Assessment of Nitrogen Source, Sulfur, and Fall Fungicide Applications on the Management of Spring Dead Spot of Bermudagrass

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Abstract

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Spring dead spot, caused by species of *Ophiosphaerella*, is the most serious disease of bermudagrass (*Cynodon* spp.) in regions where cold temperatures induce winter dormancy. Previous research indicates that soil pH reduction may reduce spring dead spot severity. Of the 165 isolates collected from 16 sites in Missouri and surrounding regions, *Ophiosphaerella herpotricha* was the most prominent spring dead spot pathogen found, with 154 confirmed isolates. Ten isolates were identified as *O. korrae*, being detected at a low incidence at 6 of 16 sites. In vitro assays, most mycelial growth of both species occurred from pH 5 to 6,

with more growth on calcium-nitrate-amended media than ammonium sulfate. In a naturally infested field study, nitrogen source alone did not affect spring dead spot severity. Less spring dead spot severity ($P < 0.05$) was observed in plots receiving tebuconazole but no treatment provided more than 38% control after 1 year. Three sulfur applications (each at 98 kg ha^{-1}) provided as much control as a single fall tebuconazole application ($0.28 \text{ kg a.i. ha}^{-1}$) in the second year; however, significant phytotoxicity was observed in sulfur-treated plots thereafter. The suppression obtained from one fall tebuconazole treatment was as effective as two.

Spring dead spot is a destructive disease of bermudagrass (*Cynodon dactylon* (L.) Pers.) and hybrid cultivars (*C. dactylon* × *C. transvaalensis* Burt-Davy) in geographic locations where freezing temperatures induce bermudagrass dormancy. Spring dead spot pathogens are soilborne fungi that colonize bermudagrass roots, rhizomes, and stolons (Tredway et al. 2009b; Walker and Smith 1972). Although pathogen infection and colonization occurs in cool soil temperatures ($<21^{\circ}\text{C}$) in fall and winter, symptoms do not appear until spring (Crahaay et al. 1988; Endo et al. 1985; Tredway et al. 2009b; Walker et al. 2006). Sunken, bleached circular patches (3 cm to >1 m in diameter) are prominent as uninfected bermudagrass resumes normal growth and comes out of winter dormancy. Belowground, dark runner hyphae are evident on infected roots and rhizomes, resulting in necrotic, black, and brittle plant structures. Symptoms are often more severe on highly maintained, 3- to 5-year-old bermudagrass swards (Tredway et al. 2009b).

Spring dead spot is caused by three species in the genus *Ophiosphaerella*: *Ophiosphaerella herpotricha* (Fr.) J. Walker; *O. korrae* (J. Walker & A. M. Sm. bis.) Shoemaker and C. E. Babc.; and *O. narmari* (J. Walker & A. M. Sm. bis.) H. C. Wetzel, Hubert & Tisserat. Distribution of these pathogens is not uniform and may vary by geographical region in the United States. *O. herpotricha* is primarily found in the Midwest region, while *O. korrae* is the main spring dead spot pathogen species in the southeastern region of the United States and California (Crahaay et al. 1988; Endo et al. 1985; Iriarte et al. 2004; Tisserat et al. 1989; Wetzel et al. 1999b). *O. narmari* has been occasionally isolated from infected bermudagrass tissue in Oklahoma, Kansas, North Carolina, and California but is not prevalent in the United States (Iriarte et al. 2004; Wetzel et al. 1999a). Although each species appears to have its primary location, some overlapping distribution does exist. *O. herpotricha* and *O. korrae* are often identified from the same site, and occasionally isolated from the same individual patch (Iriarte et al. 2004; Walker and Smith 1972; Wetzel et al. 1999b). Additionally, all three species have been detected from the same site in Oklahoma (Tredway et al. 2009b; Wetzel et al. 1999b).

Spring dead spot control is difficult because the infections occur belowground several months before symptom expression. Fungicides must be delivered below the thatch layer, an area of undecomposed plant material between leaves and soil surface (Iriarte et al. 2004). Once the disease becomes established, a combination of cultural and chemical practices is necessary in a multiyear approach to achieve acceptable spring dead spot control (Butler and Tredway 2006). Cultural practices include use of cold-tolerant bermudagrass varieties, core aeration, verticutting, soil pH manipulation, and nitrogen fertilization (Dernoeden et al. 1991; Tredway et al. 2009b; Vincelli 2014).

Reducing soil pH has been successful in suppression of soilborne diseases such as summer patch of Kentucky bluegrass (*Poa pratensis* L.) and take-all of wheat (*Triticum* spp.) of creeping bentgrass (*Agrostis stolonifera* L.) (Heckman et al. 2003; Smiley and Cook 1973; Thompson et al. 1995); Tredway et al. (2009a) reported ammonium sulfate applications reduced spring dead spot symptoms caused by *O. herpotricha* but not *O. korrae*. Conversely, calcium nitrate applications suppressed spring dead spot symptoms caused by *O. korrae* but not *O. herpotricha*. Dernoeden et al. (1991) also reported a reduction of spring dead spot symptoms caused by *O. korrae* as a result of ammonium sulfate applications, indicating that soil acidification may reduce severity. Nitrification inhibitors (dicyandiamide and others) reduce nitrate leaching by slowing the conversion of NH_4 to NO_3 (Mosdell et al. 1986). Therefore, nitrification inhibitors may be beneficial in cases where ammonium uptake and soil acidification suppress soilborne plant diseases (Hill et al. 2003; Mosdell et al. 1986; Smiley and Cook 1973).

