Genetic Diversity and Aggressiveness of *Ophiosphaerella korrae*, a Cause of Spring Dead Spot of Bermudagrass

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ABSTRACT

Iriarte, F. B., Wetzel, H. C., III, 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.

The distribution of three *Ophiosphaerella* spp. that cause spring dead spot (SDS) of bermudagrass was studied by sampling at 24 locations in the southeastern United States. *O. korrae* was isolated from 73% of the 204 bermudagrass cores collected and was the only SDS pathogen recovered at most sites. *O. herpotricha* was isolated at three locations in Kentucky and one in North Carolina, and *O. narmari* was found at two locations in North Carolina. Most *O. korrae* isolates collected from Alabama, Kentucky, Mississippi, Tennessee, and Virginia clustered in an amplified fragment length polymorphism group (AFLP group II) that was distinct from Kentucky bluegrass isolates collected throughout North America and similar to bermudagrass isolates from Kansas and Oklahoma (AFLP group I). A third AFLP group (III) consisting of bermudagrass isolates from Mississippi and Virginia was identified. Isolates representing AFLP groups II and III grew more rapidly on potato dextrose agar at 25 and 30°C than those in group I. *O. korrae* isolates differed in their aggressiveness to two bermudagrass cultivars in greenhouse studies, but these differences were not associated with AFLP group, location, or host from which the isolate was collected.

Additional keywords: *Leptosphaeria korrae*, *L. narmari*, root rot, turfgrass

Spring dead spot (SDS) is an important disease of common bermudagrass (*Cynodon dactylon*) and its hybrids (*C. dactylon* × *C. trasvaalensis*) in North America (1,3,7) and Australia (5,11). The disease results in the formation of circular patches of bleached, dead grass in the spring as the turfgrass breaks winter dormancy. Roots and stolons of affected plants are dark brown to black and are severely rotted. Patches tend to reappear in the same spot for three or more years. In Australia and California, patches may be visible on slow-growing, but not dormant, bermudagrass in autumn and winter following wet, cold weather.

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Contribution of The Kansas Agricultural Experiment Station 04-274-J.

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Accepted for publication 6 July 2004.

Three closely related fungi cause spring dead spot symptoms. *Ophiosphaerella korrae* (J. Walker & A.M. Smith) Shoemaker & C.E. Babcock (= *Leptosphaeria korrae* J. Walker & A.M. Smith) was first reported as a cause of SDS in Australia (11,16) and was subsequently found in California, Maryland, and other regions of the United States (1,3,18). *O. korrae* also causes necrotic ring spot of Kentucky bluegrass (*Poa pratensis*), annual bluegrass (*Poa annua*), and creeping red fescue (*Festuca rubra*) in North America (2,6,9,10,19). *O. narmari* (J. Walker & A.M. Smith) Wetzel, Hulbert & Tisserat (= *L. narmari* J. Walker & A.M. Smith) is the most common cause of SDS in Australia (5,16) and has been recovered from diseased bermudagrass in California, Kansas, and Oklahoma (11,17). *O. herpotricha* (Fr.:Fr.) J. Walker is the most abundant SDS pathogen in Kansas and Oklahoma (14,18). It also causes SDS symptoms on buffalograss (12).

Wetzel et al. (18) studied the genotypic variation in *O. herpotricha* and *O. korrae* using amplified fragment length polymorphism (AFLP) analysis. They observed that a majority of the *O. herpotricha* isolates collected from a golf course fairway in Oklahoma were distinct haplotypes, suggesting that sexual recombination was occurring. The level of diversity among *O.*

herpotricha isolates collected throughout the United States was similar to that found among isolates collected at a single golf course. However, a few of the isolates collected from different SDS spots at this same golf course had identical haplotypes based on the AFLP analysis. The authors concluded that the fungus also was propagating asexually and probably was dispersed over longer distances as mycelium on infected stolons or sod.

