

## RESEARCH

# Insights into the Biology of *Sphaerobolus stellatus* as a Causal Agent of Thatch Collapse in Golf Turfs

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## ABSTRACT

Symptoms consistent with thatch collapse have been observed on golf courses in New Zealand and the United States. Symptoms include circular patches ranging from 8 to 46 cm in diameter, which result in a depression in golf turf stands of creeping bentgrass (*Agrostis stolonifera*), annual bluegrass (*Poa annua*), and browntop bentgrass (*Agrostis capillaris*). Commonly associated with the thatch degradation was *Sphaerobolus stellatus*, an agaricomycete not previously reported within turfgrass stands. The primary objectives of this study were to confirm *S. stellatus* as a causal agent of thatch collapse; to morphologically compare isolates; and to determine cardinal temperatures for isolate growth and glebae production. Isolates were obtained from symptomatic turf from five golf courses in the United States and two golf courses in New Zealand. Colonies were creamy white, and mycelia were either appressed, stringy, or aerial. Mycelia growth increased with increasing temperature from 5 to 25°C, and more glebae were produced at 10, 15, and 20°C than at 25°C. Amplification of the ribosomal internal transcribed spacer region (ITS) with primer set ITS4/ITS5 resulted in 695 to 724 bp sequences from all isolates that had 98 to 99% similarity to known isolates of *S. stellatus*. Thatch degrading ability of *S. stellatus* was determined using the loss on ignition method and by direct measurements of thatch depth. When compared with the noninfested control, plugs infested with *S. stellatus* exhibited a 20 and 25% decrease of organic matter and thatch depth, respectively. Koch's postulates were fulfilled, confirming that *S. stellatus* is a causal agent for organic matter degradation resulting in thatch collapse on golf turfgrass.

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**Abbreviations:** AOA, antibiotic oatmeal agar; ITS, internal transcribed spacer; OA, oatmeal agar; USGA, United States Golf Association.

**I**N 2010, a new disease believed to be caused by an agaricomycete (i.e., fungus with typical mushroom fruiting bodies) was reported on *Festuca* spp. on a golf course in Scotland (Dernoeden et al., 2011). Because of the symptoms expressed on finely mown turf, the disease has been referred to as thatch collapse. Thatch degradation within golf greens and fairways can create depressed patches from the decay of organic matter in the thatch layer between the soil surface and green vegetation (Beard, 1973; Danneberger et al., 1984). Thatch collapse appears as 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 mushroom odor (Dernoeden et al., 2011). Morphological characteristics of mycelia associated with affected areas include clamp connections, typical of basidiomycotan fungi (Fig. 1).

Thatch collapse symptoms are distinct from those caused by other mushroom or puffball-forming basidiomycotan fungi commonly associated with turfgrass. Fairy ring is a similar disorder,

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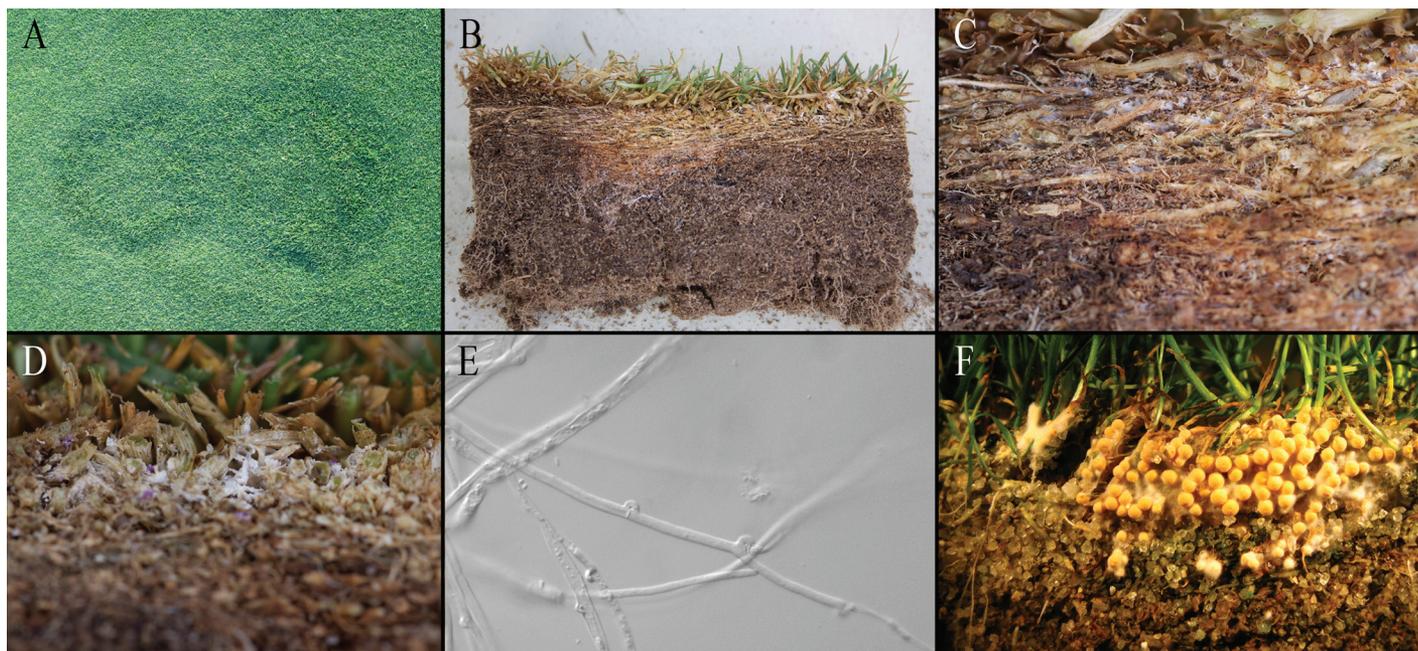