Another method to reduce soil pH is through elemental sulfur applications. Vincelli (2014) reported sulfur applications reduced spring dead spot severity but may also delay emergence from winter dormancy (spring greenup). Lowering soil pH may also be important for increasing plant-available manganese (Mn^{2+}) in the soil. Manganese plays a crucial role in plant defense systems through cell detoxification and synthesis of cell wall lignin and phenolics (Alscher et al. 2002; Brown et al. 1984; Hill et al. 1999). The relationship between soil pH and spring dead spot occurrence may vary between site locations depending on soil type (Vincelli 2014).

Factors affecting fungicide efficacy include fungicide selection, application timing, and application method. Fungicide applications must be applied preventively during fall, before or during the infection period (Butler and Tredway 2006). Research indicates that fungicides with the ability to suppress spring dead spot include tebuconazole,

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propiconazole, benomyl, azoxystrobin, and myclobutanil (Butler and Tredway 2006 ; McCarty et al. 1992 ; Tredway et al. 2009b). Walker (2009) that reported two fall or one fall and one spring fungicide application of propiconazole offered the most effective and cost-effective disease control. Because spring dead spot pathogens are soilborne, the fungicide application must be either watered-in with post application irrigation or rainfall or applied in a high volume of water carrier.

A better understanding of the factors affecting spring dead spot occurrence and management is needed to develop more effective management practices for turfgrass managers. The objectives of this research were to provide additional evidence for the geographic distribution of spring dead spot pathogens and to investigate the impact of cultural practices and fungicide applications for spring dead spot control. The specific objectives were to (i) sample and assess the diversity of spring dead spot pathogens in the Midwest region, (ii) assess the impact of pH and nitrogen source on *O. herpotricha* and *O. korrae* in vitro, and (iii) evaluate the impact of nitrogen source, sulfur, nitrification inhibitors, and the fungicide tebuconazole on field spring dead spot symptoms caused by *O. herpotricha*.

Materials and Methods

Sampling and identification. Bermudagrass samples were collected at 16 locations exhibiting spring dead spot symptoms on golf course fairways, greens, and athletic fields in Missouri, Arkansas, and Kansas. At each site, the symptomatic area was defined, and samples were obtained from individual patches randomly selected along diagonal transects of the area. At each location, 10 to 20 cup cutter-sized samples (10.75 cm in diameter by 10.16 cm in depth) were taken from the margin of necrotic spring dead spot patches.

Infected bermudagrass roots and rhizomes were cut into sections (5 to 10 mm in length), wrapped in cheesecloth, and continually rinsed with water for 30 min. After rinsing, the sections were surface sterilized in a 0.06% NaOCl solution for 5 min followed by submersion in an ethanol solution (70% vol/vol) for 30 s. The sections were then rinsed once with sterile water, blotted dry with a sterile paper towel, and plated on one-fourth-strength (9.75 g/liter) potato dextrose agar (PDA; Difco Laboratories, Detroit) amended with chloramphenicol (50 µg ml⁻¹), tetracycline (50 µg ml⁻¹), and streptomycin sulfate (50 µg ml⁻¹). For long-term storage prior to DNA extraction, isolates were placed on PDA slants (39 g/liter), incubated at room temperature for 7 days, covered with 5 ml of light mineral oil (Thermo Fisher Scientific, Waltham, MA), and stored at 2°C. Reference isolates of *O. herpotricha* and *O. korrae* were also obtained from the collection of L. Tredway (North Carolina State University, Raleigh).

Genomic DNA was extracted from 165 collected isolates and 2 reference isolates with the Easy-DNA kit (Invitrogen Corp., Carlsbad,

CA). Polymerase chain reaction (PCR) amplification of ribosomal (r) DNA internal transcribed spacer (ITS) regions ITS1, 5.8S rRNA, and ITS2 was conducted with the primer set ITS1f and ITS4 on all DNA extracts, according to previous protocols (Gardes and Bruns 1993; Henrion et al. 1992). Each PCR was 50 µl in volume and consisted of 10 µl of MyTaq red reaction buffer (Bioline Inc., Taunton, MA), 200 nM each primer, 0.25 µl of *Taq* polymerase (Bioline Inc.), and 50 ng of genomic DNA. Thermal cycling conditions involved an initial denaturation step at 94°C for 1 min; followed by 33 cycles of 94°C for 30 s, 65°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 2 min. Purification of amplified isolate DNA was performed using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Purified amplicons were taken to the University of Missouri DNA sequencing facility (Bond Life Sciences Center, University of Missouri, Columbia) for cleanup, electrophoresis, and sequencing.

The DNA sequence of each isolate was determined using the consensus of two reactions, one for each primer set. A BLAST search of GenBank was performed with each consensus sequence. For each sample, one or two of the most similar sequences based on maximal identity percentage were downloaded for comparison. All sequences were aligned using the MUSCLE method in MegAlign (12.2; DNASTAR Inc., Madison, WI) and adjusted by visual examination. A phylogenetic tree was constructed in MEGA 5.2 (Tamura et al. 2011) using the neighbor-joining algorithm from genetic distances calculated using the Kimura two-parameter model analysis. Bootstrap support at branch nodes was based on 1,000 random samples of the data set. Due to the similarity in sequences, *Vaccellum curtisii* HQ235045 was used as an outgroup for trees examining the ITS region.

In vitro growth assays. The radial mycelial growth response to a range of pHs and two different nitrogen sources was determined for a subset of 42 isolates of *O. herpotricha* and 8 isolates of *O. korrae*. The affect of two different nitrogen sources on mycelial yield of *O. herpotricha* was also assessed. For all growth assays, petri plates (100 by 15 mm; Thermo Fisher Scientific) and conical tubes (50 ml; Corning Inc., Corning, NY) were arranged in four replications of a complete randomized design. The growth response for all isolates was determined in two repeated experimental runs.