The pattern of genotypic diversity for *O. korrae* appears to be markedly different from that found in *O. herpotricha*. Raffle and Hsiang (8) found only six haplotypes among 71 North American isolates collected from Kentucky bluegrass exhibiting symptoms of necrotic ring spot. Similarly, Wetzel et al. (18) found that *O. korrae* isolates collected from bermudagrass at a golf course in Oklahoma were represented by just a few haplotypes, and that these were nearly identical by AFLP analysis to bermudagrass isolates from Australia and to Kentucky bluegrass isolates collected throughout North America (8,18). The low level of genetic diversity among these isolates indicated that a limited number of genotypes were distributed over a wide geographic region. During their survey, Wetzel et al. (18) identified a group of 10 isolates of *O. korrae* collected from the southern United States that clustered into an AFLP group distinct from isolates collected in the northern and western regions of North America. There was a high degree of genetic similarity among the southern isolates, even though they were collected from widely dispersed geographic regions. This suggested that another distinct genetic population of *O. korrae* was present in the United States. However, this conclusion was based on a relatively low number of these southern isolates.

There is limited information on differences in aggressiveness of SDS pathogens to bermudagrass. In Australia, Smith (11) found that an isolate of *O. korrae* was more aggressive than one of *O. narmari*. Nevertheless, *O. narmari* is the more common cause of SDS in that country (5,16). Tisserat et al. (14) showed that two isolates of *O. herpotricha* caused more bermudagrass root discoloration and weight loss than an isolate of *O. korrae* in greenhouse experiments. They also

showed that a Kentucky bluegrass isolate of *O. korrae* was pathogenic to bermudagrass. However, more detailed experiments on aggressiveness of isolates collected from different hosts, locations, or genetic populations have not been conducted.

The objectives of our research were to determine the distribution of SDS pathogens in the southern United States, examine the genetic diversity and in vitro growth of *O. korrae* isolates collected in this region, and compare aggressiveness of these isolates to those collected from the Great Plains and northern regions of the United States.

MATERIALS AND METHODS

Sample collection and DNA isolation.

Bermudagrass samples exhibiting SDS symptoms were collected in 1998 to 2000 from locations listed in Table 1. An 11-cm-diameter × 10-cm-deep turfgrass core was removed from the margin of each SDS patch, and putative isolates of *Ophiosphaerella* spp. were cultured from roots as previously described (14). At some sites, multiple turf cores were collected by sampling

individual SDS patches that were separated by at least 6 m. Isolates were grown in modified yeast extract broth, and DNA was extracted and resuspended for amplification in polymerase chain reaction (PCR) assays as described by Wetzel et al. (17). Isolates were identified to species by PCR using species-specific oligonucleotide primers (13,17). All isolates were maintained in a 10% glycerol solution at -80°C for long-term storage.

AFLP analysis. Isolates collected from Alabama, Kentucky, Mississippi, Tennessee, and Virginia were initially compared by AFLP analysis to two isolates representing AFLP group I (California isolates CA47 and CO32 from bermudagrass) and II (Arkansas AR202 and Georgia GA 149 isolates from bermudagrass) as described by Wetzel et al. (18). Representative isolates from each site were then selected and reexamined by AFLP analysis.

AFLP reactions were done following the procedure developed by Vos et al. (15) and modified by Zeller et al. (20). All buffers and DNA modifying enzymes were used according to manufacturer's instructions or

standard protocols. Selective amplification was done using the primer pairs *Eco*RI+AA and *Mse*I+CA. The selective *Eco*RI primer was end labeled with [³³P] ATP, and the resulting DNA fragments were separated in 6% denaturing polyacrylamide gels (Bio Technologies Inc., GIBCO BRL Sequencing System, Rockville, MD) in 1× Tris borate EDTA buffer (pH 8.3). Gels were then blotted to 0.33 mm gel blot paper (Midwest Scientific, Valley Park, MO) and dried on a water aspirator vacuum pump (National Labnet Co., Woodbridge, NJ) for 1 h at 80°C. Autoradiographs were obtained by exposing Classic blue BX film (Midwest Scientific) to the dried gel for 3 to 7 days at room temperature. Films were developed in a Kodak X-OMAT1000A Processor (Eastman KODAK Co., Rochester, NY).