Figure 1. (A) Symptoms of thatch collapse appear as circular patches of dark-green turf ranging from 8 to 46 cm; (B) degraded organic matter creates sunken depressed areas; (C) decomposing thatch contains a fawn color and a mushroom odor; and (D) signs contain profuse mycelia within the upper 2.5 cm of the soil–thatch interface; (E) clamp connections on mycelia; and (F) fruiting bodies develop within thatch and the turfgrass canopy.

in which basidiomycotan fungi create disease-like symptoms of dark green, lush-growing rings and arcs of turf while decomposing organic matter, but the fungi associated with fairy ring are not pathogenic on turf (Fidanza, 2009). However, fairy ring fungi can result in symptoms, including necrotic rings and arcs, or growth of basidiocarps in rings or arcs of turf (Miller, 2010), which are different from the consistent sunken patches of thatch collapse. Other turf diseases caused by basidiomycotan fungi growing in soil or thatch include superficial fairy ring [*Trechispora cohaerens* (Schwein) Julich and Stalpers, *T. farinacea* (Pers.:Fr.) Libert, and *Coprinus kubickae* Pilat and Svrcek] and yellow ring [*Trechispora alnicola* (Bourd. and Galzin) Libert] (Jackson, 1972; Smiley et al., 2005). Superficial fairy rings are found in golf turfs, and symptoms and signs include white circular patches infested with fungal mycelium on the surface and infused in thatch (Smiley et al., 2005). Yellow ring is only known to occur in *Poa pratensis* L., and symptoms and signs include chlorotic rings in turf and thatch infested with whitish fungal mycelium (Wilkinson, 1987). In late 2011 and 2012, the agaricomycete *Sphaerobolus stellatus* (Tode) Persoon was found in association with thatch collapse symptoms in New Zealand, California, Michigan, Montana, and South Dakota (Baetsen et al., 2012). This consistent association has identified *S. stellatus* as one possible causal agent of thatch collapse.

The genus *Sphaerobolus* was first recorded almost 300 yr ago as *Carpobolus* by Micheli (1729). Tode renamed the genus to *Sphaerobolus* and further identified the species

*stellatus* (Tode, 1790). *Sphaerobolus stellatus* is an agaricomycete that decomposes lignin in thatch, mulch, and other sources of organic matter. It is classified in the family Geastraceae (formerly Gastereromycetes), whose members are distinguished by the fact that spore-bearing tissues are produced within a fruiting body (i.e., puffball) and not on the exposed surfaces of gills. On maturity, the outer wall (i.e., peridium) of the spherical to oblong-shaped, 1- to 3-mm-diameter fruiting body splits and ejects sticky spore-bearing tissue known as glebae. Glebae may be projected distances of a few centimeters to up to several meters (Buller, 1958), hence the common name “artillery fungus” (Brantley et al., 2001a). On multiple temperate continents this fungus is commonly found inhabiting woody substrates, dung, and forest floors (Buller, 1958; Geml, 2004). The large expansion of landscaping mulch has provided an optimal substrate for *S. stellatus*, making this fungus a common entity in home landscapes (Brantley et al., 2001a,b; Davis and Fidanza, 2011).

There have been no published reports describing the basic biology of *S. stellatus* colonizing managed turfgrass systems or causing thatch collapse. The purpose of this study was to (i) morphologically and molecularly identify and confirm *S. stellatus* as a causal agent of thatch collapse; (ii) document and compare morphologically representative *S. stellatus* isolates from distinct geographic areas; (iii) elucidate temperatures favoring mycelia growth of *S. stellatus*; and (iv) determine optimal temperature for glebae production.

**Table 1. Isolate designation, location, date received, and turfgrass species from nine golf courses affected by thatch collapse or *Sphaerobolus stellatus*, 2011 to 2012.**

Isolate	Golf course	City, region or state, country	Date received	Species <sup>†</sup>	Collector
ICMP 18282	Jacks Point G.C.	Lake Wanaka, Otago, NZ	13 Nov 2009	BTBG	Christensen
ICMP 18279	Cape Kidnappers G.C.	Hawke's Bay, Hawke's Bay, NZ	13 Nov 2009	BTBG	Christensen
ICMP 18280	Cape Kidnappers G.C.	Hawke's Bay, Hawke's Bay, NZ	13 Nov 2009	BTBG	Christensen
ICMP 18281	Cape Kidnappers G.C.	Hawke's Bay, Hawke's Bay, NZ	13 Nov 2009	BTBG	Christensen
SS-SD	Spearfish Canyon G.C.	Spearfish, SD, USA	10 Oct 2011	CBG, ABG	Miller
SS-CA-1	Harding Park G.C.	San Francisco, CA, USA	16 Sept 2011	CBG, ABG	Baetsen
SS-MI	Little Traverse Bay G.C.	Harbor Springs, MI, USA	20 Nov 2011	CBG, ABG	Baetsen
SS-CA-2	Franklin Canyon G.C.	Hercules, CA, USA	27 Jul 2012	CBG	Baetsen
SS-MT	Larchmont G.C.	Missoula, MT, USA	13 Aug 2012	CBG, ABG	Baetsen

<sup>†</sup> CBG = creeping bentgrass (*Agrostis stolonifera* L.); ABG = annual bluegrass (*Poa annua* L.); and BTBG = browntop bentgrass (*Agrostis capillaris* L.).

## MATERIALS AND METHODS

### Sample Collection and Isolation

Symptomatic turf samples were submitted to our laboratory from five golf courses in California, Montana, Michigan, and South Dakota and two golf courses in New Zealand (Table 1). Turf samples consisted of plugs from putting greens and approaches containing living plants, thatch, and soil and were incubated in a plastic container lined with moist paper towels. When present, fruiting bodies were surface disinfested by using a 1-min rinse in 10% sodium hypochlorite (Bleach Clorox) followed by three 30-sec washes in sterilized distilled water. To isolate the fungus, fruiting bodies were dried on sterilized filter paper and transferred to antibiotic oatmeal agar (AOA) amended with 0.5 g L<sup>-1</sup> penicillin G (TCI) and 0.5 g L<sup>-1</sup> streptomycin sulfate (Sigma Aldrich Corp.). Growth medium was prepared by autoclaving single grain baby oatmeal (20 g L<sup>-1</sup>, Gerber) and bacteriological agar (15 g L<sup>-1</sup>, Amresco) for 25 min at 121°C. The medium was allowed to cool to 55°C before the addition of antibiotics and was poured into 100- × 15-mm dishes. Fruiting-body-seeded petri dishes were sealed with parafilm (American National Can), and cultures were incubated for 2 wks at 21°C in constant darkness (Alasoadura, 1963).