For assessment of nitrogen impact on radial growth, a modified Melin-Norkrans (MMN) medium was utilized. The media contained 15 g of agar, 5 g of glucose, 0.5 g of KH₂PO₄, 0.15 g of MgSO₄, 50 mg of CaCl₂, 25 mg of NaCl, and 12 mg of FeCl₃ per liter of distilled water delivered in a 1% (wt/vol) solution (Marx 1969). Yeast extract, malt extract, and ammonium phosphate were omitted to avoid utilization of nontarget nitrogen sources. Ammonium sulfate or calcium nitrate were amended as sole nitrogen sources to the

Table 1. Collection date, location, bermudagrass cultivar, and ribosomal DNA (rDNA) clade for sequences of *Ophiophora* spp. obtained from bermudagrass exhibiting spring dead spot symptoms

Collection date	Location	Bermudagrass cultivars	Number of sequenced isolates	Clade ^a
21 May 2011	Columbia, MO	Riviera, Patriot, Cody, SWI 2000, Sunrise	44	A (n = 43), C (n = 1)
28 April 2012	Overland Park, KS	Unknown	4	A (n = 3), B (n = 1)
30 April 2012	St. Louis, MO	Unknown	14	A (n = 11), B (n = 3)
9 May 2012	Osage Beach, MO	Cheyenne	11	A (n = 10), B (n = 1)
15 May 2012	Branson, MO	Mirage	4	A (n = 2), B (n = 2)
15 May 2012	Branson, MO	Midlawn	4	A (n = 3), B (n = 1)
15 May 2012	Branson, MO	Savannah	4	A
16 May 2012	Fayetteville, AR	Patriot, Princess, Tifway, Riviera	6	A
16 May 2012	Fayetteville, AR	Tifeagle	6	4 A, 2 B
27 May 2012	St. Louis, MO	Westwood	10	A
12 June 2012	Columbia, MO	Riviera	12	A
21 June 2012	St. Joseph, MO	Patriot	7	A
25 June 2012	St. Louis, MO	Unknown	8	A
21 May 2013	Arnold, MO	Westwood	12	A
21 May 2013	Park Hills, MO	Quickstand	6	A
6 June 2013	Fenton, MO	Riviera	13	A
Total	...	...	165	A (n = 154), B (n = 10), C (n = 1)

^a Clade designated in phylogenetic tree found in Figure 1; A = *O. herpotricha*, B = *O. korrae*, and C = *O. narmari*.

medium prior to autoclaving at concentrations of 50, 100, 200, 400, or 800 $\mu\text{g ml}^{-1}$. MMN media containing no nitrogen source was used as a control. All media were autoclaved for 30 min, cooled to 55°C, and poured into 100-by-15-mm polystyrene petri plates. To assess pH impact, full-strength PDA (39 g/liter) was adjusted to a pH range of 3 through 9 prior to autoclaving using lactic acid ($\text{C}_3\text{H}_6\text{O}_3$) or sodium hydroxide (NaOH). All pH measurements were recorded from 20-ml aliquots mixed at 100 rpm in a 50-ml beaker with a 2.5-cm magnetic stirring bar. Measurements were taken at room temperature with a precalibrated Accumant AB15 pH meter (Thermo Fisher Scientific, Waltham, MA). All chemicals and supplies were obtained from Thermo Fisher Scientific.

Hyphal plugs (6 mm in diameter) from the edge of actively growing *O. herpotricha* and *O. korrae* colonies were homogenized in 5 ml of sterile potato dextrose broth to provide a uniform suspension of mycelial fragments. A 5-mm-diameter agar plug was removed from the center of each plate to form a well in the solidified medium. Mycelial suspension (50 μl) was transferred to the well in each of four replicate plates. Cultures were incubated at room temperature (25°C), with radial growth measured at 12 days

after plate inoculation. Radial growth was assessed as the average diameter of each colony measured in two perpendicular directions.

To assess nitrogen source impact on mycelial yield, hyphal plugs (6 mm in diameter) were excised from the edge of actively growing *O. herpotricha* isolates grown on 20 ml of sterilized full-strength PDA. Plugs were placed in 50-ml polypropylene conical tubes (BD Biosciences, Durham, NC) containing 20 ml of MMN media with the same ingredients as above, excluding agar. Nitrogen sources and concentrations were the same as those used in the solid media growth assay. Inoculated tubes were shaken at room temperature at 140 rpm for 12 days. Mycelial plugs were harvested after 12 days by vacuum filtration onto preweighed filter paper (7 mm in diameter; Thermo Fisher Scientific). Filter paper and mycelial plugs were dried at 40°C for 4 h and weighed. Initial filter paper weight was subtracted from final weight to obtain a yield of mycelial biomass. After several attempts, isolates of *O. korrae* would not grow in the liquid media. To assess potential pH changes from autoclaving, the pH of a 20-ml aliquot of each treatment was measured before and after autoclaving. Measurements of pH were also obtained at day 12 to assess the impact of mycelial growth on solution pH.

Fig. 1. Neighbor-joining phylogenetic tree of *Ophiosphaerella* spp. produced from sequences of ribosomal DNA (rDNA) regions: partial nuclear small-subunit rDNA, internal transcribed spacer (ITS)1, 5.8S, ITS2, and partial nuclear large subunit rDNA. Isolates either were recovered from Missouri and surrounding regions from bermudagrass exhibiting spring dead spot symptoms or were reference isolates obtained from L. Tredway (North Carolina State University, Raleigh). Letters indicate the clades containing known *Ophiosphaerella* spp. found in this study. Additional sequences from recovered isolates ($n = 139$) are collapsed into clade A and not shown. Scale bar indicates the horizontal distance corresponding to genetic distance as calculated by the Kimura two-parameter model. Bootstrap values are indicated adjacent to nodes and are based on 1,000 resamplings of the data set.

