Multiplex End-Point PCR for the Detection of Three Species of *Ophiosphaerella* Causing Spring Dead Spot of Bermudagrass

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Abstract

A multiplex end-point polymerase chain reaction (PCR) assay was developed for identifying the three-fungal species in the genus *Ophiosphaerella* that cause spring dead spot (SDS), a devastating disease of bermudagrass. These fungi are difficult to identify by morphology because they seldom produce pseudothecia. To achieve species-specific diagnosis, three pairs of primers were designed to identify fungal isolates and detect the pathogen in infected roots. The internal transcribed spacer region, the translation elongation factor 1- α , and the RNA polymerase II second-largest subunit were selected as targets and served as templates for the design of each primer pair. To achieve uniform melting temperatures, three to five random nucleotide extensions (flaps) were added to the 5' terminus of some of the designed specific primers. Temperature cycling conditions and PCR components were standardized

to optimize specificity and sensitivity of the multiplex reaction. Primers were tested in multiplex on DNA extracted from axenic fungal cultures and from field-collected infected and uninfected roots. A distinct amplicon was produced for each *Ophiosphaerella* sp. tested. The DNA from *Ophiosphaerella* close relatives and other common bermudagrass pathogens did not amplify during the multiplex assay. Metagenomic DNA from infected bermudagrass produced species-specific amplicons while DNA extracted from noninfected roots did not. This multiplex end-point PCR approach is a sensitive and specific molecular technique that allows for correct identification of SDS-associated *Ophiosphaerella* spp. from field-collected roots.

Keywords: fungi, pathogen detection, turf

Bermudagrasses are widely used as turf in warm regions throughout the world. The two most common types are *Cynodon dactylon* (L.) Pers. and interspecific hybrids of *C. dactylon* $\times$ *C. transvaalensis* (Burt-Davy). The fine-textured, dark-green interspecific hybrids are widely used on golf courses, lawns, and sport fields due to their high shoot density, aggressive growth, and pest and disease tolerance (Tredway et al. 2009).

Spring dead spot (SDS) is one of the most damaging diseases of bermudagrass where cold temperatures induce plant dormancy. The disease is caused by three fungal species in the ascomycete 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, and Tisserat (Endo et al. 1985; Smiley et al. 2005; Smith 1965; Tisserat et al. 1989; Wetzel et al. 1999). Genus *Ophiosphaerella* is a member of the Pleosporales family, which includes a wide variety of plant pathogens and facultative saprophytes. During periods of cool temperatures, the fungi colonize the roots, stolons, and rhizomes of the grass, which can lead to necrosis and lesions prior to cold temperature-induced dormancy. In the spring, once temperatures increase, plant regrowth originates from stolons and rhizomes; however, stolons and rhizomes that were colonized by the pathogenic fungi do not survive winter dormancy. As healthy grass resumes growth in the spring, SDS symptoms appear as dead plants in circular or semicircular patches, extending from a few centimeters to more than a meter in diameter (Wadsworth and Young 1960). Field evaluations have shown that *O. herpotricha* is the most aggressive of the three species, followed by *O. korrae*, and

O. narmari (Iriarte et al. 2005). For smaller dead patches, reestablishment of turf may be achieved in a few months; however, for larger patches, this may take the entire growing season. In addition to the dead grass, weed establishment in the dead areas is common, requires additional herbicide applications, and can compete with the regrowth of bermudagrass.

Researchers, diagnosticians, and turfgrass managers may need to know the species of *Ophiosphaerella* present at a given location because differences in virulence among species have been reported (Iriarte et al. 2005; Tisserat et al. 1989; Tredway et al. 2008). Traditional identification requires the fungi to be directly isolated from colonized living plant tissue. *Ophiosphaerella* spp. are slow-growing fungi (Iriarte et al. 2004) and other common, faster-growing fungi associated with bermudagrass make recovery of pure cultures difficult. When isolates of *Ophiosphaerella* are recovered, traditional morphological-based identification and discrimination among species is not possible, given that the fungi rarely produce ascospores or other diagnostic structures. Therefore, DNA sequence analysis of ribosomal DNA (rDNA) genes is routinely used for species identification.

