

Isolation and morphological and metabolic characterization of common endophytes in annually burned tallgrass prairie

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Abstract: Dark septate endophytes (DSE) are common and abundant root-colonizing fungi in the native tallgrass prairie. To characterize DSE fungi were isolated from roots of mixed tallgrass prairie plant communities. Isolates were grouped according to morphology, and the grouping was refined by ITS-RFLP and/or sequencing of the ITS region. Sporulating species of *Periconia*, *Fusarium*, *Microdochium* and *Aspergillus* were isolated along with many sterile fungi. Leek resynthesis was used to quickly screen for DSE fungi among the isolates. *Periconia macrospinosa* and *Microdochium* sp. formed typical DSE structures in the roots; *Periconia* produced melanized intracellular microsclerotia in host root cortex, whereas *Microdochium* produced abundant melanized inter- and intracellular chlamydospores. To further validate the results of the leek resynthesis growth responses of leek and a dominant prairie grass, *Andropogon gerardii*, were assessed in a laboratory resynthesis system. Leek growth mainly was unresponsive to the inoculation with *Periconia* or *Microdochium*, whereas *Andropogon* tended to respond positively. Select *Periconia* and *Microdochium* isolates were tested further for their enzymatic capabilities and for ability to use organic and inorganic nitrogen sources. These fungi tested positive for amylase, cellulase, polyphenol oxidases and gelatinase. *Periconia* isolates used both organic and inorganic nitrogen sources. Our study identified distinct endophytes in a tallgrass prairie ecosystem and indicated that these endophytes can use a variety of complex nutrient sources, suggesting facultative biotrophic and saprotrophic habits.

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INTRODUCTION

Dark septate endophytes (DSE) are a miscellaneous group of ascomyceteous root-colonizing microfungi characterized by melanized cell walls and intracellular colonization of healthy plants (Jumpponen and Trappe 1998, Addy et al. 2005). Although DSE fungi are taxonomically unrelated and vary in ecological or physiological functions (Addy et al. 2005) many of these fungi form similar morphological structures in host roots (Jumpponen and Trappe 1998). Irrespective of the host plant species the characteristics include superficial mycelium, hyphal penetration into the cortex and formation of melanized microsclerotia (Jumpponen and Trappe 1998, Yu et al. 2001).

DSE fungi colonize a variety of hosts and appear to have a global distribution (Jumpponen and Trappe 1998). To evaluate the abundance of DSE fungi across ecosystems Mandyam and Jumpponen (2005) compared published estimates of the proportion of plant species colonized by different mycorrhizal and DSE fungi and concluded that DSE and mycorrhizal fungi were equally abundant. A 2 y study on the seasonal variation in the root colonization by arbuscular mycorrhizas (AM) and DSE fungi at Konza Prairie Biological Station, a Long-Term Ecological Research (LTER) site, showed that DSE colonization in a tallgrass prairie was equal to AM colonization and occasionally even exceeded AM colonization (Mandyam and Jumpponen 2008). So far only a limited number of DSE fungi have been identified (see Addy et al. 2005 for the most common DSE fungi from different habitats).

Not only is the diversity of DSE fungi poorly understood, but only few of these taxa have been metabolically characterized (Currah and Tsuneda 1993; Caldwell et al. 2000; Caldwell and Jumpponen 2003a, b; Wilson et al. 2004). As reviewed in Mandyam and Jumpponen (2005) DSE fungi studied thus far are known to use organic and inorganic nutrient pools. Nitrogen is the most limiting nutrient in a tallgrass prairie and its availability can vary with fire, grazing, soil texture and topography (Blair et al. 1998). Because DSE are abundant at Konza Prairie, where N is a limiting nutrient, it is imperative to

understand whether DSE fungi metabolize both organic and inorganic N and thus have the potential to aid plant N acquisition from these sources. DSE fungi produce a variety of extracellular enzymes, and some DSE fungi have been tested for their enzymatic capabilities (Mandyam and Jumpponen 2005). These enzymatic capabilities suggest the DSE potential to access detrital C, N and P with a potential to aid host nutrient uptake.

Much of the present understanding on the diversity and function of the root-associated endophytes is based on a limited number of taxa and strains from alpine, arctic and antarctic environments. Among these fungi *Phialocephala fortinii* is the most studied DSE taxon. In contrast studies on endophytes from grasslands are few (but see Kovács and Szigetvári 2002). The objectives of this study were: (i) to isolate and identify DSE fungi from roots of mixed plant communities of a mesic tallgrass prairie, (ii) to evaluate growth responses of the nonnative leek (*Allium porrum* L.) and the dominant native grass (*Andropogon gerardii* Vitman) to DSE fungi, and (iii) to evaluate the metabolic and enzymatic capabilities of the isolated endophytic fungi.