The reproducibility of the radial mycelial growth and mycelial yield assay procedures was also assessed. For the radial growth assay, two isolates of *O. herpotricha* and *O. korrae* were arbitrarily selected from the collection of 42 and 8 isolates, respectively. For the mycelial yield assay, the same two *O. herpotricha* isolates were used. Response to calcium nitrate at 100 $\mu\text{g ml}^{-1}$ and ammonium sulfate at 100 $\mu\text{g ml}^{-1}$ was determined for all isolates in five separate repeated runs. The mean values, standard deviation, variance, coefficient of variance, and 95% confidence intervals were calculated for each isolate.

Least square means for radial mycelial growth and mycelial yield were subjected to analysis of variance (ANOVA) using the PROC MIXED procedure in SAS (version 9.2; SAS Institute, Cary, NC). Least square means were separated by pairwise comparisons performed using the LSmeans command ($\alpha = 0.05$).

Naturally infested field trial. A 3-year field experiment was initiated in 2011 to assess the impact of curative applications of nitrogen source, sulfur, and tebuconazole applications on spring dead spot severity. The experiment was conducted at the University of Missouri Turfgrass Field Research Farm in Columbia. The study

was performed on a 'Riviera' bermudagrass research plot on a Mexico silt loam soil with an initial pH of 5.5 to 5.7, as measured from six random samples taken throughout the plot area in 2010. A natural occurrence of spring dead spot symptoms was observed across the plot area prior to trial initiation. The site was mowed at 1.9 cm three times a week and irrigated as needed to prevent drought stress. Plots were 1.5 by 3 m, and arranged in a strip-split plot design with five replications. Nitrogen source was the main plot (strips) and sulfur and fungicide treatments were the subplots. Plots were not rerandomized between years to assess multiyear treatment affects. To determine the *Ophiophora* spp. causing symptoms in the experimental plot, six cup-cutter-sized samples (10.75 cm wide by 10.16 cm in depth) were collected at random from each replication for a total of 30 samples. Samples were then speciated as stated previously by ITS sequence analysis.

On 20 October 2010, initial bulk soil pH and nutrient analysis was conducted on six samples taken randomly across the plot in diagonal transects. Bulk soil pH was determined on 5 September 2011, 30 May 2013, and 22 August 2013 by pulling one soil core (1.9 cm wide by 7.62 cm deep) from each plot, and bulking the five replicates together to obtain one representative soil sample for each treatment ($n = 30$). Soil pH was measured for each sample in a 1:2 (wt/vol) suspension of 10 g of soil in 0.01 M CaCl_2 . Measurements were taken at room temperature with a precalibrated Accumant AB15 pH meter (Thermo Fisher Scientific). Samples were also sent to MicroMacro Analytical Laboratory (Athens, GA) for bulk soil pH measurement and soil elemental analysis with inductively coupled plasma emission spectrometry (Soltanpour et al. 1996). To determine irrigation water pH, six samples (50 ml) of irrigation water were collected on 8 June 2012 in 50-ml polypropylene conical tubes (BD Biosciences) directly from irrigation heads and measured.

Treatments were initiated 10 June 2011. Nitrogen source treatments included urea, ammonium sulfate, calcium nitrate, UMAXX, and UFLEXX applied at a rate of N at 49 kg ha^{-1} once a month in June, July, and August using a 91.4-cm-wide drop spreader (The Gandy Company, Owatonna, MN). UMAXX and UFLEXX (Agrotain International, St. Louis) contain the urease inhibitor N-(n-butyl) thiophosphoric triamide and the nitrification inhibitor dicyandiamide. Flowers of sulfur (MFA Inc., Columbia, MO) were applied at 98 kg ha^{-1} using a hand broadcast spreader at the same time as nitrogen. Tebuconazole (Torque; Cleary Chemicals LLC, Dayton, NJ) was applied at 0.82 kg a.i. ha^{-1} either once per annum on 14 October or twice per annum on 16 September and 14 October. Tebuconazole was applied in water equivalent to 7.57 liters per 92.9 m^2 with a CO_2 -powered sprayer calibrated to 172 kPa using TeeJet 8008 nozzles. All nutrient and fungicide treatments were watered in with 5 to 6 mm of overhead irrigation water immediately after application.

To establish a baseline disease severity for each plot, disease severity was assessed on 10 Jun 2011 before the trial was initiated (Y_0). Disease symptoms were most evident in the plot area during complete greenup of uninfected bermudagrass, which occurred on 22 May or 23 May in all 3 years. Percent control was calculated as $Y_0 - Y_i/Y_0$, where Y_i = disease severity on 22 May 2012, 23 May 2013, or 23 May 2014.

Disease assessment. Visual disease severity ratings were taken for all field trials by estimating the percentage of foliar spring dead spot symptoms in a single plot. Digital images were also obtained on 10 June 2011, 22 May, 8 June, and 22 June 2012; 23 May, 10 June, and 24 June in 2013; and 23 May, 6 June, and 20 June in 2014. Images of each plot were taken with a D90 Nikon DSLR camera (Nikon Inc., Melville, NY) mounted on a monopod constructed of 3.1-cm-wide vertical and horizontal square aluminum tubing. The vertical tubing was 3 m high and the horizontal section (0.8 m long) was fastened to the top and center of the vertical section at a 90° angle so the camera was centered directly above the plot area. A 30.5-cm horizontal guide was attached at the bottom so pictures would be taken at the same location in each plot. Horizontal and vertical levels were mounted to the vertical tubing of the monopod to ensure the monopod was directly upright. The camera was

Table 2. Mycelial growth of *Ophiophora* spp. as affected by pH and nitrogen source^a

Growth assessment, treatment ^b	Concentration ($\mu\text{g ml}^{-1}$) ^c	pH, PA ^d	pH, PH ^e
pH			
3	...	2.86	...
4	...	3.81	...
5	...	4.94	...
6	...	6.06	...
7	...	6.95	...
8	...	7.64	...
9	...	8.37	...
Nitrogen source: Solid (NH_4) ₂ SO ₄	0	6.14	...
	50	6.12	...
	100	6.12	...
	200	6.08	...
	400	5.96	...
	800	...	—
CaNO ₃	0	...	—
	50	...	—
	100	...	—
	200	...	—
	400	...	—
	800	...	—
Nitrogen source: Liquid (NH_4) ₂ SO ₄	0	5.66	5.52
	50	5.42	4.13
	100	5.47	4.16
	200	5.41	3.60
	400	5.36	3.54
	800	5.52	3.56
CaNO ₃	0	5.66	5.52
	50	5.48	5.73
	100	5.57	5.88
	200	5.63	5.93
	400	5.43	6.00
	800	5.49	6.16

^a Measurements of pH in growth media taken before and after autoclaving, and post mycelium harvest of *O. herpotricha* in the amended liquid media.