Development of polymerase chain reaction (PCR)-based methods to quickly identify species of *Ophiosphaerella* have been previously conducted (O'Gorman et al. 1994; Tisserat et al. 1994; Wetzel et al. 1999) and targeted conserved rDNA regions. Primers LK17S and 5.8SC developed by O'Gorman et al. (1994), targeted conserved regions of the 18S and 5.8S rDNA of *O. korrae*. Primers OHITS1 and OHITS2 for *O. herpotricha* and OKITS1 and OKITS2 for *O. korrae* were based on the internal transcribed spacer (ITS) region and can discriminate these two species (Tisserat et al. 1994). Wetzel et al. (1999) developed the primers OnITS1 and OnITS2 from the ITS1 and ITS2 regions to distinguish *O. narmari* from *O. herpotricha* and *O. korrae*.

Although the primers developed by O'Gorman et al. (1994) and Tisserat et al. (1994) were able to amplify the respective fungal DNA from naturally infected and inoculated plants, they require separate reactions for each primer pair, making screening of large numbers of plant samples costly. One methodological alternative to

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reduce diagnostic costs and effort is multiplex end-point PCR. This PCR variant includes two or more primer pairs per reaction, allowing detection and discrimination of multiple target organisms in a single reaction (Henegariu et al. 1997).

Multiplex end-point PCR primers are designed with similar or very close melting temperatures and PCR conditions are optimized in order to obtain specific target amplification using the same annealing temperature for all the primer sets included in the reaction (Abd-Elmagid et al. 2013; Elnifro et al. 2000; Henegariu et al. 1997). PCR targets are usually resolved in agarose or polyacrylamide gels and visualized using either UV-light or visible-light photography. By reducing sample processing time and effort, this practical approach can also reduce overall diagnostic costs. Multiplex end-point PCR has been successfully used for identification of diverse plant pathogens, including fungi (Abd-Elmagid et al. 2013; Côté et al. 2004; Elnifro et al. 2000; Schaad et al. 2003). The objective of this study was to develop primers that could discriminate the three species of *Ophiosphaerella* in plants using multiplex end-point PCR.

Materials and Methods

Primer design. Different loci were targeted for the design of each primer pair: the ITS region for *O. korrae*, the translation elongation factor 1- α (EF1 α) for *O. herpotricha*, and the RNA polymerase II second-largest subunit (RPB2) for *O. narmari*. Geneious V. 6.0.6 (Kearse et al. 2012) was used for DNA sequence alignments. Sequences from SDS-causing species and closely related fungi were obtained from the National Center for Biotechnology Information's GenBank. For each individual locus, a minimum of 80 sequences from diverse sources was aligned to identify conserved motifs that were exclusive for each species. Primers that included such motifs at the 3' end were designed. The online bioinformatics tools Multiple Primer Analyzer (<https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html>) and Oligoanalyzer (<https://www.idtdna.com/site/account/login?returnurl=%2Fcalc%2FAnalyzer>) were used for examining the potential formation of secondary structures and determining the optimal annealing temperature of specific primers in silico. To achieve uniform melting temperatures, three to five random nucleotide extensions (flaps) were added in the 5' end of some of the designed specific primers. Specificity of the primers was initially assessed by blasting them against the GenBank database using Primer-BLAST (Ye et al. 2012).