MATERIALS AND METHODS

Site.—Konza Prairie Biological Station (KPBS, 39°05'N, 96°35'W) represents a native mesic tallgrass prairie in the Flint Hills of eastern Kansas, USA. The site spans 3487 ha and remains undisturbed by agriculture. The vegetation is dominated by C₄ grasses including *A. gerardii*, *Sorghastrum nutans* (L.) Nash., *Schizachyrium scoparium* (Michx.) Nash. and *Panicum virgatum* L. (see Towne 2002 for all vascular plants at Konza Prairie). The soil parent material is chert-bearing limestone with the soil bulk density of 1.0 g/cm³. January mean temperature is -3 C (range -9 to 3 C) and the July mean is 27 C (range 20 to 33 C). Annual precipitation is 835 mm ~75% of which falls in the growing season. Our research site was subjected to an annual spring burning, a typical grassland management practice in this area.

Sample collection.—Roots of mixed plant communities were collected for isolating root colonizing fungi. More than 98% of root length was colonized by DSE fungi at our site (Mandyam and Jumpponen 2008). Twelve permanent plots (4 m²) in a lowland watershed at KPBS were selected for sampling. The chosen watershed well represents the silt-clay loams formed from deep alluvial and colluvial soils characteristic of lowland topographic positions and is strongly dominated by the common C₄ grasses (above). The isolations were performed twice, in Jun and Aug 2002. Two random soil cores from each plot were pooled and manually homogenized. We made no attempt to identify the host taxa because that would have required excessively destructive sampling within the permanent plots and as a result our sample consisted of a random root community

likely composed of dominant grasses. Roots were immediately washed free of soil under running tap water and processed immediately. A subsample of roots was selected arbitrarily for isolating fungi, whereas another subsample was stained for microscopic examination for DSE structures.

Staining of field-collected roots.—Roots were cleared and stained as described by Barrow and Aaltonen (2001). In brief roots were cleared in 2.5% KOH, followed by staining with Sudan IV and destained overnight in acidified glycerol. Inter- and/or intracellular melanized hyphae and micro-sclerotia in the root cortex and hyaline hyphae stained by Sudan IV in the cortex and stele were examined microscopically and recorded.

Isolation of root-inhabiting fungi.—Roots were surface sterilized with bleach (0.5% active ingredient sodium hypochlorite) 1 min, washed with sterile water and treated with 70% alcohol 2 min followed by several washings with sterile distilled water. The roots were plated on water agar (1.5%) and incubated at 25 C in dark. Roots were observed routinely under a dissecting microscope, and the emerging fungi were transferred onto cornmeal agar and potato dextrose agar (CMA; PDA; Becton Dickinson & Co, Maryland). Colony morphology was recorded and sporulation studied on CMA and PDA. The isolates were maintained by routine subculturing with single-spore isolations.

Identification of fungal isolates.—Sporulating fungi were identified based on colony morphology, conidiospore and conidiophore characteristics (Ellis 1971). Many isolates failed to sporulate; they were broadly grouped by their macromorphological characteristics. The morphological groups were refined further by restriction fragment length polymorphism of the internal transcribed spacer (ITS-RFLP; Gardes and Bruns 1996) after genomic DNA extraction from pure culture following a protocol by Gardes and Bruns (1993) or with UltraClean™ soil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, California). ITS-region was amplified with primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) and digested with restriction enzymes *Hinf*I and *Alu*I (New England Biolabs, Ipswich, Massachusetts) as described in Gardes and Bruns (1996). Similar RFLP patterns were considered an approximation of conspecific groups.