DNA bands were scored from AFLP autoradiographs as described by Wetzel et al. (18). AFLP fingerprints were scored manually as "1" for the presence of a band and "0" for absence, assuming that the bands with the same molecular size (same mobility on the gel) in different isolates

Table 1. Frequency of *Ophiosphaerella korrae*, *O. herpotricha*, and *O. narmari* isolated from bermudagrass spring dead spots collected at 27 locations in southeastern United States

Location	Collection year	Turf cores collected	Turf cores with species present ^a						Turf cores where species not recovered
			<i>O. herpotricha</i>	<i>O. narmari</i>	<i>O. korrae</i> (AFLP group)				
					I	II	III	ND ^b	
Alabama									
Vestavia Hills	1999	20	0	0	0	16	0	2	2
Kentucky									
Henderson	1998	20	14	0	0	5	0	0	1
Lexington	1999	7	4	0	0	0	0	0	3
Mayfield	1998	15	2	0	1	5	0	7	0
Paducah	1998	19	0	0	0	15	0	2	2
Mississippi									
Starkville	1999	20	0	0	0	7	9	0	4
North Carolina									
Anson County	1999	2	0	0	0	0	0	2	0
Cornealis	1999	1	0	0	0	0	0	1	0
Fuquay Varina	1999	1	0	0	0	0	0	1	0
Goldsboro	1999	3	0	0	0	0	0	3	0
Goldsboro	2000	5	0	0	0	0	0	5	0
Laurinburg	1999	1	0	1	0	0	0	1	0
Raleigh	1999	2	1	1	0	0	0	1	0
Raleigh	2000	21	9	0	0	0	0	6	6
Rolesville	1999	1	0	0	0	0	0	1	0
Rolesville	2000	1	0	0	0	0	0	1	0
Southern Pines	1999	3	0	0	0	0	0	3	0
Wilmington	1999	4	0	0	0	0	0	4	0
Winston Salem	1999	1	0	0	0	0	0	1	0
South Carolina									
Columbia	1999	5	0	0	0	0	0	5	0
Florence	1999	1	0	0	0	0	0	1	0
Myrtle Beach	1999	3	0	0	0	0	0	3	0
Santee	1999	2	0	0	0	0	0	2	0
Tennessee									
Knoxville	1999	16	0	0	0	15	0	0	1
Virginia									
Charlottesville	1999	7	0	0	0	0	0	5	2
Virginia Beach	1999	20	0	0	0	12	2	3	3
West Virginia									
Huntington	1999	3	0	0	0	0	0	3	0
Total	0	204	30	2	1	75	11	61	24

^a Based on the number of turfgrass cores sampled from each location. *Ophiosphaerella* spp. were positively identified by polymerase chain reaction using species-specific primer pairs.

^b Not determined.

were the same allele and unresolved bands or missing data were scored as ambiguous. AFLP amplification was repeated one or two times with the same results. Band sizes were estimated against BRL Low-Mass ladder (Life Technologies, Rockville, MD) according to the migration pattern from 1,200 to 100 bp in each gel. The Unweighted Pair Grouping by Mathematical Averages (UPGMA) method of Phylogenetic Analysis Using Parsimony (PAUP) V.8.0 was used to determine the relatedness between and among *O. korrae* isolates and to construct the dendrograms and the supporting 1,000 parsimony bootstrap replications implemented in PAUP.

Growth rates of SDS pathogens on PDA. Growth rates of 30 *O. korrae* isolates were compared in culture. Thirteen of the isolates were from AFLP group I, and 17 isolates were from AFLP group II. Five of the AFLP group I isolates (CO352, MI18, MI44, NE95, and OH161) were collected from Kentucky bluegrass, whereas the rest were isolated from bermudagrass. A 5-mm-diameter disk of potato dextrose agar (PDA) containing mycelium of each isolate was removed from the margin of actively growing colonies and placed in the center of each of four replicate PDA plates. Plates were incubated in darkness at 25 or 30°C in a randomized complete block design, and colony diameters were measured in two directions after 18 days. In a similar experiment, growth rates of six representative isolates of *O. korrae* from AFLP group I (Kentucky bluegrass isolates CO32, CO352, OH161, and MI44, and bermudagrass isolates OK-A11-84A and OK-A16-3-2), group II (bermudagrass isolates AR155, AR157, AR158, VA5, KY10R6, and TN2), and group III (bermudagrass isolates MS3, MS9, MS19, MS11, MS12, and MS13) were compared. Experiments at each temperature were repeated once, and data were combined for analysis.

