Techniques

Selective Amplification of rDNA Internal Transcribed Spacer Regions to Detect *Ophiosphaerella korrae* and *O. herpotricha*

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ABSTRACT

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The internal transcribed spacer (ITS) regions of the rDNA of *Ophiosphaerella herpotricha* and *O. korrae* (= *Leptosphaeria korrae*) were amplified with the universal primers ITS4 and ITS5. Amplifications of genomic DNA from *O. herpotricha* isolates always resulted in a single 590-bp fragment, whereas amplifications of *O. korrae* isolates resulted in a single fragment of either 590 or 1,019 bp. Primers specific for *O. herpotricha* (OHITS1 and OHITS2) and *O. korrae* (OKITS1 and OKITS2) were derived from sequence analyses of the ITS regions. The OHITS primers amplified a 454-bp fragment from DNA of *O. herpotricha* but not from DNA of *O. korrae* or 29 other fungal or bacterial species. Similarly, the OKITS primers amplified a 454-bp fragment from DNA of *O. korrae* isolates only. The OHITS and OKITS primers also detected *O. herpotricha* or *O. korrae*, respectively, in total DNA preparations from greenhouse-inoculated or naturally infected bermudagrass roots. These primers can be used to rapidly diagnose turfgrass patch diseases caused by *O. herpotricha* and *O. korrae* without culturing the fungi from diseased tissue.

Ophiosphaerella korrae (J. C. Walker & A. M. Sm.) R. Shoemaker & C. Babcock (= *Leptosphaeria korrae* J. C. Walker & A. M. Sm.) and *O. herpotricha* (Fr.:Fr.) J. C. Walker are ectotrophic, root-rotting fungi that cause spring dead spot on bermudagrass (*Cynodon dactylon* (L.) Pers.) (1,3,16,18,19). *O. korrae* is also responsible for a disease on Kentucky bluegrass (*Poa pratensis* L.) called necrotic ringspot (15). Spring dead spot symptoms of bermudagrass in California and Maryland are caused by *O. korrae* (1,3), while those in Kansas, Oklahoma, and Texas are caused by *O. herpotricha* (12). Both *O. korrae* and *O. herpotricha* have been isolated from diseased bermudagrass in North Carolina and Kentucky, suggesting that these fungi have overlapping distributions in the southeastern United States. Identification of the cause of spring dead spot is complicated by the facts that *O. korrae* and *O. herpotricha* are difficult to isolate from rotted roots and are morphologically similar in culture and do not readily produce ascocarps on artificial media.

To facilitate identification of these fungi, highly repetitive, species-specific DNA sequences were cloned from genomic DNA of *O. korrae* and *O. herpotricha* (12,17). These clones were successfully used to identify isolates of each fungus and to detect the pathogens in infected root samples following DNA extraction and dot hybridization assays. Although the DNA probes were highly specific, hybridization assays on DNA extracted from diseased bermudagrass roots took several days to complete and required the use of radioactive isotopes. Therefore, this technique may have limited use for routine diagnostics.

Another method for rapid identification of fungal pathogens associated with spring dead spot is amplification of selected DNA sequences by polymerase chain reaction (PCR) techniques. Schesser et al (13) developed a set of nested primers that could

be used to amplify DNA from the mitochondrial genome of *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *graminis*. This fungus is associated with spring dead spot and summer decline symptoms of bermudagrass in the southern United States (2,8). Oligonucleotide primer pairs were derived from the multicopy clones pOH29 from *O. herpotricha* (12) and pLK66 from *O. korrae* (11). These primer pairs amplified multiple-sized DNA fragments in *O. herpotricha, O. korrae,* and other soilborne fungi associated with turfgrass diseases. Because of the lack of specificity, these primers could not be used for taxonomic identification of *O. herpotricha* and *O. korrae.*

The objective of our current research was to develop species-specific primers from the internal transcribed spacer (ITS) regions of rDNA for the identification of *O. korrae* and *O. herpotricha* in infected root tissue. The ITS regions were selected for primer development because 1) they occur as multiple copies within the fungal DNA; 2) they tend to be similar within but variable between fungal species; and 3) species-specific oligonucleotides for other plant pathogens have been identified within the ITS regions (7,10).