Resynthesis with leek and Andropogon.—Because *Periconia macrospinosa* Lefebvre and Johnson constituted about 45% of isolates we chose five representative isolates and three isolates from other species or conspecific groups to confirm whether they were endophytic. Instead of native grasses or forbs, leek was chosen for quick screening of endophytes because of its many advantages: availability of endophyte-free leek seeds, 98% germination rate and nearly transparent root system. Seeds (W. Atlee Burpee & Co, Warminster, Pennsylvania) were surface sterilized with a 30% solution bleach (5.25% sodium hypochlorite before dilution) 1 min, followed by 3 min in 70% ethanol, washed several times with sterile distilled water and dried on sterile No. 1 Whatman filter paper. Sterilized seeds were germinated on 1/10 Murashige Skoog (MS) basal salt mixture medium without any organic additives (Sigma, Missouri) a week in a growth

chamber under 12 h light-dark cycle (ca. 250 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PAR at 20 C). A small opening in the Petri dish was made by making overlapping cuts both in the lid and the dish containing MS (1/10 strength) medium. A sterile seedling was transferred onto this plate so that the shoot emerged through the opening while the roots were contained within the Petri dish. The dish was sealed with parafilm except at the opening and placed upright in the growth chamber. Seedlings were allowed to stabilize 4 d in the chamber before inoculation with a 6 mm fungal plug. Fungal plugs were cored from isolates grown on PDA at 25 C for 15 d. A total of 15 replicates for each fungal treatment were incubated upright in the chamber under the above conditions. Fungus-free controls were mock-inoculated with sterile PDA plugs. Plants were observed periodically and harvested after 6 wk. Shoots of all and roots of 10 replicates were harvested, dried at 60 C and dry weight recorded. Roots from the remaining five replicates were used for microscopic confirmation of colonization, and the fungal morphology within the roots was recorded.

Roots were cleared and stained as described above. A fungal endophyte was confirmed if the fungus produced melanized microsclerotia and/or chlamydospores in the root cortex. In addition roots were examined for presence of inter- and/or intracellular melanized hyphae and hyaline hyphae stained by Sudan IV.

Fungal isolates confirmed to be DSE also were inoculated to *A. gerardii* (U.S. Department of Agriculture, Natural Resources Conservation Service, Manhattan Plant Materials Center, Manhattan, Kansas). Seeds were sterilized 10 min in full strength H₂O₂ followed by 10 min in 90% alcohol and rinsed several times in sterile water. Seeds were germinated on water agar, and *Andropogon* resynthesis was carried out in the resynthesis system as described above. A total of 15 replicates per treatment were incubated along with fungus-free controls. Shoots of all and roots of 10 replicates were harvested after 6 wk, dried at 60 C for 3 d and dry weights recorded. Roots from the remaining five replicates were used for microscopic confirmation of DSE structures.

DNA sequencing.—Fungi that produced typical DSE structures in leek and *A. gerardii* roots were selected for sequencing to further confirm their taxonomic affinities. The PCR-amplified DNA from cultures of *Microdochium* and *Periconia* isolates were purified with UltraCleanTM PCR clean up kit (MoBio Inc., Carlsbad, California) and sequenced on a Beckman-Coulter CEQ 8000 DNA Analysis System with primers ITS1F and ITS4 at Kansas State Veterinary Diagnostic Laboratory (GenBank accession numbers are *P. macrospinosa* FJ536207-209 and *Microdochium* sp. FJ536210). Sequence similarities within the two taxa were estimated after alignment with Sequencher (Genecodes Inc., Ann Arbor, Michigan) and their taxon affinities confirmed by BLAST (Altschul et al. 1997). All sequenced *Periconia* isolates were 98–99% similar to *P. macrospinosa* and *Microdochium* was 93% similar to *Microdochium* sp.

Tests for enzymatic activities.—Eight *P. macrospinosa* (KS0019, KS0025, KS0045, KS0054, KS0060, KS0082, KS0093 and KS0100) and two *Microdochium* (KS0012 and KS0014) isolates were tested for hydrolytic capabilities.

Enzymes hydrolyzing complex carbon molecules, amylase, cellulase, polyphenol oxidases including laccase and tyrosinase, were tested. Confirmed endophytes also were tested for gelatinase. Each test medium was inoculated with a 6 mm fungal plug cored from CMA, and each isolate was incubated in triplicates 2 wk at room temperature. Tyrosinase tests were incubated 3 wk. A basal medium (Caldwell et al. 1991) composed of mineral salts was used for amylase and cellulase evaluation.

(i) *Amylase*. The basal medium was amended with starch (1%) as the sole carbon source in the medium (Caldwell et al. 2000). Iodine (10%) was used to view the zone of hydrolysis, and the strength of activity was classified based on the diameter of the hydrolytic zone.

(ii) *Cellulase (endoglucanase or CMCase)*. The basal medium was amended with carboxy methyl cellulose (CMC, 1%; Caldwell et al. 2000). The zone of hydrolysis was viewed by flooding the plate with an aqueous solution of Congo red (1 mg/mL) 15 min. The plate was flooded with 1M NaCl 15 min after draining the dye, followed by stabilization with 1 M HCl (Teather and Wood 1982). The strength of activity was classified based on the diameter of the hydrolytic zone.