In vitro testing. Sensitivity and specificity of the primers were assayed in vitro. Specificity was tested using isolates of each SDS-causing species of *Ophiosphaerella*, as well as closely related fungal species and common fungal pathogens of bermudagrass. Pure cultures of each species that were previously identified using PCR were obtained from the culture collection of the turfgrass pathology laboratory at Oklahoma State University. Fungi were grown on semiselective medium containing potato dextrose agar amended with rifampicin at 10 mg/liter, ampicillin at 250 mg/liter, and fenpropatrin at 5 mg/liter (Walker et al. 2006). Hyphal tips of fungal colonies were transferred to potato dextrose broth and incubated at room temperature for 2 weeks. Mycelia were washed by filtration on Whatman 40 filter paper and lyophilized. Fungal DNA was extracted using a

cetyltrimethylammonium bromide method (Weising et al. 1994), with some modifications. Extraction buffer (1 ml), heated in a microwave for 10 s, was added to 30 to 50 mg of lyophilized mycelium in a 2-ml plastic tube containing one 5-mm and three 3-mm zirconia beads. Samples were bead-beaten four times for 20 s to disrupt cells and incubated at 58°C and 600 rpm for 30 min. Samples were centrifuged at 14,000 $\times$ g for 20 min at 4°C; 1.5 volumes of cold isopropanol were added to the supernatant and the samples were incubated at 4°C for 2 h. The precipitated DNA was pelleted by centrifugation at 14,000 $\times$ g for 20 min at room temperature, the supernatant was discarded, and the pellet was washed twice with 70% ethanol and resuspended with Tris-acetate-EDTA buffer.

Multiplex PCR final volumes of 25, 50, or 60 μ l were assayed, each reaction contained final concentrations of 5 to 10 μ M each for one of six primers, 1 $\times$ GoTaq Green Master Mix (Promega Corp., Madison, WI, U.S.A.), and up to 35 ng of template DNA. The optimal final volume and concentrations were established in 60 μ l of 10 μ M primers because this combination had the brightest bands with the least amount of primer-dimers in the electrophoresis gel. A gradient of annealing temperatures was tested under the following cycling conditions: 97°C for 5 min; 30 cycles of 95°C for 45 s, 52, 54.3, 56, 57.3, or 58°C for 45 s, and 72°C for 45 s; followed by a final extension at 72°C for 5 min. Therefore, the sensitivity was defined as the lowest amount of detectable DNA. In order to determine whether the assay can detect more than one *Ophiosphaerella* sp. in a single sample, an equimolar mixture of DNA from the three species that cause SDS was also tested.

The specificity of the multiplex was tested using DNA from fungi that are closely related to genus *Ophiosphaerella* or may live in the rhizosphere of bermudagrass, including *Phaeosphaeria nodorum*, *Clarireedia jacksonii* (syn. *Sclerotinia homoeocarpa*), *Bipolaris cynodontis*, *B. sorokiniana*, and *Gaeumannomyces graminis*. Serial dilutions of DNA, decreasing by one order of magnitude from 10 to 10⁻⁶ ng/ μ l, were prepared, and served as templates for testing the sensitivity of the reaction on each species. For all cases, amplicons were resolved in 2% agarose gels using sodium borate (SB) electrophoresis buffer at 200 V for 30 min. Cycling conditions and PCR components were standardized to optimize the concentration and quality of the amplicons.

Validation. The standardized multiplex PCR was validated on field-collected plant samples. Since samples were obtained in autumn, plants were asymptomatic (SDS manifests itself exclusively in spring). Plants were obtained in Stillwater and Broken Bow, OK in October 2015 using a 5-cm-diameter soil core. In Stillwater, samples were collected from a stand of bermudagrass at three locations where *O. herpotricha* was previously inoculated and at three locations in the same stand where disease was not present previously. Additional samples were collected in Broken Bow from a golf fairway with a history of severe SDS caused by both *O. korrae* and *O. herpotricha*. Roots were surface sterilized; the DNA was obtained as described above and used as template for the multiplex PCR assay.