When fruiting bodies were not present, samples were incubated to induce their production. Samples were placed within a sealed plastic container lined with moist paper towels and incubated under constant fluorescent light at 12°C for 2 wks or until fruiting bodies developed.

Hyphae development was monitored microscopically on symptomatic turf and thatch samples. The presence of intracellular hyphae in plant tissues, including leaves, roots, crowns, and stolons, was examined by staining with methyl blue (Sigma Aldrich Corp.). Infested organic matter also was microscopically assessed for intracellular colonization using methyl blue stain (Chermisvathana, 1952).

### DNA Sequencing of Isolates and Comparison with Known Fungal Species

Autoclaved potato dextrose broth (PDB) containing 24 g L<sup>-1</sup> of Difco agar was aseptically poured into 20- × 116-mm test tubes in 9-mL aliquots. Mycelial fragments of each isolate were seeded separately and grown in stationary PDB for 3 wks at 22 ± 1°C in ambient light. The DNA was extracted using a DNEasy isolation kit (QIAGEN, Inc.) 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 internal transcribed spacer (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<sub>2</sub>, 1 μL reverse primer ITS4 and 1 μL forward primer ITS5, 1 μL DNA template (100 ng μL<sup>-1</sup>), and 0.1 μL of Taq DNA polymerase (White et al., 1990). The PCR reactions were performed in a thermocycler (PTC-100 Programmable Thermal Controller, MJ Research, Inc.) using the following program: one cycle at 94°C for 5 min; followed by 35 cycles of 94°C for 1 min; one cycle at 56°C for 1 min; one cycle at 72°C for 1 min; and one cycle at 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). Purified amplification products were sequenced at the Pennsylvania State University Nucleic Acid Facility on an ABI 3730XL automated DNA sequencer (Life Technologies Corporation).

Consensus sequence data were assembled and edited using Sequencer 3.1 (Gene Codes) from both forward and reverse DNA sequences. Consensus 20 sequences were scanned through NCBI BLASTN (National Center for Biotechnology Information) 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, Biodesign Institute), along with sequences of *S. stellatus* SS31 (Accession: AY487999.1), *S. iowensis* SS391 (Accession: AY650235.1), and *S. ingoldii* (Accession: AF139975.1) for genetic comparisons. Sequences were aligned using ClustalW (European Bioinformatics Institute; Sievers et al., 2011; McWilliam et al., 2013). Phylogenetic trees were constructed using the neighbor-joining method, and a bootstrap consensus tree was inferred by 1000 replicates (Saitou and Nei, 1987). The evolutionary distances were computed using the P-distance method (Nei and Kumar, 2000). To confirm the neighbor-joining reconstructed tree, maximum likelihood method analyses also were performed on the basis of the Tamura-Nei model (Tamura et al., 2011). A bootstrap consensus tree was inferred from 1000 replicates as described previously (Felsenstein, 1985; Tamura et al., 2011). The initial tree for the heuristic search was obtained by applying the neighbor-joining 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 (Tamura et al., 2011).

## Confirmation of the Causal Agent

To confirm a cause and effect relationship between thatch collapse and *S. stellatus*, Koch's postulates were completed with *S. stellatus* isolated from turf (Agrios, 1997). A thatch-infested inoculum was developed and used for the procedure. Organic matter for the inoculum was obtained from the Joseph E. Valentine Turfgrass Research Center located in University Park, PA, in November of 2011. Living leaf and sheath tissue from a mature stand of 'A-4' creeping bentgrass grown on a sand-based putting green was removed by mowing to a height of 2.0 mm. Organic matter then was obtained by vertically slicing into the thatch layer with a Ryan Mataway slicer (Ryan), and all organic fragments were collected. Organic matter was dried at 65°C for 7 d and stored in dry plastic containers before use.

Flasks (250 mL) containing 1.0 g of dried organic matter were stopped with cotton and covered with aluminum foil before sterilization at 121°C for 90 min on three consecutive days. Three glebae from one culture of *S. stellatus* obtained from a golf course in Spearfish, SD (SS-SD), were equally spaced using aseptic forceps on AOA in a 15-cm petri dish. One gram of sterile organic matter moistened with 3 mL of sterilized distilled water was placed atop seeded AOA petri dishes. Dishes were sealed with parafilm and incubated under continuous fluorescent light at 22 ±1°C until fruiting bodies formed. Infested organic matter with developed fruiting bodies was used to seed healthy 15-cm-diameter plugs of bentgrass turf.

In May and July of 2012, 12 plugs (15-cm diameter) of mature Penn 'A-1' creeping bentgrass were removed from a research green with no prior history of thatch collapse at our field research facility. Four plugs were seeded with 0.6 g of *S. stellatus*-infested organic matter (isolate SS-SD) at the soil-thatch interface (2-cm depth) within the center on top of the plug. Seeding of noninfested, sterilized organic matter as well as noninfested plugs served as two controls. A plug from each treatment was placed in plastic containers and incubated at 12°C under 24-h light and monitored for fruiting body development in the thatch layer. Upon fruiting body development, *S. stellatus* was reisolated onto AOA by the aforementioned fruiting body isolation technique, and thatch degradation was quantified by measurements of thatch depth. Thatch layer depth was determined by placing a 1-kg weight on the surface of a vertically bisected plug to compress the thatch as described by Linde et al. (1999). Compressed thatch depth was measured with a ruler from the crowns of the turf to the beginning of the soil layer.