Aggressiveness of *O. korrae* and *O. herpotricha* isolates. Bermudagrass cultivars Midlawn and Tifgreen were started from stolons and grown in 6.4 × 6.4 cm plastic pots containing a steamed soil: perlite:peat mixture (1:1:1, vol/vol/vol). Cultivars were grown in the greenhouse for 4 months prior to inoculation. Plants were inoculated with one of 10 isolates of *O. herpotricha*, 12 isolates of *O. korrae* from AFLP group I, or 16 isolates of *O. korrae* from AFLP group II by removing the soil and root plug from the pot, uniformly inserting four sterile or infested oat seeds approximately 0.5 cm into the sides of the plug 2 cm below the soil surface, then repotting the turfgrass. Oat inoculum was prepared as previously described (14). There were four replicate pots of each treatment. Pots were placed in the greenhouse at 21 to 28°C in a split-plot design with cultivars as the main plot and fungal isolates as subplots. Turf was watered and

fertilized as needed. After 1 month, pots were placed in a growth chamber at 4°C with a 12-h photoperiod (light intensity 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and incubated for 1 month. Pots were then placed in a freezing chamber at -1°C. The temperature was lowered 1°C/h until the temperature reached -4°C and then held for 1 h (4 h total freezing time). Because of the small size of the freezing chamber, replicate pots (blocks) were exposed to the freezing procedure on four consecutive days. Frozen turf was

placed at 4°C for 24 h to thaw and then moved to the greenhouse for recovery. The percentage of living shoots in each pot was determined after 1 month in the greenhouse by dividing the number of live shoots by the total number of shoots in each pot. Data were analyzed as a split plot with cultivars as a main plot and isolates as subplots using the mixed procedure of SAS (version 6.11 for Windows; SAS Institute, Cary, NC). Data from two experiments were combined for analysis.

Fig. 1. Unweighted Pair Grouping by Mathematical Averages (UPGMA) dendrogram of *Ophiostoma korrae* isolates based on amplified fragment length polymorphism (AFLP) using the selective primer pair EAA/MCA. Bermudagrass isolates from Alabama (AL), Kentucky (KY), Mississippi (MS), Tennessee (TN), and Virginia (VA) were compared with representative isolates of AFLP groups I (California CA47 and Colorado CO32 collected from Kentucky bluegrass) and II (Arkansas AR202 and Georgia GA149 collected from bermudagrass), as described by Wetzels et al. (18). Not all isolates listed in Table 1 are included in this dendrogram. Numbers at branch nodes represent bootstrap values (1,000 replicates).

RESULTS

Abundance of SDS pathogens. *O. korrae* was isolated from 73% of the 204 turf cores collected from 24 locations in eight states (Table 1). It was the only SDS pathogen recovered at most sites. *O. herpotricha* was isolated at three locations in Kentucky and one in North Carolina. *O. narmari* was found at two locations in North Carolina, and in both cases *O. korrae* was recovered from the same core. This is the first report of *O. herpotricha* and *O. narmari* in North Carolina.

Genetic diversity of *O. korrae* isolates. AFLP analysis was conducted on bermudagrass isolates collected in Alabama, Kentucky, Mississippi, Tennessee, and

Virginia (Table 1, Fig. 1). Sixteen of the 18 Alabama isolates tested had genetic similarities ranging from 72 to 92% and clustered with isolates of AFLP group II. Placement of the other two isolates in one of the AFLP groups was not strongly supported by bootstrap analysis (<50%). Twenty-five *O. korrae* isolates from Kentucky representing 19 haplotypes and with genetic similarities ranging from 88 to 100% were placed with AFLP group II isolates. One isolate from Mayfield, KY, clustered with isolates of AFLP group I with a bootstrap support of 100% (data not shown). Seven of 16 Mississippi isolates had genetic similarities ranging from 84 to 99% and were placed with AFLP group II

isolates. Nine other Mississippi isolates representing eight haplotypes and with genetic similarities ranging from 62 to 100% were placed in AFLP group III, although the bootstrap support for this group (61%) was not as strong as the other groups. All 15 Tennessee isolates, representing seven haplotypes and with genetic similarities ranging from 83 to 100%, clustered with isolates in AFLP group II. Twelve Virginia Beach, VA, isolates, representing 10 haplotypes and with genetic similarities ranging from 70 to 100%, clustered with isolates in AFLP group II. Two isolates representing the same haplotype were placed in AFLP group III, whereas three other isolates could not be placed in any AFLP group because of low bootstrap support (51%).