MATERIALS AND METHODS

Primer selection. Oligonucleotide primers specific for *O. korrae* and *O. herpotricha* were identified by sequence analyses of the ITS regions of the nuclear rDNA. Genomic DNAs of *O. herpotricha* (ascospore isolates KS27 and KS66) and *O. korrae* (isolates KS25 and KS59) were initially amplified with the universal rDNA primers ITS4 and ITS5 described by White et al (20). The ITS5 primer sequence is located near the 3′ end of the 18s rDNA, and ITS4 primes synthesis of the opposite DNA strand near the 5′ end of the 28s rDNA. Used together, the two primers amplify a fragment that includes the transcribed spacer region between the 18s and 5.8s rDNA (ITS1 region), the 5.8s rDNA, and the transcribed spacer region between the 5.8s and 28s rDNA (ITS2 region).

Amplifications of the ITS regions of *O. herpotricha* and *O. korrae* were performed in a programmable thermal cycler (MJ Research, Inc., Watertown, MA). A 25-μl reaction mixture contained 25 pmol of each primer (ITS4 and ITS5), 0.25 U of Replitherm DNA polymerase (Epicentre Technologies, Madison, WI), 200 μM 2′-deoxynucleotide 5′-triphosphates (U.S. Biochemical Corp., Cleveland, OH), and 10–100 ng of fungal DNA. Reactions were amplified for 30 cycles (1.5 min at 94 C, 45 s at 58 C, and 1.5 min at 72 C).

DNA fragments amplified by the ITS4 and ITS5 primers were separated by electrophoresis in a 1% low-melting point agarose gel at 60 V for 3–4 h in TBE buffer (0.44 M boric acid, 0.44 M Trizma base, and 10 mM EDTA), stained with ethidium bromide, and viewed under ultraviolet light. Amplified DNA from each isolate was cut from the gels and purified with Magic minipreps (Promega, Madison, WI). DNA fragments were then ligated into the *Sma*I-cut BluescriptII SK$^-$ plasmid vector and used to transform *Escherichia coli* strain DH5α by the procedure of Hanahan (4). The ITS clones from *O. korrae* and *O. herpotricha* were sequenced at the Nucleic Acids Research Facility (Iowa State University, Ames) and compared for variation in the oligonucleotide sequences.

Primers specific for *O. korrae* (OKITS1 and OKITS2) and *O. herpotricha* (OHITS1 and OHITS2) were selected from regions of the ITS clones with variable nucleotide sequences. Primers were synthesized at Operon Technologies, Inc., Alameda, CA.

Species-specific amplifications of fungal DNA. Isolates of *O. korrae, O. herpotricha,* and other fungal and bacterial species used in PCR amplification studies have been previously listed (12,17). DNA was extracted from fungal isolates by the method described by Tisserat et al (17) and by the boiling technique reported by Henson et al (5). Amplifications of fungal DNA (1–10 ng) with ITS primers specific to *O. korrae* and *O. herpotricha* were performed as described above, except that reactions were amplified at a higher stringency (94 C for 1.5 min, 65 C for 45 s, and 76 C for 1.5 min).

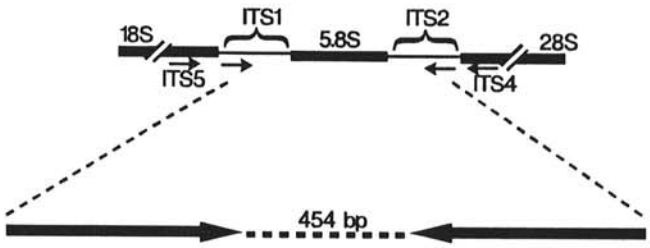
Detection of *O. herpotricha* and *O. korrae* in turfgrass roots. Bermudagrass cultivar Arizona common was vegetatively propagated in the greenhouse and then inoculated with either *O. herpotricha* or *O. korrae* by the method described by Tisserat et al (18). Inoculated bermudagrass (five pots for each fungus) was incubated for at least 90 days and then washed with water to remove soil from the roots. Root samples from each inoculation were prepared for DNA amplification as described below. Root segments (0.5-cm) were also surface sterilized and placed on potato-dextrose agar acidified with lactic acid to confirm the presence of each fungus and to insure that there was no cross-contamination in the pots.