Results

Primer design. In total, 45 primers with species-specific polymorphisms were designed and tested with DNA of *Ophiosphaerella*. Three species-specific primer pairs that did not show cross-reactivity

Table 1. Primers designed for the identification of *Ophiosphaerella* spp. that cause spring dead spot of bermudagrass^a

<i>Ophiosphaerella</i> spp.	Primer	5'–3' Sequence	Tm (°C) ^b	GC (%)	Product size (bp)	Target region ^c
<i>O. herpotricha</i>	OHERFW	CGTAATCTCCAAGATGGCCAA	55.2	45.5	247	EF1 α
	OHERRV	CACGCAGTTGGTAGAAACGT	55.5	50	...	...
<i>O. korrae</i>	OKORFW	GGACACCCATTGAACCTWTTT	55.1	45.5	272	ITS
	OKORRV	GTTATCWGACGCAGTGGAGTG	55.4	52.4	...	...
<i>O. narmari</i>	ONARFW	GGACTCTTCGATAGGGATATCAG	53.7	47.8	205	RPB2
	ONARRV	GCCACTCATCAAGATCTTCCG	55.6	52.4	...	...

^a Nucleotides shown in bold are not complementary to target sequences and were added to achieve uniform melting temperatures among primers.

^b Melting temperature.

^c Translation elongation factor 1- α (EF1 α), internal transcribed spacer (ITS) region, and RNA polymerase II second-largest subunit (RPB2).

were chosen for further experimentation (Table 1). Guanine-cytosine-rich flaps, of 3 to 5 bases, were added to primers OHERFW, OHERRV, OKORRV, and ONRWF to achieve a uniform melting temperature for all the primers, which was close to 55°C.

Secondary structure analysis showed that primers were not likely to form hairpins at temperatures above 46.6°C. Only OHERFW and ONARFW showed a strong potential for homodimer formation with changes of Gibbs free energy of bonding (ΔG s) of -13.19

and -11.71 kcal/mol, respectively. Potential heterodimers with ΔG below -9 kcal/mol were not found among the six primers. In silico, all primers hybridized only with sequences corresponding to the target species. Primers for *O. herpotricha*, *O. narmari*, and *O. korrae* matched specifically with 43, 8, and 25 sequences, respectively, in GenBank.

In vitro testing. The reaction resulted in specific amplicons for each species. Products were 272, 247, and 205 bp in size for *O. korrae*, *O. herpotricha*, and *O. narmari*, respectively, and the three bands could be resolved simultaneously in an agarose gel when a DNA mixture from all three species was used as template (Fig. 1). The highest annealing temperature at which clear bands were observed was 56°C; at higher temperatures, the band corresponding to *O. narmari* started to fade (Fig. 2). The multiplex PCR was tested with DNA from several *Ophiophora* isolates previously identified to the species level and it amplified products of the expected size in all cases (Fig. 3). Reactions using DNA from closely related fungi as template resulted in no amplification (Fig. 4). The highest sensitivity was attained with a reaction volume of 60 μ l and a final concentration of 5 μ M for each of the six primers used in multiplex, detecting the presence of *O. korrae* DNA down to a concentration of 1 pg/ μ l while the detection limit for *O. herpotricha* and *O. narmari* was 10 pg/ μ l. The simultaneous detection of the three species using DNA mixtures was not possible at concentrations below 10 ng/ μ l.

Validation. The DNA extracted from plants at concentrations between 25 and 35 ng/ μ l was obtained from partially homogenized root tissues. The multiplex assay produced the expected 247-bp amplicons for the six samples taken from locations that were previously inoculated with *O. herpotricha* (Fig. 3). *Ophiophora* spp. were detected in three of the seven samples from a fairway with history of SDS; two of these samples contained *O. herpotricha* and one *O. korrae*. There was no amplification for samples collected from locations with no history of SDS.