Total organic matter content across treatments was compared 6 wks postseeding by quantifying the decrease in sample weight through loss-on-ignition (Ben-Dor and Banin, 1989). One 1.5-cm diameter × 4.0-cm long core was taken 3 cm from the seeding point on each plug. Cores were air-dried and ground to a particle size <0.4 mm using a mortar and pestle. Crucibles were heated to 100°C for 2 h and placed in a desiccator over CaCl<sub>2</sub> to cool. A subsample of 2.5 g of each ground sample was placed in a tared crucible and then placed inside a drying oven for 24 h 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.) at 400°C and combusted for 16 h and then cooled in a desiccator over CaCl<sub>2</sub>. The subtraction method was used to calculate the weight of the 400°C samples (Ben-Dor and Banin, 1989). Loss-on-ignition (LOI) content of the sample was calculated as:

$$\text{LOI \%} = (\text{Weight}_{105} - \text{Weight}_{400} / \text{Weight}_{105}) \times 100$$

The experiment was arranged as a completely randomized design with four replications and repeated twice. Thatch depth and percentage total organic matter data across treatments were statistically compared using PROC MIXED of SAS v. 9.3 (SAS Institute). Means were separated at  $P \leq 0.05$  using Tukey's least significant difference test.

To complete Koch's postulates the aforementioned *S. stellatus* isolation procedure was completed on fruiting bodies and mycelia fragments removed from the infested plugs. The cultures were monitored for colony morphology and fruiting body development.

## 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 the aforementioned oatmeal agar without antibiotics (OA) and placed in the dark for 3 wks. Cultures were incubated at room temperature (22 ±1°C) under 24-h fluorescent lighting to induce fruiting body production. Ejected glebae growing on OA were randomly collected within petri dishes with sterilized forceps and characterized. Ten basidiospores were randomly selected within a crushed gleba for measurement and repeated with five glebae. Length and width of 50 basidiospores and 30 glebae were measured using an Axio Cam HRC (Carl Zeiss Microscopy, LLC) at 100X for glebae and 400X for basidiospores using Axio Vision software AxioVs40V4.8.0.0 (Carl Zeiss Microscopy) (Geml et al., 2005a). Mean length and width of basidiospores among the isolates were statistically compared using PROC MIXED of SAS v. 9.3 (SAS Institute). Means were separated at  $P \leq 0.05$  using Tukey's least significant difference test.

## Temperatures Favoring Mycelia Growth of *Sphaerobolus stellatus* in Culture

In vitro growth rates of three *S. stellatus* isolates (SS-CA-1, SS-MI, and SS-SD) were assessed at 5, 10, 15, 21, 25, and 30°C. A 5-mm circular plug taken from the edge of an actively growing colony that was multiplied from a gleba was placed in the center of a petri dish containing 15 to 20 mL OA. Dishes were sealed with parafilm and incubated in the dark at one of the six aforementioned temperatures. Colony diameters (centimeters) were measured twice weekly in two perpendicular directions until *S. stellatus* reached the edge of the petri dish (Ingold, 1972). The experiment within each calibrated growth chamber was arranged as a completely randomized design with four replications, and the experiment was repeated twice in rerandomized growth chambers. Average daily growth data were statistically compared across temperatures for each isolate, and daily growth was compared among isolates at each temperature using PROC

MIXED of SAS v. 9.3 (SAS Institute). Means were separated at  $P \leq 0.05$  using Tukey's least significant difference test.

## Temperatures Favoring Glebae Production in Culture

Glebae production was evaluated at 10, 15, 21, and 25°C using an isolate from each of the three aforementioned golf courses (i.e., SS-CA-1, SS-MI, and SS-SD) (Ingold, 1972). One surface-sterilized gleba from each isolate was placed in the center of a petri dish containing 15 to 20 mL OA. Dishes were sealed with parafilm and incubated in the dark for 5 wks at 21°C before incubation in 12 h of fluorescent light at one of four temperatures. Glebae production was assessed weekly for 11 wks by counting the number of glebae discharged within petri dishes (Ingold, 1972). The experiment within each calibrated incubator was arranged as a completely randomized design with four replications and the experiment repeated twice in rerandomized growth chambers. Total glebae production of all isolates was statistically compared at each temperature; total glebae production over the four temperatures was combined and compared among isolates using PROC MIXED of SAS v. 9.3 (SAS Institute). 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 ( $n = 1$ ). Four isolates also were received from two separate golf courses in New Zealand ( $n = 4$ ) and were originally isolated from stands off browntop bentgrass (Table 1).

The South Dakota isolate, obtained from L. Miller (University of Missouri, Columbia), was isolated from a modified United States Golf Association (USGA) green in which the organic mixture component was comprised of wood fibers from a local sawmill (Davis, 1973). This isolate produced large quantities of fruiting bodies and glebae when grown on OA. Four isolates provided by M. Cushnahan (NZ Sports Turf Insitiute, Palmerston North, N.Z.) were isolated by M. Christensen (AgResearch Grasslands Research Centre, Palmerston North, N.Z.) from symptomatic turfgrass at two locations in Lake Wanaka ( $n = 1$ ; Lake Wanaka) and Hawkes Bay ( $n = 3$ ; Hawkes Bay), New Zealand. The Michigan isolate was obtained from a fairway with excessive thatch (~3 cm). A large, dense area of creamy-orange mycelia within the thatch was noted in the MI sample, but no fruiting bodies were present on submission to the lab. After 3 wks of exposure to 24 h of fluorescent light, fruiting bodies developed within the thatch layer, but few developed in culture.