Field samples of bermudagrass showing symptoms of spring dead spot were collected from Kansas, Kentucky, Maryland, and Oklahoma. Kentucky bluegrass samples with symptoms of necrotic ringspot were collected from Washington. Isolations were attempted from each sample to confirm the presence of *O. korrae* or *O. herpotricha.* Isolates were identified with species-specific DNA probes (12,17).

Infected root tissue (200 mg) from greenhouse and field bermudagrass or Kentucky bluegrass samples was frozen in 1.5-ml microfuge tubes by the addition of liquid nitrogen and then ground with a steel rod (12). The steel rod was flame heated between samples to avoid possible DNA contamination. Frozen samples were suspended in 600 μl of 2× cetyltrimethylammonium bromide (CTAB) buffer, incubated at 65 C for 15 min, extracted twice with chloroform, and precipitated by the addition of 0.8 volume of isopropanol. DNA was resuspended in 40 μl of Tris-EDTA buffer, and 10-fold dilutions to 10^{-3} were prepared for PCR amplification. All PCR assays contained samples of primers without template DNA to serve as negative controls, and all PCR reactions were repeated.

RESULTS

Selection of primers. The ITS regions of the rDNA of *O. herpotricha* and *O. korrae* were initially amplified with the universal primers ITS4 and ITS5. Amplifications of genomic DNA from 26 isolates of *O. herpotricha* always resulted in a single 590-bp fragment. The nucleotide sequences of the amplified products from two *O. herpotricha* isolates (KS27 and KS66) were identical. In contrast, amplifications of DNA from 29 isolates of *O. korrae* resulted in a single, 590- or 1,019-bp fragment. Approximately half the isolates had the smaller 590-bp fragment. The nucleotide sequences of the 590-bp fragment in isolate KS59 and the 1,019-bp fragment in isolate KS25 were identical except for a 429-bp insertion in the larger 1,019-bp amplified product of isolate KS25. The insertion was located 2 bp downstream from



Primer Sequences 5′-3′

OKITS1 CCAAGTGCAGCACAAACTGCATG
OHITS1 CCAAGTGTAGAACAAACTACGC

OKITS2 AAGAGGCTTAATGGGTGCCCAC
OHITS2 AAAAGGCTTATTGGGTGCCTAT

Fig. 1. Development of selective oligonucleotide primers from the internal transcribed spacer (ITS) regions of rDNA of *Ophiosphaerella korrae* and *O. herpotricha.* The ITS regions were first amplified with universal primers ITS4 and ITS5 and sequenced. Sequence differences between the two fungi were identified, and species-specific oligonucleotide primers were prepared. The complete *O. korrae* (U04862) and *O. herpotricha* (U04861) sequences are available from GenBank.

the ITS5 primer sequence.

The primers OHITS1 (22 nucleotides [nt]) and OKITS1 (23 nt) were selected from regions of variable nucleotide sequence 48 bp downstream from the ITS5 primer sequence in *O. herpotricha* and *O. korrae,* respectively. The primer sequences of OHITS1 and OKITS1 differed by six bases (Fig. 1). Similarly, the primers OHITS2 (22 nt) and OKITS2 (23 nt) were selected from variable regions 46 bp downstream from the ITS4 sequence and differed by 4 nt.

Species-specific DNA amplification. The OHITS1 and OHITS2 primers amplified a 454-bp fragment in 29 isolates of *O. herpotricha* tested. This fragment included part of the ITS1 region, the 5.8s rDNA, and part of the ITS2 region. The OHITS1 and OHITS2 primers did not amplify purified, genomic DNA from *O. korrae, L. narmari* J. C. Walker & A. M. Sm., or from 28 other fungal and bacterial isolates tested (Fig. 2A).

The primers OKITS1 and OKITS2 also amplified a single 454-bp product from the DNA of all 26 isolates of *O. korrae,* including those with a 1,019-bp fragment following amplifications with ITS4 and ITS5 primers. The 429-bp insert in the ITS region of some *O. korrae* isolates did not interfere with amplification because this sequence resided upstream from the OKITS1 primer. The primers did not amplify genomic DNA from *O. herpotricha* or from other fungal and bacterial isolates tested (Fig. 2B).

DNA extracted from mycelium of *O. herpotricha* and *O. korrae* by the boiling technique described by Henson et al (5) was consistently amplified by the OHITS and OKITS primers, respectively (data not shown).

