mixture and 1.25 mL of distilled water. A standard curve of 0 to 4 mg of glucose was used to determine the breakdown of cellulose (Adney et al., 1996). All reactions were completed in duplicate.

**Xylanase**

Xylanase was measured through the reduction of xylan from the crude extract to form a colorimetric change through the production of reducing sugars. The reaction mixture contained 0.38 mL of 1% xylan substrate in 50 mM sodium acetate buffer (pH 4.5) and 0.2 mL of crude enzyme extract. The reaction mixture was incubated at 30°C for 10 min and the reaction stopped.
by adding 0.4 mL of copper solution (16 mM copper sulfate, 1.3 M sodium sulfate, 226 mM sodium carbonate, 190 mM sodium bicarbonate and 43 mM sodium potassium tartrate) followed by 10 min in a boiling water bath. The addition of 0.4 mL of arsenic-molybdic acid solution (40 mM molybdic acid, 19 mM Arsenic acid and 756 mM sulfuric acid) to the reaction mixture for development of color was followed by vortexing and 4 min of centrifugation. The reaction solution was transferred to a cuvette and absorbance read at 540 nm. A standard curve was created with a range of 0 to 4 μmol of 1 mg mL\(^{-1}\) of xylose standard solution (Alfa Aesar, West Hill, MA). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugars per min (Chen et al., 1986). All reactions were completed in duplicate.

**Results**

**Tetramethylammonium hydroxide thermochemolysis**

The SS-SD-infested and sterilized thatch samples were analyzed by TMAH thermochemolysis/GC/MS. The digested SS-SD samples showed a significant increase in G6 and S6 concentrations when compared to the uninfested, sterilized thatch. The ratio of G6/G4 increased from 0.61 to 1.60 \((P < 0.0001)\) and ratio of S6/S4 from 1.06 to 1.94 \((P = 0.0009)\) (Table 1 and Figure 3).

**Detection of Enzymes**

Assaying the crude enzyme extract revealed detectable levels of peroxidases, laccase and xylanase. Both isolates of *S. stellatus* contained 1 to 2 mU g\(^{-1}\) of organic matter of manganese independent peroxidases at 30 to 90 days after inoculation (Figure 4A). The *Panarolina* spp. had negligible amounts of manganese independent peroxidases similar to the level of activity found in the control (Figure 4A). Manganese dependent peroxidases were variable across all treatments and no trend of enzyme activity was found. Over the experiment, manganese dependent activity of all fungal species decreased and increased with the most stable levels observed within the uninfested control treatment (Figure 4B).
Laccase activity was detected within the SS-SD isolate at 30 and 60 days after inoculation (Figure 5). At 90 days no laccase activity was found for SS-SD. Laccase activity was not detected from S. stellatus isolate from mulch (SS-8), Panaeolina spp., or the uninfested control. Xylanase was detected in all treatments, but was the highest in SS-SD, SS-8 and Panaeolina isolates. Xylanase production was found from 60 and 90 days after inoculation with activity levels between 50 to 130 mU mL$^{-1}$ of crude enzyme extract for all three treatments (Figure 6).

Low cellulase activity was detected in the control treatment. S. stellatus and Panaeolina spp. produced lower levels than the control and therefore conclusions will not be further drawn about detection of cellulase activity (Figure 7).

**Discussion**

The β-O-4 linkages of the aromatic subunits composing lignin were cleaved when exposed to TMAH and all present ring hydroxyls were methylated creating S4 and G4 products. When fungi evaluated in this study enzymatically altered the structure of lignin through demethylation, ring hydroxylation and side chain oxidation, the end products changed (Geib et al., 2008). Using unlabeled TMAH thermochemolysis, the effects of fungal side chain oxidation were observed on lignin. Side chain oxidation causes C$_{\alpha}$-C$_{\beta}$ cleavage, leaving a hydroxyl group adjacent to a carbonyl. In unaltered lignin, a hydrogen is adjacent to the carbonyl. The C$_{\alpha}$-C$_{\beta}$ cleavage causes a depolymerization of lignin and the side chain oxidation increases the ratio of benzoic acids (S6 and G6 products) to benzaldehydes (S4 and S6 products) (Geib et al., 2008; Filley et al., 2000).