Discussion

From 43 primers designed that were specific for either one of the three *Ophiophora* spp. under study, only three pairs showed no cross-reaction in vitro. Cross-reaction was significantly reduced when each pair of specific primers targeted different loci (results

Fig. 1. Polymerase chain reaction products of the multiplex reaction for the identification of *Ophiophora* spp. that cause spring dead spot of bermudagrass. DNA used as template from lane 1, *Ophiophora herpotricha*; lane 2, *O. korrae*; lane 3, *O. narmari*; and lane 4, *O. herpotricha* + *O. korrae* + *O. narmari*. Lane L = 50-bp ladder and NTC = no-template control.

Fig. 2. Annealing temperature gradient for the multiplex reaction for the identification of *Ophiophora* spp. that cause spring dead spot of bermudagrass (50-bp ladder). For each temperature, left to right, DNA template from *Ophiophora herpotricha* (OH), *O. korrae* (OK), *O. narmari* (ON), *O. herpotricha* + *O. korrae* + *O. narmari* (MIX), and no-template control (NTC).

not shown). Single mismatches around or near to 3' end prevent primer annealing and affected the efficiency of the reaction (Kwok et al. 1990). A minimum of three complementary nucleotides at the 3' end of the primer are required for successful amplification of a target sequence (Sommer and Tautz 1989). Even though SDS-causing *Ophiosphaerella* spp. are closely related, and there are few species-specific polymorphisms that can be exploited for primer design, the three primer pairs selected for the multiplex PCR assay targeted sequences that included at least one polymorphic site at the 3' end to avoid mispriming. Complementarity at the primer 5' end was shown to be unnecessary for specificity, and the addition of noncomplementary nucleotides at this end helped achieve uniform annealing temperatures for all primers. The addition of flaps also rendered primers within the recommended length and annealing temperature range for oligonucleotides used in a multiplex reaction (Henegariu et al. 1997).

O. korrae primers were 100-fold more sensitive than *O. herpotricha* and *O. narmari* primers because the former amplify part of the rDNA, for which fungal genomes can have hundreds of copies (Black et al. 2013), while the latter target single-copy loci RPB2 and EF1 α . The rDNA has been the sole target of previously reported species-specific primers for detection of *Ophiosphaerella* spp. (O'Gorman et al. 1994; Tisserat et al. 1994; Wetzel et al. 1999), which had sensitivity limits similar to the ones we report for *O. korrae*. A multiplex assay for the detection of *Ophiosphaerella* spp. was not available until now due to the lack of sequence data on different loci from this genus. Previously described specific primers targeted the same polymorphic sites for the different species, rendering products of the same size (Tisserat et al. 1994); hence the need for separate assays.

This multiplex assay was able to detect and discriminate all three *Ophiosphaerella* spp. simultaneously when the total target DNA concentration was at least 10 ng/ μ l. Phylogenetic analysis of *Ophiosphaerella* revealed the presence of two distinct clades within *O. korrae* (Flores et al. 2017b). Specific primer hybridization to the ITS region of individuals from both clades was achieved by including a single degenerate nucleotide on primers OKORFW and OKORRV. In silico analysis showed that, during the annealing step at 56°C, the oligonucleotides in the reaction mix should be linearized and would hybridize with the target DNA efficiently. Primers were designed to avoid hetero- and homodimer formation and are unlikely to form hairpins at temperatures above 50°C.

Among the oligonucleotides used in the multiplex reaction, *O. korrae* primers showed the lowest capacity to form dimers, which can also explain their enhanced sensitivity. Although primers OHERFW and ONARFW showed strong potential for homodimer formation, none were observed at the optimized PCR assay conditions with one target DNA. Faint unspecific fragments were visible in some reactions that included DNA mixtures of the three *Ophiosphaerella* spp. when highly concentrated products were loaded to agarose gels (not shown). Unspecific fragments were not observed during assay validation.

SDS symptoms appear when bermudagrass comes back from dormancy but the causal agent persists in the root system all year long (Perry et al. 2010; Walker 2006). Using multiplex PCR in a diagnostic test may detect different species of *Ophiosphaerella* in a single reaction from whole DNA extracted from field samples even if they are asymptomatic. In addition, the actual host–fungus association host range for *Ophiosphaerella* spp. is cryptic and not fully characterized (Flores et al. 2017a). This diagnostic test could be used on other grass species to further define the range of plants *Ophiosphaerella* colonizes.