Detection of *O. korrae* and *O. herpotricha* in root tissue. The ability of the OKITS and OHITS primers to detect *O. korrae* and *O. herpotricha* in diseased plants was tested by amplifying total DNA extracted from greenhouse- and field-grown bermudagrass and Kentucky bluegrass roots (Fig. 3 and Table 1). The OKITS primer pairs consistently amplified a 454-bp fragment from DNA extracted from bermudagrass roots grown in a steamed soil mix and inoculated with *O. korrae* (Fig. 3). The primers did not amplify DNA extracted from bermudagrass roots inoculated with *O. herpotricha.* Similarly, the OHITS1 and OHITS2 primers amplified a 454-bp product only from roots inoculated with *O. herpotricha* (Fig. 3).

The OKITS and OHITS primers were also effective in detecting *O. korrae* or *O. herpotricha* in field samples of bermudagrass with symptoms of spring dead spot (Table 1). The OHITS primers amplified a 454-bp fragment in all DNA samples extracted from bermudagrass roots in which *O. herpotricha* was isolated and in three cases where *O. herpotricha* was not isolated. Results were similar when DNA from field samples infected with *O. korrae* were amplified with the OKITS primers. Both *O. korrae* and *O. herpotricha* were detected in one apparently healthy field sample from Lexington, Kentucky. The OKITS primers also amplified DNA in Kentucky bluegrass samples infected with *O. korrae.*

DISCUSSION

We have identified oligonucleotide primer sequences from the ITS region of the rDNA that can be used to selectively amplify DNA of *O. herpotricha* and *O. korrae.* Primer selection from rDNA sequences has certain advantages for fungal identification and detection in plant tissue. The rDNA generally exists in excess of 100 copies per haploid genome, allowing for easier detection in preparations where DNA is in very low concentrations or where pathogen DNA is a small fraction of the total DNA, e.g., a mixture of fungal and plant DNA from diseased plant extracts. The ITS regions are relatively similar within a fungal species, as was the case in our studies, but can vary sufficiently among species for identification of unique primer sequences. In addition, the ITS regions are small enough to be easily amplified by PCR and are flanked by highly conserved sequences for which nonspecific primers have been identified (20).

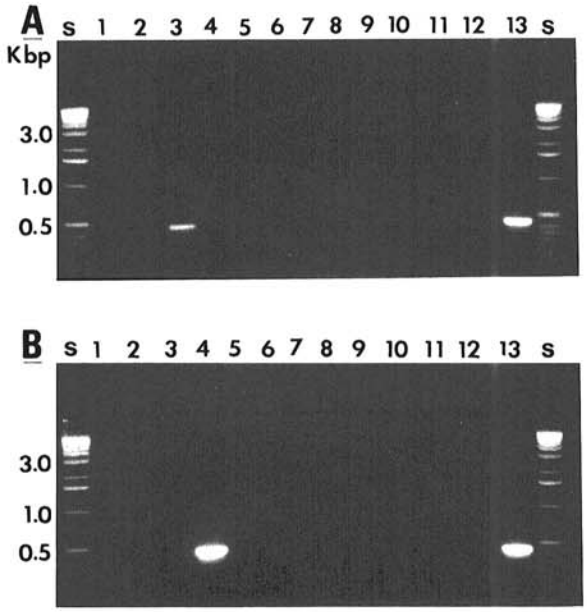


Fig. 2. A, Polymerase chain reaction (PCR) amplification with primers OHITS1 and OHITS2. Lane 1, primers without DNA template; lane 2, *Ophiosphaerella herpotricha* without primers; lane 3, *O. herpotricha* KS10; lane 4, *O. korrae* WI25; lane 5, *Gaeumannomyces incrustans;* lane 6, *G. graminis* var. *tritici;* lane 7, *G. g. avenae;* lane 8, *G. g. graminis;* lane 9, *Rhizoctonia solani* AG1; lane 10, *R. cerealis;* lane 11, *Pythium aphanidermatum;* lane 12, *Fusarium graminearum;* and lane 13, *O. herpotricha* KS66. **B,** PCR amplification with primers OKITS1 and OKITS2. Lane 1, primers without DNA template; lane 2, *O. korrae* without primers; lanes 3–12, same as in **A**; and lane 13, *O. korrae* AUS59.