Growth rates of SDS pathogens on PDA. There were differences in growth ($P = 0.001$) among *O. korrae* isolates within the same AFLP group at both 25 and 30°C, but growth rate was not associated with the specific location or, in the case of AFLP group I, the host (i.e., bermudagrass or Kentucky bluegrass) from which isolates were collected (Figs. 2 and 3). For example, MS-73 and MS-19 had one of the fastest and slowest growth rates at 25°C, respectively, among AFLP group II isolates even though they were collected from bermudagrass at the same location in Mississippi (Fig. 3). Growth rates of all isolates were reduced at 30°C (Figs. 2 and 3), but this inhibition was most pronounced in isolates from AFLP group I, where almost no radial growth occurred (Fig. 2). When isolates from AFLP group I were returned to 25°C at the conclusion of the experiment, they resumed normal growth. Growth of six representative isolates from AFLP groups I and II were compared with six isolates from AFLP group III. Mean colony diameters of AFLP group II (80.6 mm) and III (80.2 mm) isolates after 18

Fig. 2. Colony diameters of *Ophiosphaerella korrae* isolates in amplified fragment length polymorphism (AFLP) group I grown at 25°C (dark bars) and 30°C (clear bars) for 18 days. Isolates CO352, KS86, KS123, MI18, MI44, NE95, and OH161 were collected from Kentucky bluegrass, and the other isolates were recovered from bermudagrass.

Fig. 3. Colony diameters of *Ophiosphaerella korrae* isolates in amplified fragment length polymorphism (AFLP) group II grown at 25°C (dark bars) and 30°C (clear bars) for 18 days. All isolates were collected from bermudagrass.

days at 25°C were larger ($P = 0.0001$) than group I isolates (65.3 mm). At 30°C, the mean colony diameter of AFLP group III isolates (40.0 mm) was larger ($P = 0.0001$) than those of group II (28.8 mm) or group I (7.0 mm) isolates.

Aggressiveness of SDS pathogens. Aggressiveness of isolates on each cultivar was analyzed separately because of a significant ($P = 0.004$) cultivar $\times$ isolate interaction. Shoot survival on the relatively susceptible cultivar Tifgreen ranged from 69% following inoculation with isolate NE-95 to less than 10% for isolate MS-4 (Fig. 4). In contrast, isolate NE-95 was aggressive (40% shoot survival) on the more resistant cultivar Midlawn. Differences in aggressiveness among *O. korrae* isolates were not associated with AFLP group or with the specific location or host (bermudagrass versus Kentucky bluegrass) from which isolates were collected.

There were also differences in aggressiveness among *O. herpotricha* isolates (Fig. 5). In general, all isolates of *O. herpotricha* were aggressive (<50% shoot survival) on the two cultivars. Isolate TX-75 was less aggressive on Tifgreen compared with all other isolates. In contrast, TX-75 was one of the most aggressive isolates on Midlawn.

DISCUSSION

Our results indicate there are regional differences in the distribution of SDS pathogens in the United States. *O. korrae* was the only SDS pathogen isolated from most of the collection sites and appears to be the most abundant SDS pathogen in the southern United States. *O. herpotricha* was recovered from the North Carolina State University turfgrass research facility in Raleigh, where bermudagrass selections from throughout the United States are tested, but not in other locations in this or more southern regions. This is in contrast to a survey conducted in Oklahoma and Kansas, where *O. herpotricha* was the most frequently isolated SDS pathogen (18). The collection of both *O. herpotricha* and *O. korrae* at sites in Kentucky indicates that both pathogens may be widely distributed in this state.