Fig. 4. Cross reactivity of the primers tested with closely related fungi. Lane L, 100-bp ladder; lane 1, *Ophiosphaerella narmari* isolate AUS58; lane 2, *Gaeumannomyces graminis*; lane 3, *Phaeosphaeria nodorum*; lane 4, *Bipolaris sorokiniana*; lane 5, *Bipolaris cynodontis*; lane 6, *Claviceptis jacksonii*; and lane NTC, no-template control; L, 50-bp ladder; L', 100-bp ladder.

Fig. 3. Validation of the multiplex polymerase chain reaction designed to detect *Ophiosphaerella* spp. that cause spring dead spot (SDS) of bermudagrass. The multiplex was tested with samples from lanes 1 to 7, a field with history of SDS; lanes 8 to 13, locations previously inoculated with *Ophiosphaerella herpotricha*; and lanes 14 to 16, locations with no history of SDS. Lane L = 50-bp ladder and NTC = no-template control.

Results show the multiplex end-point PCR reported herein is capable of producing a unique amplicon for each one of the three SDS-associated *Ophiosphaerella* spp. There is no cross-reaction with closely related fungi or fungi commonly found associated with bermudagrass and the test is sensitive enough to detect the pathogens directly from field samples, making this method a very useful tool for timely diagnosis.