The results of undegraded and SS-SD colonized organic matter by thermochemolysised TMAH-GC/MS revealed a significant increase in G6 and S6 in the SS-SD colonized organic matter. The degraded lignin products were converted to G6 and S6, which decreased quantities of G4 and S4. The increased acid/aldehyde ratios for G and S illustrate the lignin degrading
capabilities of *S. stellatus* from turf (SS-SD) through side chain oxidation over the duration of 6 weeks. These findings correlate to other studies utilizing TMAH thermochemolysis to quantify lignin degradation through lignin altering enzymes or fungi (Chen, 2009; Geib et al., 2008; Vane et al., 2001). Chen (2009) found an increase in S6 and a significant increase in G6 through treating ensiled corn (*Zea mays* L.) stover with laccase in comparison to untreated corn stover. The same techniques were used to show a decay in wheat straw (*Triticum aestivum* L.) by *Agaricus bisporus* (Vane et al., 2001).

Lignin degradation by *S. stellatus* from turf after 6 weeks of colonization was illustrated by TMAH-GC/MS. Detection of laccase within the *S. stellatus* turf isolate (SS-SD), but not in any other fungi assayed supports the findings by TMAH-GC/MS of SS-SD degraded lignin. The detection of laccase from crude enzyme extracts produced by SS-SD confirms the rapid breakdown of organic matter by this isolate. Levels of manganese independent peroxidase were detected in both *S. stellatus* isolates (Figure 4A). However, manganese dependent peroxidases were not detected in any fungal crude enzyme extracts (Figure 4B). Certain fungi do not produce a complex of lignin degrading enzymes. For example, *Pycnoporus cinnabarinus* (Jacq.) Fr. only produces a single enzyme (i.e., laccase) while others (e.g., *Ganoderma lucidum* (Curtis) P. Karst) produce only two enzymes (D’Souza et al., 1999; Eggert et al., 1996).

Xylanase production was noticed in all three fungal extracts. Xylanase is utilized after lignin degradation, exposing xylose to the environment. The production of this enzyme accounts for the reduction of organic matter on golf courses and not just the lignin within the organic matter (Hammel, 1997). Cellulase levels for all fungal extracts did not increase above the control treatment. Therefore, it was concluded no cellulase activity was present in the three fungal treatments within the 90-day experiment.
Manganese peroxidase and laccase activities were previously detected from *S. stellatus* cultivated into contaminated sawmill soil and scots pine (*Pinus sylvestris* L.) bark in an effort to biodecontaminate the soil (Valentin et al., 2009). Based on the activity levels of the enzymes, Valentin et al. (2009) concluded that *S. stellatus* was a potential degrader of soil organic matter. It was noted within their study that pine bark was a good promoter of manganese peroxidase activity. Thus, substrate may be correlated to specific enzyme production. *S. stellatus* was isolated from organic matter on various golf course turfgrasses, which may be producing only laccase and manganese independent peroxidases. However, further research needs to be conducted to draw conclusions for *S. stellatus* enzyme production based on specific substrates.

The production of laccase and manganese independent peroxidase by *S. stellatus* isolate SS-SD further supports the role of this fungus as a lignin degrader. Therefore, *S. stellatus* isolated from turf effectively degrades lignin within organic matter through laccase and peroxidase production resulting in the development of thatch collapse.