Regional differences in the distribution of *O. herpotricha* and *O. korrae* might reflect native ranges of these fungi. *O. herpotricha* causes SDS on buffalograss in landscape plantings in Kansas and Oklahoma (12), and this grass is native to the Great Plains. Thus, bermudagrass may have been planted in soils that were naturally infested with *O. herpotricha*, and this could

explain the prevalence of this fungus as a cause of SDS in the Great Plains Region. Nevertheless, *O. herpotricha* has not been isolated from native stands of buffalograss or other native grasses in Kansas (*unpublished data*), and its origin remains unclear.

It is unlikely that *O. korrae* is native to North America, since bermudagrass and other known hosts of this fungus are not indigenous to the continent. Alternatively, *O. korrae* could have been introduced and disseminated on infected plant material. Movement of SDS pathogens on infected bermudagrass stolons and roots has previously been demonstrated (7). Many of the early improved bermudagrass selections were vegetatively propagated from stolons. For example, common and hybrid bermudagrass varieties developed in Georgia in the 1950s (e.g., the Tif series) were widely planted throughout the southern United States (4). If these cultivars were infested with *O. korrae* and then propagated, the fungus could easily have been spread throughout the southern region of the United States. Our AFLP analysis supports this hypothesis. Many of the bermudagrass isolates collected over an extensive area in the southern United States (AFLP group II) are genetically similar.

Fig. 4. Shoot survival of Tifgreen and Midlawn bermudagrass cultivars following inoculation with *Ophiophora korrae* isolates representing amplified fragment length polymorphism (AFLP) groups I (clear bars) and II (dark bars). Isolates NE-95, KS123, KS86, MI 18, MI-44, CO-352, and OH161 were collected from Kentucky bluegrass, and all other isolates were recovered from bermudagrass.

Bermudagrass isolates belonging to AFLP groups II and III collected in the southern United States are genetically distinct from Kentucky bluegrass isolates (AFLP group I) that are distributed throughout North America. Furthermore, these southern isolates tolerate higher temperatures and appear to be better adapted to warmer regions where bermudagrass grows. This suggests that *O. korrae* may have been independently introduced on bermudagrass in the southern United States and on Kentucky bluegrass in more northern regions. However, we found one isolate from Kentucky, and Wetzal et al. (18) identified numerous bermudagrass isolates from Oklahoma and Kansas, that clustered in AFLP group I. This inconsistency might be explained by the fact that Kansas, Oklahoma, and Kentucky are transitional climatic zones where both Kentucky bluegrass and bermudagrass are grown, sometimes in the same turfgrass sward. Thus, it is possible that infection of bermudagrass by isolates originating from Kentucky bluegrass could have occurred. Further information concerning the origin and possible dispersal of SDS pathogens might be obtained by surveying for *Ophiosphaerella* spp. in the native ranges of these turfgrasses.

It is probable that additional genetic populations will be discovered as more *O. korrae* isolates are collected and catego-

rized. For example, isolates from Mississippi and Virginia were loosely clustered into a third AFLP (III) group. Individual isolates from Alabama and Virginia also appear to be genetically distinct. Nevertheless, the relatively low amount of genetic diversity in *O. korrae* we and others (8,18) have found suggests that establishment of this fungus over a wide geographic area resulted from the introduction of a relatively small number of genotypes.

We observed differences in aggressiveness among *O. korrae* isolates to bermudagrass, but they were not associated with AFLP group or host from which the isolates were collected. In some cases, Kentucky bluegrass isolates were as aggressive as those from bermudagrass. Our inoculation studies were conducted at extended periods of cool temperatures (4°C) to optimize disease development under controlled conditions. Studies in which infected plants are exposed to higher temperatures, similar to what might occur in more southern regions of the United States during summer months, could give different results. Field inoculations including *O. korrae* isolates representing different genetic populations may provide a more accurate assessment of aggressiveness.

ACKNOWLEDGMENTS

This research was sponsored in part by the United States Golf Course Association and the Kansas Turfgrass Foundation. We thank Channa

Rajashekar for advice on freezing experiments and Kurt Zeller for help on AFLP analysis.

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Fig. 5. Shoot survival of Tifgreen and Midlawn bermudagrass cultivars following inoculation with *Ophiosphaerella herpotricha* isolates collected from bermudagrass.