In response to a blog posting on Turfdiseases.org in September 2011 regarding known thatch collapse symptoms, the following samples were submitted. In January

**Table 2. Gleba and basidiospore dimensions of two *Sphaerobolus stellatus* isolates from California (SS-CA-1) and South Dakota (SS-SD) grown on oatmeal agar in a controlled environment chamber.**

Isolate	Gleba <sup>†</sup>		Basidiospore <sup>†</sup>	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)
SS-SD	830.31 ±20.12 <sup>‡</sup>	654.54 ±11.67	7.83 ±1.02	4.45 ±0.06
SS-CA-1	837.12 ±20.12	718.04 ±22.41	7.42 ±0.11	5.09 ±0.07
<i>P</i> value	0.817	0.017	0.701	<0.0001

<sup>†</sup> Length and width of 30 glebae and 50 basidiospores measured with a Zeiss Axio Cam HRC at 100X for gleba and 400X for basidiospores in program AxioVs40V4.8.0.0.

<sup>‡</sup> Mean ± standard error.

2012, a symptomatic turf sample from Harding Park Golf Course in San Francisco, CA, was obtained and contained fruiting bodies and glebae within the turf canopy. The sample was from a mixed creeping bentgrass and annual bluegrass green with a Californian construction (Davis, 1973). Once in culture, very few fruiting bodies developed on OA. In late July, a symptomatic sample was submitted from Franklin Canyon Golf Course, located in Hollister, CA. No fruiting bodies were found in the sample, but sparse mycelia with clamp connections were observed. After incubating for 4 mo, however, fruiting bodies developed in the creeping bentgrass canopy. In August 2012, a symptomatic turf sample from Larchmont Golf Course in Missoula, MT, was received and contained numerous fruiting bodies and glebae within the canopy. The sample was from a modified USGA green containing a high percentage of fine sand. When the sample was exposed to florescent light for 4 mo, no further fruiting bodies developed and few were produced in culture.

### DNA Sequencing

In general, DNA sequencing of the ITS region in most isolates revealed a 99% sequence similarity to a known *S. stellatus* mulch isolate (Accession number: AY487975.1). One exception was isolate SS-MT, which was 98% similar to the known isolate. 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 (Fig. 2). The first clade included *S. stellatus* from New Zealand (i.e., ICMP 18280, 18281, and 18279), SS-SD, SS-CA-1, 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 (Fig. 3). Bootstrap values were greater than 50% for all subclades of *S. stellatus*.

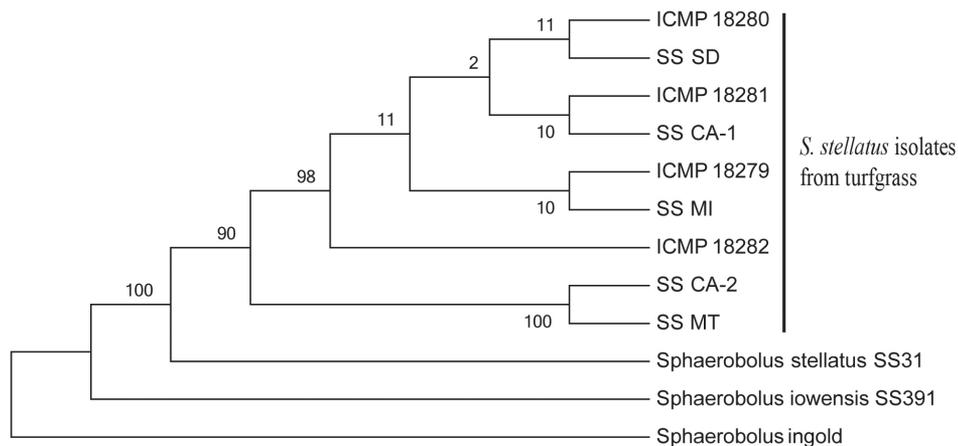


Figure 2. Evolutionary relationships between internal transcribed spacer (ITS) sequences from *Sphaerobolus stellatus* isolates, *S. iowensis*, and *S. ingoldii* conducted in MEGA5. Evolutionary history was inferred using the neighbor-joining method. Percentages of the replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) and are shown 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. 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.

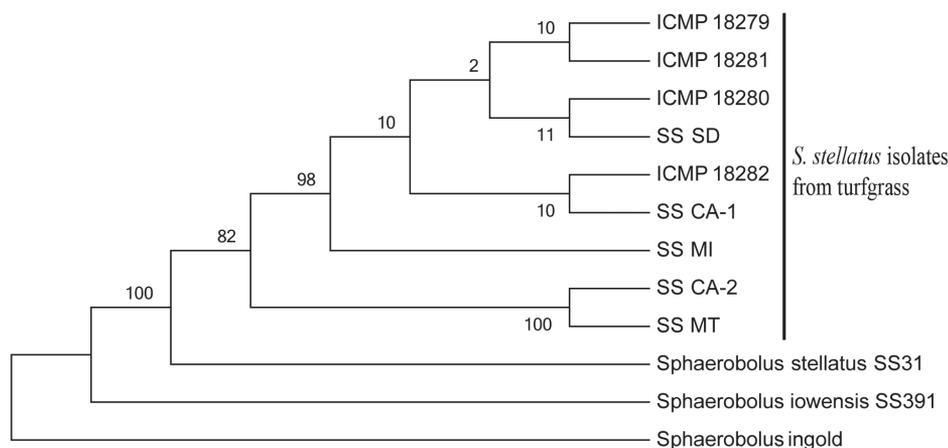


Figure 3. Evolutionary relationships among internal transcribed spacer (ITS) sequences from *Sphaerobolus stellatus* isolates, *S. iowensis*, and *S. ingoldii* conducted in MEGA5. Evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model. Bootstrap consensus trees were inferred from 1000 replicates and were taken to represent the evolutionary history of the taxa analyzed. Percentage of replicate trees are clustered together in the bootstrap test (1000 replicates) and are shown next to the branches. 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.