^b For pH treatments, lactic acid or sodium hydroxide was added to potato dextrose agar to target level pH prior to autoclaving. Ammonium sulfate or calcium nitrate was added to either solid or liquid media prior to autoclaving at the noted concentration.

^c Concentration of either ammonium sulfate or calcium nitrate.

^d Measurement of media solution pH post autoclaving.

^e Measurement of media solution pH post mycelium harvest (liquid media only).

mounted to the top horizontal section facing down with a standard tripod screw.

Before digital photography was conducted, all plot borders were outlined with cotton string. The camera was set to fully automatic mode; therefore, flash and shutter speed adjusted according to light conditions. Pictures were taken from 0800 to 1130 h to avoid shadows cast from the monopod. Images were analyzed using SigmaScan Pro 5.0 (Systat Software, Inc., Point Richmond, CA) using image editing and batch analysis macros designed by Karcher and Richardson (2003, 2005). Before analysis, all pictures were manually cropped to the plot borders and set to the same pixel size (1,600 by 1,200), color hue (40 to 100), and saturation (0 to 100) values.

Data were subject to ANOVA performed in SAS 9.2 (SAS Institute) using the PROC MIXED procedure, with year as a fixed effect. Least

square means were separated with Tukey's test ($\alpha = 0.05$). Area under the disease progress curve (AUDPC) was calculated with the formula $\sum[(y^i + y^{i+1})/2][t^{i+1} - t^i]$, where $i = 1, 2, 3, \dots, n-1$; y^i is the percent diseased plot area, and t^i is the time of the i th rating (Shaner and Finney 1977). Correlation analysis was conducted using the PROC CORR procedure in SAS 9.2 to compare the two disease estimation methods.

Results

Species distribution. Of the 213 fungi isolated from symptomatic tissue, 165 were putative *Ophiosphaerella* spp. and speciated by sequence similarity of the ITS region to GenBank accessions and reference isolates (Table 1; Fig. 1). Sequenced isolates had ITS amplicons of 535 to 600 bp in size and were 98 to 99% similar to the reference isolates of *O. herpotricha* and *O. korrae*, and the GenBank accessions of either *O. herpotricha* (KC841073, U04861), *O. korrae* (AF486626, U04862), or *O. narmari* (KC848508). *O. herpotricha* was discovered at each site and made up 93% of the isolates ($n = 154$). *O. korrae* was isolated from six locations and made up 6% of the isolates ($n = 10$). Only one isolate of *O. narmari* was found, originating from a 'Patriot' bermudagrass research plot at the University of Missouri Turfgrass Research Farm in Columbia. *O. herpotricha* and *O. korrae* were isolated once from the same turfgrass sample.

In vitro mycelial growth assay. Radial mycelial growth was greater for *O. korrae* than *O. herpotricha* in pH and solid nitrogen media assessments ($P < 0.0001$). The pH of the modified PDA used in the pH experiment changed ± 0.14 to 0.63 after autoclaving. The pH of MMN media used in the solid nitrogen source assay was similar across all treatments before and after autoclaving. Similar to solid media, the pH of amended liquid MMN media was similar across all treatments at the beginning of the experiment. At harvest, however, pH increased as calcium nitrate concentration increased and decreased as ammonium sulfate concentrations increased (Table 2).

For the two isolates of *O. herpotricha* and *O. korrae* evaluated in five repeated runs of the radial growth assay, the coefficients of variance for each isolate ranged from 0.01 to 0.07% (Table 3). The 95% confidence intervals ranged from 94 to 106% of the mean radial growth value for individual isolates subjected to the assay. For the two *O. herpotricha* isolates evaluated in five repeated runs of the mycelial weight assay, the coefficient of variance for each isolates ranged from 0.01 to 0.06%. The 95% confidence intervals ranged

Table 3. Reproducibility of the growth assays performed for determining nitrogen source impact on radial growth of *Ophiosphaerella herpotricha* and *O. korrae*, and mycelial yield of *O. herpotricha*

Assay	Isolate ^a	Diameter and weight		
		Mean ^b	CI ^c	CV (%) ^d
Radial growth (mm) (NH ₄) ₂ SO ₄	OPTF4 (O.h.)	26.20	24.64–27.75	0.06
	CTC8 (O.h.)	22.10	21.31–22.88	0.04
	2LOG3 (O.k.)	24.80	23.51–26.08	0.05
	OPTF2 (O.k.)	23.70	22.22–25.17	0.07
	CaNO ₃			
	OPTF4 (O.h.)	48.8	46.58–51.01	0.02
	CTC8 (O.h.)	52.3	51.44–53.15	0.01
Mycelial yield (mg) (NH ₄) ₂ SO ₄	2LOG3 (O.k.)	71.6	70.28–72.91	0.01
	OPTF2 (O.k.)	57.3	56.7–57.91	0.01
	WW9f (O.h.)	6.92	6.52–7.36	0.06
	DH10 (O.h.)	6.86	6.57–7.11	0.04
	CaNO ₃			
	WW9f (O.h.)	15.24	14.62–15.86	0.04
	DH10 (O.h.)	15.42	15.22–15.61	0.01

^a O.h. = *Ophiosphaerella herpotricha* and O.k. = *O. korrae*.