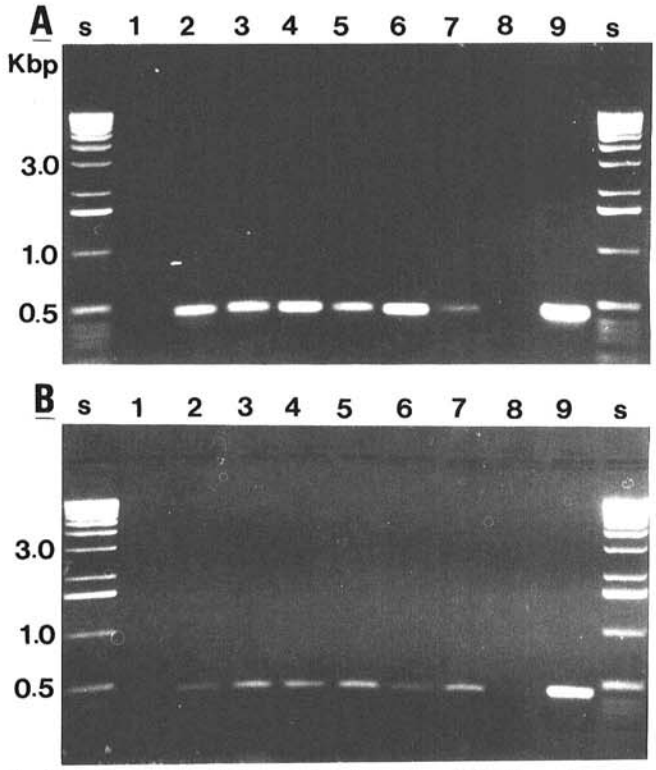


Fig. 3. A, Polymerase chain reaction (PCR) amplification with primers OHITS1 and OHITS2. Lane 1, primers without DNA template; lanes 2–7, DNA extracted from bermudagrass roots infected with *Ophiosphaerella herpotricha;* lane 8, DNA extracted from bermudagrass roots infected with *O. korrae;* and lane 9, genomic DNA of *O. herpotricha.* **B,** PCR amplification with primers OKITS1 and OKITS2. Lane 1, primers without DNA template; lanes 2–7, DNA extracted from bermudagrass roots infected with *O. korrae;* lane 8, DNA extracted from bermudagrass roots infected with *O. herpotricha;* and lane 9, genomic DNA of *O. korrae.*

Sequence analyses of the ITS regions in *O. herpotricha* and *O. korrae* can be used in developing species-specific oligonucleotide primers for other fungal pathogens of turfgrasses. For example, the ITS regions of ectotrophic root-infecting fungi such as *Magnaporthe* or *Gaeumannomyces* spp. could be amplified with the ITS universal primers and sequenced. These sequences could then be compared to those of *O. herpotricha* and *O. korrae* for nonhomologous regions where species-specific primers could be selected. This method of primer selection for a number of ectotrophic fungi could be more routine and efficient than using random primers, particularly if the same sequencing primers and PCR techniques could be used.

Detailed sequence analyses of the ITS regions may provide a means for estimating phylogenetic relationships among ectotrophic root-infecting fungi of turfgrasses. This technique has previously been used to study the phylogenetic relationships of several *Phytophthora* spp. (6). In our studies, the ITS1 regions in *O. herpotricha* and *O. korrae* were 91% homologous (145 of 159 nt were identical), whereas only 11 nucleotide changes were noted in the ITS2 region. In contrast, the ITS sequences in *O. korrae* were <70% homologous to those reported for *L. maculans* (Desmaz.) Ces. & De Not. (9) . Our sequence analysis supports the disposition of *L. korrae* to *O. korrae* as proposed by Shoemaker and Babcock (14) on the basis of their assessment of ascocarp and ascospore morphology. Preliminary sequence analyses of the ITS regions in *L. narmari* also suggest that this fungus may be more closely related to *O. korrae* and *O. herpotricha* than to other *Leptosphaeria* spp. (S. H. Hulbert and N. A. Tisserat, *unpublished data*).