Literature Cited

- Abd-Elmagid, A., Garrido, P. A., Hunger, R., Lyles, J. L., Mansfield, M. A., Gugino, B. K., Smith, D. L., Melouk, H. A., and Garzon, C. D. 2013. Discriminatory simplex and multiplex PCR for four species of the genus *Sclerotinia*. *J. Microbiol. Methods* 92:293-300.
- Black, J., Dean, T., Byfield, G., Foadre, K., and Menetrez, M. 2013. Determining fungi rRNA copy number by PCR. *J. Biomol. Tech.* 24:32-38.
- Côté, M.J., Tardif, M.C., and Meldrum, A. J. 2004. Identification of *Monilinia fructigena*, *M. fructicola*, *M. laxa*, and *Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. *Plant Dis.* 88:1219-1225.
- Elnifro, E. M., Ashshi, A. M., Cooper, R. J., and Klapper, P. E. 2000. Multiplex PCR: Optimization and application in diagnostic virology. *Clin. Microbiol. Rev.* 13:559-570.
- Endo, R. M., Ohr, H. D., and Krausman, E. M. 1985. *Leptosphaeria korrae*, a cause of the spring dead spot disease of bermudagrass in California. *Plant Dis.* 69:235-237.
- Flores, F. J., Marek, S. M., Anderson, J. A., Mitchell, T. K., Moreno-Zambrano, M., and Walker, N. R. 2017a. Reactive oxygen species production and alternative hosts of spring dead spot-causing fungi. *Int. Turfgrass Soc. Res. J.* 13:213-224.
- Flores, F. J., Marek, S. M., Orquera, G., and Walker, N. R. 2017b. Molecular identification and multilocus phylogeny of *Ophiosphaerella* species associated with spring dead spot of Bermudagrass. *Crop Sci.* 57(S):249-261.
- Henegariu, O., Heerema, N., Dlouhy, S., Vance, G., and Vogt, P. 1997. Multiplex PCR: Critical parameters and step-by-step protocol. *Biotechniques* 23:504-511.
- Iriarte, F. B., Wetzel, H. C., Fry, J. D., Martin, D. L., and Tisserat, N. A. 2004. Genetic diversity and aggressiveness of *Ophiosphaerella korrae*, a cause of spring dead spot of bermudagrass. *Plant Dis.* 88:1341-1346.
- Iriarte, F. B., Wetzel, H. C., Fry, J. D., Martin, D. L., Vincelli, P., Dixon, E. W., and Tisserat, N. A. 2005. Aggressiveness of spring dead spot pathogens to bermudagrass. *Int. Turfgrass Soc. Res. J.* 10:258-264.
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., and Drummond, A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647-1649.
- Kwok, S., Kellogg, D. E., McKinney, N., Spasic, D., Goda, L., Levenson, C., and Sninsky, J. J. 1990. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. *Nucleic Acids Res.* 18:999-1005.
- O'Gorman, D., Xue, B., Hsiang, T., and Goodwin, P. H. 1994. Detection of *Leptosphaeria korrae* with the polymerase chain reaction and primers from the ribosomal internal transcribed spacers. *Can. J. Bot.* 72:342-346.
- Perry, D., Tomaso-Peterson, M., and Baird, R. 2010. Seasonal variation in frequency of isolation of *Ophiosphaerella korrae* from bermudagrass roots in Mississippi and pathogenicity and optimal growth of selected isolates. *Mycopathologia* 169:395-402.
- Schaad, N. W., Frederick, R. D., Shaw, J., Schneider, W. L., Hickson, R., Petrillo, M. D., and Luster, D. G. 2003. Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. *Annu. Rev. Phytopathol.* 41:305-324.
- Smiley, R. W., Dernoeden, P. H., and Clarke, B. B. 2005. Compendium of Turfgrass Diseases, 3rd ed. American Phytopathological Society, St. Paul, MN, U.S.A.
- Smith, A. M. 1965. *Ophiobolus herpotrichus* a cause of spring dead spot in couch turf. *Agric. Gaz. N. S. W.* 76:753-758.
- Sommer, R., and Tautz, D. 1989. Minimal homology requirements for PCR primers. *Nucleic Acids Res.* 17:6749.
- Tisserat, N. A., Hulbert, S. H., and Sauer, K. M. 1994. Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiosphaerella korrae* and *O. herpotricha*. *Phytopathology* 84:478-482.
- Tisserat, N. A., Pair, J. C., and Nus, A. 1989. *Ophiosphaerella herpotricha*, a cause of spring dead spot of bermudagrass in Kansas. *Plant Dis.* 73:933-937.
- Tredway, L. P., Butler, E. L., Soika, M. D., and Bunting, M. L. 2008. Etiology and management of spring dead spot of hybrid bermudagrass in North Carolina, USA. *Acta Hort.* 783:535-546.
- Tredway, L. P., Tomaso-Peterson, M., Perry, H., and Walker, N. R. 2009. Spring dead spot of bermudagrass: A challenge for researchers and turfgrass managers. *Plant Health Prog.* 10. doi:10.1094/PHP-2009-0710-01-RV
- Wadsworth, D. F., and Young, H. C. 1960. Spring dead spot of bermudagrass. *Plant Dis. Rep.* 44:516-518.
- Walker, N. R. 2006. Viability of dormant bermudagrass in spring dead spot patches caused by *Ophiosphaerella herpotricha* during winter months. (Abstr.) *Phytopathology* 96:S119.
- Walker, N. R., Mitchell, T. K., Morton, A. N., and Marek, S. M. 2006. Influence of temperature and time of year on colonization of bermudagrass roots by *Ophiosphaerella herpotricha*. *Plant Dis.* 90:1326-1330.
- Weising, K., Nybom, H., Pfenninger, M., Wolff, K., and Meyer, W. 1994. DNA Fingerprinting in Plants and Fungi. CRC Press, Boca Raton, FL, U.S.A.
- Wetzel, H. C., Hulbert, S. H., and Tisserat, N. A. 1999. Molecular evidence for the presence of *Ophiosphaerella narmari* n. comb., a cause of spring dead spot of bermuda grass, in North America. *Mycol. Res.* 103:981-989.
- Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. L. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinf.* 13:134.