^b Mean radial growth values based upon values obtained in five repeated assays.

^c The 95% confidence intervals based upon values collected.

^d Coefficient of variances based upon values collected.

Fig. 2. Growth of *Ophiosphaerella herpotricha* and *O. korrae* after 12 days on potato dextrose agar adjusted to a pH range of 3 to 9 with NaOH or lactic acid. No growth occurred on media amended to pH 3. Within species, columns with the same letter are not statistically different according to pairwise comparisons of least square means ($P = 0.05$). Least square means are averaged across all isolates. Error bars represent the standard error of the means.

from 94 to 107% of the mean radial growth value for individual isolates subjected to the assay.

Effect of pH on mycelial growth. Radial mycelial growth was significantly influenced by pH ($P < 0.0001$). No growth was recorded for either species at pH 3. Growth was significantly lower for both species at pH 4 compared with higher pH treatments ($P < 0.0001$). Radial growth of *O. korrae* was significantly higher (+3.36 to 20.04 mm) at pH 6 than other pH treatments ($P \leq 0.015$; Fig. 2). Growth of *O. korrae* was not significantly different among pH treatments of 5, 7, 8, and 9 ($P > 0.05$). Radial growth of *O. herpotricha* was significantly higher (+1.76 to 13.84 mm) on pH treatments of 5 and 6 than other pH treatments ($P \leq 0.014$; Fig. 2). Growth was not significantly different for *O. herpotricha* among pH treatments of 7, 8, and 9 ($P > 0.05$).

Nitrogen source. Nitrogen source and concentration significantly affected radial growth on liquid amended media ($P < 0.0001$). Radial mycelial growth of *O. herpotricha* and *O. korrae* isolates was greater on calcium-nitrate-amended MMN media than on ammonium sulfate ($P < 0.0001$). No differences in radial growth of *O. korrae* were

observed among calcium-nitrate-amended media concentrations. Little differences in growth of *O. herpotricha* on calcium-nitrate-amended media were observed, with only growth on calcium nitrate at 800 $\mu\text{g ml}^{-1}$ being significantly lower (-2.07 to 3.16 mm) than other treatments ($P \leq 0.016$). Radial growth of *O. korrae* was significantly lower on MMN media amended with ammonium sulfate at 100, 200, 400, and 800 $\mu\text{g ml}^{-1}$ compared with ammonium sulfate at 50 $\mu\text{g ml}^{-1}$ and no nitrogen ($P < 0.0001$; Fig. 3). Similarly, radial growth of *O. herpotricha* was highest on 50 $\mu\text{g ml}^{-1}$, with growth lowest on 200, 400, and 800 $\mu\text{g ml}^{-1}$ ($P \leq 0.001$; Fig. 3).

Nitrogen source and concentration had a significant affect on mycelial yield after 12 days of incubation ($P < 0.0001$). Treatments with no nitrogen amendment weighed significantly less (-3.77 to 7.23 mg) than all nitrogen-amended treatments ($P < 0.0001$; Fig. 4). Similar to the radial growth response in solid media, *O. herpotricha* weighed significantly more (+1.45 to 3.44 mg) after 12 day of incubation in liquid amended with calcium nitrate than ammonium sulfate ($P < 0.0001$). Mycelial yield of *O. herpotricha* was greater in liquid media amended with calcium nitrate at 800 $\mu\text{g ml}^{-1}$ than

Fig. 3. Growth of **A**, *Ophiosphaerella herpotricha* and **B**, *O. korrae* on modified Melin-Norkrans medium amended with either ammonium sulfate or calcium nitrate at 0 to 800 $\mu\text{g ml}^{-1}$. Within nitrogen source, columns with the same letter are not statistically different according to pairwise comparisons performed in SAS using the LSmeans command ($\alpha = 0.05$). Least square means are averaged across all isolates. Error bars represent the standard error of the means.

unamended or with calcium nitrate treatments at 50 or 200 $\mu\text{g ml}^{-1}$ ($P \leq 0.025$) but not significantly different than calcium nitrate treatments of 100 or 400 $\mu\text{g ml}^{-1}$ ($P \geq 0.071$) (Fig. 4). Mycelial yield of *O. herpotricha* was higher in ammonium sulfate at 200 $\mu\text{g ml}^{-1}$ than at 100 $\mu\text{g ml}^{-1}$ ($P = 0.019$) but not significantly different from other ammonium sulfate treatments (Fig. 4).

Field study. Initial bulk soil pH on October 2010 averaged 5.36. In subsequent analysis, plots receiving sulfur averaged a pH of 5.52, which was slightly lower than plots not receiving sulfur (5.61). No substantial differences were observed in any soil nutrient levels between analyses conducted on 20 October 2012, 30 May 2013, or 22 August 2013 (data not shown). Irrigation water pH was slightly alkaline (7.5 to 8.0). All 30 isolates of *Ophiophora* spp. recovered from the field site had ITS amplicons of 535 to 591 bp in size and were 98 to 99% similar to the GenBank accession of *O. herpotricha* (U04861).

A significant difference between years ($P < 0.0001$) was observed for both digital image analysis and visual estimates of disease severity; therefore, data from each year were analyzed separately.

For each rating date, Pearson's correlation coefficient (r) between severity determined from visual ratings and digital imaging analysis ranged from 0.286 to 0.902 and averaged 0.640 (Table 4). Correlation became weaker at later rating dates, due to visual differentiation between sulfur phytotoxicity and spring dead spot symptoms that wasn't discerned in digital image analysis. During periods of high disease severity, visual estimates were consistently higher than estimates obtained from digital imaging analysis.