The use of these extracellular lignin degrading enzymes produced by white rot fungi to manage organic matter could potentially benefit the playability of turfgrass surfaces and decrease the necessity to physically remove organic matter. In a recent greenhouse study, applications of commercially available laccase from *Trametes versicolor* (L.) Lloyd and *Pycnoporus* spp. P. to creeping bentgrass significantly decreased organic content and thatch layer thickness (Sidhu, et al., 2012). Field studies found comparable results after 6 months of initial laccase applications (Sidhu et al., 2012). *S. stellatus* produces laccase and manganese independent peroxidases. If optimized, production of *S. stellatus* lignin degrading enzymes could provide a less disruptive means of organic matter reduction for golf turf managers.
Table 1. Ratio of acid to aldehyde forms of guaiacyl (G) and syringyl (S) for undegraded control lignin and *Sphaerobolus stellatus*-infested lignin (SS-SD).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acid/aldehyde ratio for G</th>
<th>Acid/aldehyde ratio for S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lignin</td>
<td>0.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.06</td>
</tr>
<tr>
<td><em>S. stellatus</em> infested lignin</td>
<td>1.60</td>
<td>1.94</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ratio of acid to aldehyde forms were assessed after 6 weeks of growth through gas chromatography/mass spectroscopy tetramethylammonium hydroxide thermochemolysis.

<sup>b</sup> Means were significantly different according to Tukey’s least significant difference test.
Figure 1. Products of TMAH on undegraded guaiacyl and syringyl monomers (G4 and S4), and degraded guaiacyl and syringyl monomers (G6 and S6) (Chen, 2009).
Figure 2. *Panaeolina* spp. basidocarps present on a research green with thatch collapse at the Joseph E. Valentine Turfgrass Research Facility (photo courtesy of Peter Landschoot).
Figure 3. Side-chain oxidation and hydroxylation of organic matter after colonization with *Sphaerobolus stellatus* turf isolate SS-SD. Significant changes were observed with the ratio of acid to aldehyde in guaiacyl and syringyl in degraded and undegraded samples. Bars represent means ± standard deviation. Combined means within each product followed by different letters are significantly different at $P \leq 0.05$ according to Tukey’s least significant difference test.
Figure 4. Time course development of manganese independent peroxidase activity (A) and manganese dependent peroxidase activity (B) by *Sphaerobolus stellatus* from turf (SS-SD) or mulch (SS-8), *Panaeolina* spp. (P) and an untreated control on sterilized organic matter and oatmeal agar. Bars represent means ± standard deviation.
Figure 5. Laccase activity of *Sphaerobolus stellatus* from turf (SS-SD) or mulch (SS-8), a *Panaeolina* spp. (P) and an untreated control on sterilized organic matter and oatmeal agar. Bars represents means ± standard deviation.
Figure 6. Xylanase activity of *Sphaerobolus stellatus* from turf (SS-SD) or from mulch (SS-8), a *Panaeolina* spp. (P) and uninfested control on sterilized organic matter and oatmeal agar. Bars represent means ± standard deviation.
Figure 7. B-glucosidase activity, a component of cellulase of *Sphaerobolus stellatus* from turf (SS-SD) or from mulch (SS-8), a *Panaeolina* spp. (P) and uninfested control on sterilized organic matter and oatmeal agar. Bars represents means ± standard deviation.
Literature Cited


Appendix A

Table 1. Average daily growth rate in culture of three *Sphaerobolus stellatus* isolates from California (SS-CA-1), Michigan (SS-MI) and South Dakota (SS-SD) at temperatures ranging from 5 to 30°C.