### Colony Morphology, Gleba, and Basidiospore Characteristics

Thirty glebae and 50 basidiospores from five measured glebae were characterized for isolates SS-CA-1 and SS-SD. *Sphaerobolus stellatus* isolate SS-MI produced no glebae in culture and, therefore, was excluded from the analyses. All glebae produced within petri dishes had a dark-brown to black hue and generally were spherical to rectangular in shape. Average gleba length and width for the isolates was 833.7  $\mu\text{m}$  and 686.3  $\mu\text{m}$ , respectively (Table 2). Glebae produced by SS-CA-1 were significantly ( $P = 0.017$ ) wider (718.0  $\pm 22.1$   $\mu\text{m}$ ) than SS-SD (654.5

$\pm 11.7$   $\mu\text{m}$ ), but both isolates had similar glebal lengths of 830.3  $\pm 20.1$   $\mu\text{m}$  and 837.1  $\pm 20.1$   $\mu\text{m}$  for SS-SD and SS-CA-1, respectively.

Average basidiospore length and width was 7.6  $\mu\text{m}$  and 4.8  $\mu\text{m}$ , respectively, and lengths were similar among isolates (Table 2). Widths of basidiospores were different ( $P < 0.0001$ ), with SS-CA producing wider spores (5.1  $\pm 0.1$   $\mu\text{m}$ ) than SS-SD (4.5  $\pm 0.1$   $\mu\text{m}$ ).

*Sphaerobolus stellatus* was isolated from either fruiting bodies 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. White mycelia colonizing the organic matter produced an orange hue in the thatch along the outer edges of patches (Fig. 1). However, on microscopic examination of stained tissues within samples, no hyphae bearing clamp connections were observed inside leaves, roots, crowns or stolons.

Colony morphology varied among isolates. South Dakota and California isolates produced creamy-white, stringy, aerial mycelia that extended upward toward the lid of the petri dish. The Michigan and Montana isolates formed creamy-white, appressed mycelia. All isolates produced concentric rings of mycelia across the media.

### Confirmation of the Causal Agent

Growth of *S. stellatus* isolate SS-SD was allowed to progress over 6 wks on incubated plugs. Differences between thatch depth ( $P = 0.0723$ ) or total organic matter ( $P = 0.5390$ ) were not observed between experiments and, therefore, data were combined for statistical analyses. Thatch layer depth differences were significant ( $P < 0.0001$ ) across treatments. The *S. stellatus*-infested treatment had a thatch layer depth of 1.46 cm, which was less than the noninfested control (1.95 cm) and plugs that only received sterilized organic matter control (1.88 cm) treatments (Fig. 4). Because of the lack of thatch-depth differences between the noninfested control and sterilized organic matter controls, percentage of organic matter was determined only for *S. stellatus*-infested samples and the noninfested control samples. Percentage of organic matter was less ( $P = 0.0064$ ) in the *S. stellatus*-infested treatment (1.13%) when compared with the control (1.42% or 20.4% less organic matter) (Fig. 5). In all experiments, *S. stellatus* was successfully reisolated from fruiting bodies in infested plugs.

### Temperatures Favoring Mycelia Growth of *S. stellatus* in Culture

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

#### Study I

Daily growth was analyzed for each isolate over six temperatures. Isolates SS-CA-1 and SS-MI had the largest daily growth at 21°C and 25°C ( $P < 0.0001$ ), and SS-SD had the largest daily growth at 21, 25, and 30°C ( $P < 0.0001$ ) (Table 3). All isolates had the smallest daily growth at 5°C ( $P < 0.0001$ ) (Table 3). At 25°C and 30°C, SS-SD and SS-CA-1 isolates sustained the largest and smallest daily growth, respectively, of the three isolates when daily growth was evaluated among isolates at each temperature ( $P < 0.05$ ) (Fig. 6A).

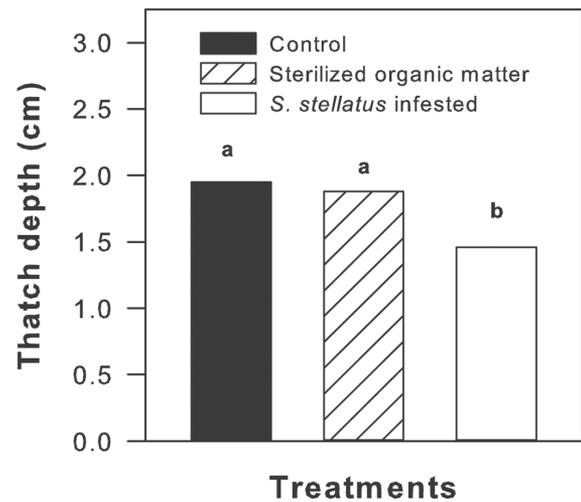


Figure 4. Organic matter depth of Penn 'A-1' creeping bentgrass plugs after 6 wks of incubation at 12°C. Seeded treatments included a noninfested control, sterilized organic matter, and *Sphaerobolus stellatus*-infested thatch. Treatment means followed by the same letter are not significantly different according to Tukey's least significant difference test at  $P \leq 0.05$ .

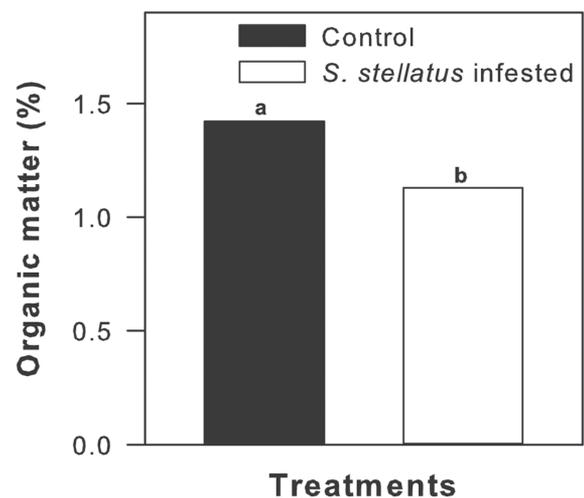


Figure 5. Total organic matter (%) of Penn 'A-1' creeping bentgrass samples as determined by the loss on ignition method following incubation at 12°C for 6 wks. Seeded treatments included a noninfested control and *Sphaerobolus stellatus*-infested organic matter. Means followed by the same letter are not significantly different according to Tukey's least significant difference test at  $P \leq 0.05$ .