Nitrogen source did not have a significant effect on spring dead spot severity. On 22 May 2012, sulfur treatments significantly reduced spring dead spot severity ($P = 0.0127$) and in 2013 reduced AUDPC ($P < 0.0001$) (Table 4). A significant interaction of nitrogen source and sulfur occurred in May 2013 and 2014, as estimated with digital image analysis (Table 4). Delayed spring greenup was observed on 23 May 2013 in plots receiving both ammonium sulfate and sulfur. On 23 May 2014, all plots receiving sulfur were delayed in spring greenup, with ammonium sulfate being most affected. In both years, digital image analysis was unable to discern between dormant and diseased turf during the spring greenup period (Fig. 5).

Fig. 4. Yield of *Ophiophora herpotricha* mycelium in liquid Melin-Norkrans medium amended with either ammonium sulfate or calcium nitrate at 0–800 $\mu\text{g ml}^{-1}$. Within nitrogen source, columns with the same letter are not significantly different according to pairwise comparisons performed in SAS using the LSmeans command ($\alpha = 0.05$). Means are averaged across all isolates. Error bars represent the standard error of the means.

Table 4. Results of analysis of variance for field experiment to determine the effects of nitrogen source, sulfur, and fungicide on curative control of spring dead spot caused by *Ophiophora herpotricha*^a

Source ^d	df	Individual rating dates ^b						AUDPC ^c					
		22 May 2012		23 May 2013		23 May 2014		2012		2013		2014	
		DIA	Visual	DIA	Visual	DIA	Visual	DIA	Visual	DIA	Visual	DIA	Visual
Nitrogen source	4	0.568	0.865	0.809	0.921	0.036	0.694	0.699	0.906	0.533	0.704	0.001	0.704
Fungicide	2	0.001	0.089	<0.001	0.007	0.200	0.606	<0.0001	<0.0001	<0.0001	<0.0001	0.037	0.022
Nitrogen source × fungicide	8	0.910	0.073	0.884	0.936	0.988	0.399	<0.0001	0.066	0.593	0.055	0.949	0.378
Sulfur (S)	1	0.013	0.004	0.418	0.010	0.003	0.092	0.057	0.007	<0.0001	<0.0001	<0.001	0.043
Nitrogen source × S	4	0.805	0.748	0.018	0.078	<0.0001	0.464	0.215	0.576	0.180	0.537	<0.0001	0.372
Fungicide × S	2	0.786	0.881	0.534	0.984	0.675	0.488	0.158	0.557	0.025	0.090	0.532	0.43
Nitrogen source × fungicide × S	8	0.810	0.749	0.436	0.697	0.995	0.541	0.763	0.883	0.711	0.974	0.372	0.458

^a P values calculated with the PROC GLIMMIX procedure. Significant values ($P < 0.05$) indicated in bold.

^b Individual ratings taken on dates when spring dead spot symptoms were first prominent after uninfected bermudagrass emerged from dormancy. Ratings were obtained through either digital image analysis (DIA) or by visual estimation of the percent spring dead spot in the plot area.

^c Area under the disease progress curve (AUDPC) for each year of the study calculated with the trapezoidal rule (Shaner and Finney 1977).

^d Factors of the field experiment. Nitrogen source = urea, calcium nitrate, ammonium sulfate, UFLEX, or UMAXX applied with N at 49 kg ha⁻¹ in June, July, and August; Fungicide = tebuconazole (0.82 kg a.i. ha⁻¹) applied either once or twice per fall; and S = flowers of sulfur applied at 98 kg ha⁻¹ in June, July, and August.

Fungicide treatments significantly reduced AUDPC in each year and on most rating dates (Table 4). Plots treated with tebuconazole either once or twice had lower AUDPC values in 2012 than untreated plots without sulfur treatment (Fig. 6). In 2013, the

AUDPC of plots treated with one tebuconazole application with and without sulfur, or two applications without sulfur, were not significantly different from plots treated with sulfur alone. In all 3 years, no significant difference in spring dead spot severity

Fig. 5. Effect of nitrogen source and sulfur on the percent reduction of spring dead spot severity caused by *Ophiosphaerella herpotricha* on 23 May 2013. Percent reduction of spring dead spot severity was calculated as $Y_0^* - Y_i/Y_0$, where Y_i = disease severity (percent green tissue) assessed by digital image analysis on 23 May 2013 and Y_0 = disease severity on 10 June 2011, before the trial was initiated. Columns with the same letter are not statistically different according to Tukey's test ($\alpha = 0.05$). Least square means were averaged across all nitrogen source and sulfur treatments. Error bars represent the standard error of the mean.

Fig. 6. Effect of nitrogen source and sulfur on area under the disease progress curve (AUDPC) values derived from digital image analysis assessments of spring dead spot caused by *Ophiosphaerella herpotricha*. AUDPC was calculated with the trapezoidal rule (Shaner and Finney 1977). UT = no fungicide treatment, 1F = one fall application of tebuconazole (0.28 kg a.i. ha⁻¹) per year on 14 October, 2F = two fall applications of tebuconazole (0.28 kg a.i. ha⁻¹) per year on 16 September and 14 October, S- = no sulfur treatment, and S+ = sulfur applied annually as flowers of sulfur at 98 kg ha⁻¹ in June, July, and August. Columns with the same letter within each year are not statistically different according to Tukey's test ($\alpha = 0.05$). Least square means were averaged across all fungicide and sulfur treatments. Error bars represent the standard error of the mean.

was detected between one and two fall applications per year (Fig. 6).