The OHITS and OKITS primers amplified DNA of *O. herpotricha* and *O. korrae* from diseased roots of bermudagrass collected in the field. The primers were specific and did not amplify other fungal or plant DNA from crude plant DNA extracts. Furthermore, the amplification products were unambiguous; i.e., only a single 429-bp fragment was amplified from positive test samples, and no amplification occurred in noninfected roots. This technique may offer advantages over the use of random primers that amplify multiple-sized DNA fragments in the fungus. The multiplicity of fragments amplified by random primers in infected plant material that contains DNA from numerous organisms could result in ambiguity in identifying a specific banding pattern for the test fungus.

In our studies, DNA was prepared by grinding root samples in CTAB buffer and extraction in chloroform and isoamyl alcohol. Dilutions of 10^{-2} to 10^{-3} of the DNA extract were needed for successful PCR reactions. PCR amplifications with DNA extracts collected from boiled roots (5) were unsuccessful. These results corroborate those of Henson et al (5), which suggested some type of polymerase inhibitor was present in bermudagrass roots or fine soil particles attached to the roots. This may explain why dilutions of crude DNA extracts were necessary for PCR amplification in our studies. Further studies on methods of decreasing inhibition of DNA amplification with soil and turfgrass samples are needed.

The OHITS and OKITS primers will be useful in rapidly screening other turfgrasses and prairie grasses as potential hosts for *O. herpotricha* and *O. korrae*. We have detected *O. korrae* on naturally infected Kentucky bluegrass and bermudagrass roots,

TABLE 1. Polymerase chain reaction (PCR) amplifications of DNA extracted from roots of symptomless or spring dead spot-affected bermudagrass or necrotic ringspot-affected Kentucky bluegrass field samples with the oligonucleotide primer pairs OHITS1 and OHITS2 and the pairs OKITS1 and OKITS2, which are specific for *Ophiosphaerella herpotricha* and *O. korrae*, respectively

Sample Location[a]	Number of samples	PCR		Isolation[d]
		O. herpotricha[b]	*O. korrae*[c]	
Bermudagrass				
Georgia				
Diseased	1	0	1	*O. korrae*
Kansas				
Independence				
Diseased	1	1	0	*O. herpotricha*
Hutchinson				
Diseased	12	12	0	*O. herpotricha* (11/12)
Healthy	5	0	0	None
Manhattan				
Diseased	36	36	0	*O. herpotricha* (34/36)
Healthy	3	0	0	None
Maryland				
Diseased	3	0	3	*O. korrae* (3/3)
Oklahoma				
Diseased	2	2	0	*O. herpotricha* (2/2)
Kentucky				
Lexington				
Site 1 diseased	2	0	2	None
Site 2 diseased	1	0	1	None
Site 3 diseased	1	0	1	*O. korrae*
Site 1 healthy	1	0	0	None
Site 2 healthy	1	0	1	None
Site 3 healthy	1	1	1	*O. herpotricha*
Henderson				
Diseased	3	3	0	*O. herpotricha* (3/3)
Healthy	3	0	0	None
Kentucky bluegrass				
Washington				
Diseased	7	0	6	*O. korrae* (6/7)

[a] Plant samples were collected from 1991 to 1993. Diseased bermudagrass samples were collected from margins of turf showing symptoms of spring dead spot; healthy samples were collected from areas showing no symptoms at the same location. Diseased Kentucky bluegrass samples were collected by C. Foss, Washington State University, Puyallop.

[b] Total DNA was extracted from 200 μg of roots and resuspended in 40 μl of Tris-EDTA. One microliter of a 10^{-2} or 10^{-3} dilution of the template DNA extract was added to the PCR reaction containing the primers OHITS1 and OHITS2, which are specific to *O. herpotricha*.

[c] One microliter of a 10^{-2} or 10^{-3} dilution of template DNA extract was added to the PCR reaction containing the primers OKITS1 and OKITS2, which are specific to *O. korrae*.

[d] Isolation of *O. herpotricha* and *O. korrae* from root samples. The proportion of samples from which these fungi were successfully isolated is in parentheses.

and we have amplified DNA of *O. herpotricha* from buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.) and zoysiagrass (*Zoysia japonica* Steud.) roots collected from noninoculated, field-grown plants. Information concerning the host range of *O. korrae* and *O. herpotricha* may give some insight into the distribution and interactions these root-infecting fungi have in natural grass ecosystems.

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