#### Study II

Data revealed that SS-MI had the largest daily growth at 21, 25, and 30°C; SS-CA-1 at 21 and 25°C; and SS-SD at 21°C ( $P < 0.0001$ ) (Table 3). Each isolate had the smallest daily growth at 5°C ( $P < 0.0001$ ) (Table 3). Evaluation of daily growth among isolates at each temperature showed SS-SD and SS-CA-1 had the largest and smallest daily growth, respectively, of the three isolates at 5, 10, 21, and 25°C ( $P < 0.05$ ) (Fig. 6B).

**Table 3. Average daily growth of three *Sphaerobolus stellatus* isolates on oatmeal agar from California (SS-CA-1), Michigan (SS-MI), and South Dakota (SS-SD) in a controlled environment chamber.**

Temperature °C	Isolate		
	SS-CA-1	SS-MI	SS-SD
	Daily growth <sup>†</sup>		
	mm		
Study I			
5	0.2 d <sup>‡</sup>	0.2 e	0.5 d
10	0.4 c	0.5 d	0.6 c
15	1.1 b	1.1 c	1.2 b
21	1.5 a	1.6 a	1.7 a
25	1.6 a	1.6 a	1.8 a
30	1.1 b	1.4 b	1.6 a
<i>P</i> value	<0.0001	<0.0001	<0.0001
Study II			
5	0.4 e	0.3 d	0.5 e
10	0.5 d	0.6 c	0.8 d
15	1.0 c	1.1 b	1.2 c
21	1.7 a	1.5 a	2.0 a
25	1.7 a	1.5 a	1.9 ab
30	1.5 b	1.5 a	1.7 b
<i>P</i> value	<0.0001	<0.0001	<0.0001

<sup>†</sup> Daily growth was determined by measuring colony diameter in two perpendicular directions biweekly and divided by total growing days.

<sup>‡</sup> Mean daily growth values followed by the same letter are not significantly different at  $P \leq 0.05$  according to the Tukey's least significance difference test.

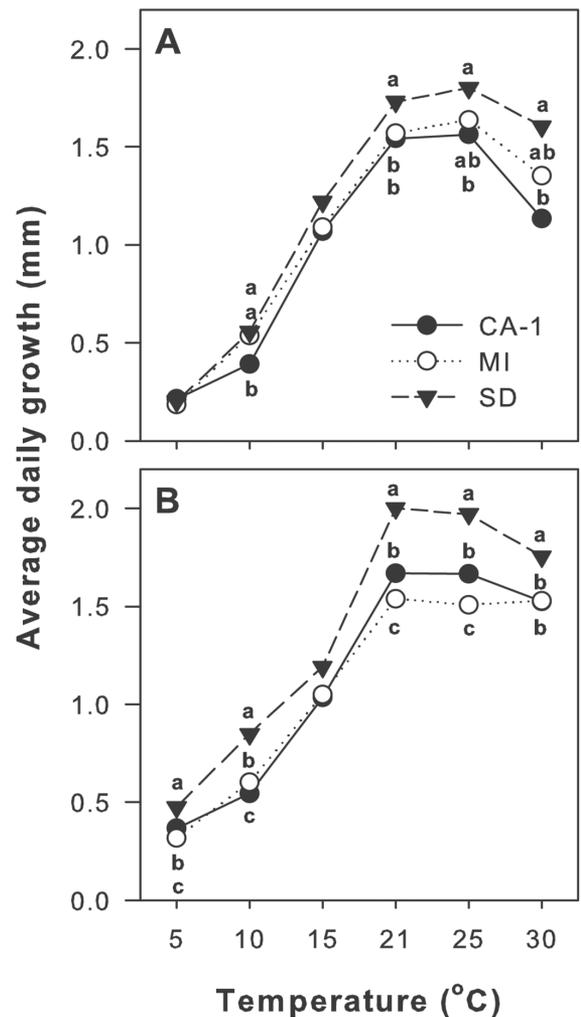
## Temperatures Favoring Gleba Production in Culture

The influence of temperature on *S. stellatus* gleba production of three isolates (SS-CA-1, SS-MI, and SS-SD) was examined for 11 wks. Significant study effects were not observed for glebae production; therefore, data were combined for statistical analyses.

Fruiting bodies developed 1 wk after exposure to light, and glebae were discharged during the second week of the study when incubated at 21 and 25°C (Fig. 7). Glebae production of isolates incubated at 10 to 15°C was not observed until approximately 3 to 4 wks after incubation. The length of time that glebae were produced also varied by temperature. At 21°C, all glebae production occurred within 4 wks. Glebae production at 10, 15, and 25°C, however, ceased after 11, 8, and 6 wks, respectively. Isolate SS-SD produced glebae at all temperatures, whereas isolate SS-CA-1 only produced glebae at 10 and 15°C, and isolate SS-MI produced glebae at 15°C (Table 4). When analyzed by temperature, SS-SD produced the most glebae at all temperatures among the isolates (Table 4).

## DISCUSSION

The detrimental effects to ball roll and firmness of golf greens (Brame, 2008) makes understanding the cause and biology behind thatch collapse of importance to the industry and for its management. Symptomatic depressions create unpredictable ball movement on playing surfaces



**Figure 6. Average daily growth of three *Sphaerobolus stellatus* isolates (SS-CA-1, SS-MI, and SS-SD) on oatmeal agar in a controlled environment chamber at six 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  $P \leq 0.05$ .**

that are held accountable for consistency. *Sphaerobolus stellatus* was isolated from fruiting bodies within the turf canopy or thatch layer from all symptomatic samples collected. Fruiting bodies developed on nonliving organic matter colonized with white- to orange-colored mycelia. Although clamp connection bearing hyphae were observed within the organic matter, no visible signs of *S. stellatus* were found within living plant tissues, indicating that the organism is not pathogenic.