Discussion

Based on sequences of the ITS region, *O. herpotricha* was the predominant spring dead spot pathogen detected in Missouri and the surrounding region. This geographic region has been suggested as a native region for this *Ophiophaerella* sp., which would explain its high frequency of isolation (Wetzel et al. 1999b). On occasion, *O. korrae* was also isolated from infected bermudagrass tissue, indicating an overlapping species distribution. *O. herpotricha* and *O. korrae* have previously been isolated from the same location and may even be isolated from the same tissue sample (Wetzel et al. 1999b). *O. narmari* was isolated from a single spring dead spot sample obtained from a 1-year-old Patriot bermudagrass block vegetatively propagated the previous year with plant material imported from Oklahoma. Therefore, this pathogen species is likely not widely distributed in Missouri but may be introduced occasionally with distributed plant material.

O. herpotricha is considered the most aggressive of the three spring dead spot pathogen species (Tredway et al. 2009b) and, in this study, was isolated from 13 different cultivars. Large spring dead spot outbreaks were observed on cold-tolerant cultivars such as Patriot and Riviera, suggesting that cold-tolerant cultivars are also susceptible to spring dead spot pathogen infection. More research is needed to screen the susceptibility of new bermudagrass cultivars to spring dead spot, and determine whether host susceptibility varies among spring dead spot species or climactic region.

Previous research indicates that nitrogen source or soil pH may play an important role in suppressing spring dead spot. Field studies report a decrease in spring dead spot symptoms when bulk soil pH is reduced below 5.5 (Dernoeden et al. 1991; Vincelli 2014). However, *O. herpotricha* and *O. korrae* grew well at pH 5 and higher in our in vitro study. Maintaining a bulk soil pH below 5 would be challenging and may lead to nutrient deficiencies but only the rhizosphere pH may need this level of acidity during the infection period, which may occur along the root surface when ammonium ions are acquired. Accurately separating and comparing bulk and rhizosphere pH in plants possessing a fibrous root system is difficult but may need to be determined to fully understand the pH interaction with the spring dead spot pathogen (Youssef and Chino 1989).

No difference was detected among *O. herpotricha* and *O. korrae* in their in vitro response to calcium nitrate or ammonium sulfate concentrations, because both grew equally well on calcium nitrate and poorly on ammonium sulfate. Hacskeylo et al. (1954) performed a similar liquid mycelial growth assay and found ammonium sulfate to be a preferred nitrogen source over calcium nitrate for 24 of 25 fungal species. However, this result only occurred when fumaric acid was used to buffer treatment pH. When fumaric acid was not used as a buffer, pH in ammonium sulfate treatments decreased, which the authors hypothesize negatively affected growth. A similar mechanism of ammonium uptake is known to occur in fungal mycelium that results in pH reduction (Morton and Macmillan 1954). Repeating these assays using a treatment pH buffer may be appropriate to demonstrate nitrogen form preference for growth among these *Ophiophaerella* spp.

Contrary to previous reports, our field study did not indicate a reduction in spring dead spot severity from nitrogen source. Dernoeden et al. (1991) observed that ammonium sulfate applications reduced spring dead spot caused by *O. korrae* in Maryland. *O. herpotricha*, which is considered to be more aggressive than *O. korrae*, was the only spring dead spot species found in this field plot, which may explain the difference between these results. In North Carolina, ammonium sulfate was demonstrated to suppress spring dead spot caused by *O. herpotricha* (Tredway et al. 2009a). The different soil type or region may be responsible for this difference in results. Soil pH at our site was initially low (approximately 5.5), and perhaps the affect of ammonium sulfate application on rhizosphere pH was not as prevalent. Irrigation water pH at our research site was also slightly alkaline (pH 7.5 to 8), which may have reduced the

potential for soil pH reduction from ammonium nitrogen forms. Finally, and perhaps most importantly, this study was conducted on a site with a widespread spring dead spot epidemic caused by *O. herpotricha*, whereas the previous study was performed on inoculated plots evaluating preventive fertility treatments. After infection, *O. herpotricha* colonizes the root cortex of susceptible cultivars (Caasi et al. 2010) and may not need to reinfect the plant from the root surface every year to reproduce symptoms. Therefore, changing rhizosphere pH with a nitrogen source may have a reduced effect or require a multiyear integrated control strategy.

Sulfur applications resulted in a reduction in spring dead spot severity similar to a single fungicide application applied without sulfur in 2013. Sulfur applications have the potential to lower soil pH; however, no considerable differences were detected in bulk soil pH in sulfur-treated plots in this study. A low initial soil pH, the buffering capacity of the soil, or the alkaline pH of the irrigation water could have prevented soil acidification by the sulfur applications. As opposed to soil acidification, sulfur applications may also be directly inhibiting the pathogen by acting as a fungicide (McCallan 1949). Delayed spring greenup and premature winter dormancy were observed in plots receiving sulfur beginning in spring and fall 2013. This effect was exacerbated in plots also treated with ammonium sulfate; therefore, sulfur rates may need to be decreased or discontinued to decrease the possibility of this effect. This observation of delayed spring greenup is in agreement with previous research (Vincelli 2014).

After 1 year, one or two fall fungicide applications reduced disease compared with the untreated plots but control was still unacceptable. Therefore, 2 years of fungicide applications may be necessary to achieve desirable control levels. Additionally, one fungicide application per annum provided statistically as much control as two fungicide applications per annum in all 3 years of the study. Therefore, one application may be more cost effective in a multiyear management system. Conversely, Walker (2009) reported an 81% reduction in spring dead spot symptoms after 1 year with two fall applications of propiconazole. This difference in fungicide efficacy may stem from the different active ingredient used, or from the higher volume of post-application irrigation (10 to 13 mm) used for fungicide delivery in the previous research.

This research indicates curative management of spring dead spot can be achieved through a multiyear integrated strategy combining sulfur and fungicide applications. Future research integrating reduced sulfur rates to minimize plant side effects, cultivation practices, and fungicide application timing and methods is needed to develop management recommendations aimed toward reducing fungicide requirements and increasing control efficacy.

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