| Isolate (Study I) | Temperature °C | Daily growth rate (mm)^
|-------------------|----------------|-----------------------------
|                   | 5              | 10                         | 15            | 21            | 25            | 30            |
| SS-CA-1           | 0.21           | 0.39 b                     | 1.07          | 1.54 b        | 1.56 b        | 1.14 b        |
| SS-MI             | 0.18           | 0.54 a                     | 1.09          | 1.57 b        | 1.63 ab       | 1.35 ab       |
| SS-SD             | 0.47           | 0.56 a                     | 1.22          | 1.73 a        | 1.80 a        | 1.61 a        |
| P - value         | 0.2449         | 0.0461                     | 0.6220        | 0.0069        | 0.0989        | 0.0217        |
| Isolate (Study II)|                |                            |               |               |               |               |
| SS-CA-1           | 0.37 c         | 0.54 c                     | 1.04          | 1.67 b        | 1.67 b        | 1.53 b        |
| SS-MI             | 0.32 b         | 0.60 b                     | 1.05          | 1.54 c        | 1.51 c        | 1.53 b        |
| SS-SD             | 0.47 a         | 0.85 a                     | 1.19          | 2.00 a        | 1.97 a        | 1.76 c        |
| P - value         | 0.0002         | <0.0001                    | 0.4249        | <0.0001       | 0.0004        | 0.0002        |

^
Growth rate was assessed by measuring colony diameter in two perpendicular directions bi-weekly divided by total growing days.

Means followed by the same letter are not significantly different at $P \leq 0.05$ according to the Tukey’s test.
Table 2. Average gleba production in culture of three *Sphaerobolus stellatus* isolates from California (SS-CA-1), Michigan (SS-MI) and South Dakota (SS-SD) after 11 weeks.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Isolate</th>
<th>Total gleba produced by week 11zy</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>SS-CA-1</td>
<td>3.0</td>
<td>0.3566</td>
</tr>
<tr>
<td>15</td>
<td>0.1</td>
<td>0.0</td>
<td>0.4092</td>
</tr>
<tr>
<td>21</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1508</td>
</tr>
<tr>
<td>25</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

*z* Gleba production was assessed by counting new gleba produced on a weekly basis in a colonized plate exposed to 24 hr. light.

*y* Means followed by the same letter are not significantly different at $P \leq 0.05$ according to Tukey’s least significant difference test.
Table 3. Influence of temperature on average gleba production of three *Sphaerobolus stellatus* isolates in culture after 11 weeks.

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>Num DF(^a)</th>
<th>Den DF(^b)</th>
<th>F Value(^c)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature(^d)</td>
<td>2</td>
<td>69</td>
<td>1.49</td>
<td>0.1304</td>
</tr>
<tr>
<td>Isolate(^e)</td>
<td>3</td>
<td>69</td>
<td>20.42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time</td>
<td>6</td>
<td>69</td>
<td>0.09</td>
<td>0.7700</td>
</tr>
<tr>
<td>Temperature*isolate</td>
<td>6</td>
<td>69</td>
<td>1.62</td>
<td>0.1553</td>
</tr>
<tr>
<td>Temperature*time</td>
<td>1</td>
<td>69</td>
<td>0.64</td>
<td>0.5904</td>
</tr>
<tr>
<td>Isolate*time</td>
<td>2</td>
<td>69</td>
<td>0.26</td>
<td>0.7701</td>
</tr>
<tr>
<td>Temperature<em>isolate</em>time</td>
<td>3</td>
<td>69</td>
<td>0.92</td>
<td>0.4885</td>
</tr>
</tbody>
</table>

\(^a\) Numerator degrees of freedom.

\(^b\) Denominator degrees of freedom.

\(^c\) Calculated F value according to the equation $F = \frac{MS_{between}}{MS_{within}}$.

\(^d\) Temperatures ranged from 5 to 30°C.

\(^e\) *Sphaerobolus stellatus* isolates from California (SS-CA-1), Michigan (SS-MI) and South Dakota (SS-SD)
Figure 1. Average daily growth in culture of three *Sphaerobolus stellatus* isolates (SS-CA-1, SS-MI and SS-SD) at 6 temperatures for 11 weeks. Duplicate experiments (A and B) were analyzed separately. Combined means followed by the same letter are not significantly different according to Tukey’s least significant difference test at $P \leq 0.05$. 