Isolation of *S. stellatus* from several locations and turfgrass species across the United States and New Zealand, and conclusive fulfilling of Koch's postulates, confirms *S. stellatus* as a causal agent of thatch collapse. Thatch collapse symptoms (i.e., sunken or depressed patches) linked to *S. stellatus* are distinct from symptoms produced by other known basidiomycetes that inhabit soil and turfgrass thatch, including fairy ring, superficial fairy ring, and yellow ring (Smiley et al., 2005).

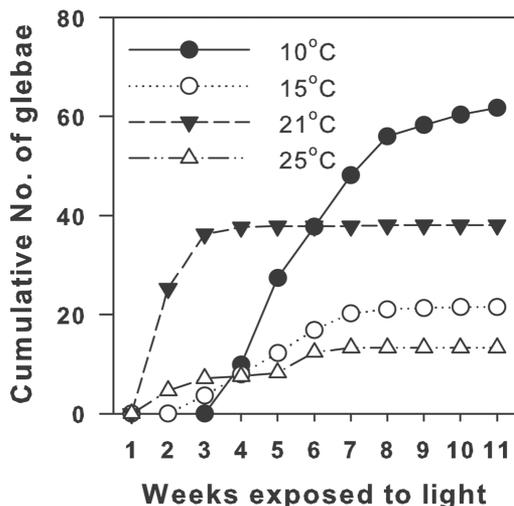


Figure 7. Average glebae production on oatmeal agar in a controlled environment chamber over 11 wks of *Sphaerobolus stellatus* isolate from South Dakota (SS-SD) at four temperatures. *Sphaerobolus stellatus* isolates from California (SS-CA-1) and Michigan (SS-MI) were not included (<5 glebae produced).

Sequencing of the ITS region revealed that the thatch collapse isolates were *S. stellatus*. Subclade development within the species of *S. stellatus* revealed subtle differences within the ITS region. The number of base pairs deleted during alignment indicated the ITS region is difficult to align among the species compared (Geiser et al., 2001; Geml and Royse 2002; Geml et al., 2004; O'Donnell et al., 1998; Peintner et al., 2003). This difficulty may be correlated to a large number of unknown taxa that could fill the phylogenetic gaps (Geml et al., 2005b).

*Sphaerobolus stellatus* isolate SS-SD induced a reduction of the thatch depth and organic matter content within seeded plugs, exemplifying the symptomatic depressions of thatch collapse. Reisolation of *S. stellatus* from symptomatic infested samples confirmed its association with, and role in, the development of thatch collapse. The degradation of organic matter by *S. stellatus* observed in this study is consistent with prior investigations (Winqvist et al., 2009). *Sphaerobolus stellatus* was found to reduce soil organic carbon by 20% over 6 mo in laboratory experiments (Winqvist et al., 2009). A 20% organic matter reduction from a *S. stellatus* isolate SS-SD occurred over 6 wk. The accelerated organic matter reduction in turfgrass by isolate SS-SD demonstrates the capabilities of this fungus to cause thatch collapse on golf playing surfaces.

The cultural morphology of *S. stellatus* was similar to two other known *Sphaerobolus* species: *S. ingoldii* and *S. iowensis* (Geml, 2004). Glebae generally were rectangular in shape. This unusual morphology likely was due to compression of glebae when forcefully discharged against the lids of petri dishes. Gleba length of the examined *S. stellatus* isolates were more closely related to the diameter of oval-shaped *S. ingoldii* (977  $\mu\text{m}$ ) gleba described by

Table 4. Average glebae 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 wks in a controlled environment chamber.

Isolate	Temperature °C			
	10	15	21	25
SS-CA-1	3 b <sup>†</sup>	14 b	0 b	0 b
SS-MI	0 b	4 b	0 b	0 b
SS-SD	53 a	21 a	38 a	13 a
P value	0.0063	0.0031	0.0064	0.0091

<sup>†</sup> Production was assessed by counting new glebae ejected within petri dish lids on a weekly basis when exposed to 24 h light.

<sup>‡</sup> Means followed by the same letter are not significantly different at  $P \leq 0.05$  according to the Tukey's least significance difference test.

Geml (2004). Basidiospore length and width data for two isolates were similar to *S. stellatus* as reported by Geml (2004); however, isolate SS-CA-1 produced slightly larger basidiospores than SS-SD. Glebae were smaller than previously reported for *S. stellatus*, which may correlate to the placement of thatch collapse isolates into a subclade of *S. stellatus* (Geml, 2004). The slight variation within speciation of *Sphaerobolus* can be attributed to the limited sporulation of isolates in this study.

*Sphaerobolus stellatus* daily growth increased with temperatures from 5 to 25°C. These average daily growth data were similar to those previously reported (Alasoadura, 1963). Mean daily growth of isolates SS-CA-1 and SS-MI at 25°C was similar to known isolates of *S. stellatus* (Geml, 2004). However, daily growth of isolate SS-SD was larger and more comparable to *S. ingoldii* (Geml, 2004).

Glebae likely serve as an important source of inoculum for spread of *S. stellatus* and development of thatch collapse, since glebae were formed over a wide range of temperatures in this study. However, only isolate SS-SD produced copious amounts of glebae in our growth chamber study. The larger delayed production of glebae observed with isolate SS-SD at 10°C also was noted with isolates studied by Alasoadura (1963). Glebae produced by isolate SS-SD followed the same trend as observed by Alasoadura (1963), with higher amounts produced at 10, 15, and 20°C versus 25°C. The SS-SD isolate produced the highest numbers of glebae at 10°C, whereas in Alasoadura's (1963) study isolates of *S. stellatus* produced the most glebae at 15°C. The decrease in gleba production at 25°C also was observed in other studies (Walker, 1927; Ingold, 1972).

This is the first formal report of the nature and biology of a thatch collapse causal agent. Other organisms may cause similar symptoms, but thus far only *S. stellatus* has been linked to thatch collapse. The information reported herein will advance our understanding of thatch collapse and future management strategies to minimize the development and impacts on ball roll and firmness of golf greens.

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