(iii) *Laccase spot test*. Fungal isolates were grown on Sabourauds' medium and PDA amended with o-dianisidine (0.01%). Laccase production can be affected by media components, and therefore it must be evaluated on different media (Hutchinson 1990). A dark brown zone around the colony was indicative of a positive reaction. Strength of the activity was based on the visibility of the brown zone.

(iv) *Tyrosinase (cresolase) spot test*. Fungal isolates were grown on 2.5% malt extract agar 3 wk. One drop of 0.1 M p-cresol dissolved in ethanol was added on the colony margin (Gramss et al. 1998). The indicator p-cresol stains red in the presence of tyrosinase.

(v) *Gelatinase liquefaction test*. Fungi were inoculated into gelatin (12%) slants and incubated 2 wk. A positive test is indicated by liquefaction in the tube after chilling 30 min. The proportion of liquefied medium indicated the strength of the activity.

Nitrogen use.—Confirmed endophytes (seven *Periconia* isolates and one *Microdochium*) were grown on liquid medium containing either an organic (alanine, arginine and glycine) or inorganic (NH₄Cl and NH₄NO₃) nitrogen source (Finlay et al. 1992). Dextrose was the carbohydrate source and media were adjusted to C:N = 39:1. Media pH was adjusted to 6.0 before fungal inoculation. N sources were omitted from control treatments. Fungal isolates were inoculated to 20 mL liquid medium of each N source in five replicates and incubated at 22 C 3 wk. The fungal biomass was extracted by filtration and dried at 60 C. The biomass was recorded as proxy for N use. The pH after N use was 2.5–7.5. The fungal biomass on N-free medium was subtracted from the observed biomass on different N media for each of the tested isolates.

Statistical analyses.—*A. gerardii* and leek dry weight data failed the assumptions of ANOVA due to severe distortions

and unequal variances. No transformation normalized and stabilized variance in all groups simultaneously. Accordingly nonparametric tests (Hettmansperger and McKean 1998, Higgins 2003) were considered necessary. In case of leek only total biomass was analyzed. All pairwise Wilcoxon rank sum tests against the control were performed with SAS 9.1. The sum of scores for rank sum statistic for each comparison was compared to critical values that were calculated with a version of Dunnett's test adapted to rank sums (Hsu 1996). To analyze *Andropogon* data a Kruskal-Wallis test was performed with PROC NPARIWAY (SAS 9.1). All pairwise comparisons were made for root, shoot and total biomass. Overall type I error rate for the set of tests was not more than 0.05. The fungal biomass comparison for N uptake fulfilled the assumptions of ANOVA. The different N sources were analyzed separately by one-way ANOVA in SAS 9.0. Pairwise differences were determined by a conservative Bonferroni's test ($P < 0.05$).

RESULTS

DSE structures in field-collected roots.—Percent root length colonized by DSE structures from roots of mixed plant communities were recorded previously from the study sites (Mandyam and Jumpponen 2008). Extensive melanized hyphae, microsclerotia and hyaline vesicles were abundant in the cortex of field-collected roots. Inter- and intracellular chlamydospores were observed routinely in the field samples.

Fungal isolates.—A total of 127 isolates were obtained, 79 of which sporulated and represented these taxa: *Aspergillus* sp. (four), *Acremonium* sp. (five), *Cladosporium* sp. (three), *Curvularia* sp. (one), *Fusarium* sp. (eleven), *Microdochium* sp. (six) and *P. macrospinosa* (49). An additional 48 nonsporulating fungi were grouped based on colony morphology and confirmed by ITS-RFLP (eight green sterile slow-growing fungi, 22 sterile white fungi and 18 sterile dark fungi).

Our *Periconia* ITS sequences were 98–99% similar to *P. macrospinosa*. However macro- and micro-morphologies of *P. macrospinosa* isolates were variable (SUPPLEMENTAL INFORMATION). The *Microdochium* isolate was 93% similar to *Microdochium* sp. (GenBank accession number FJ536210) and produced conidiospores in addition to abundant chlamydospores.

Leek resynthesis.—*Acremonium* sp., *Aspergillus* sp., *Cladosporium* sp. and *Fusarium* sp. did not produce DSE structures in the roots. Leek plants inoculated with sterile green fungus (KS0001), *Microdochium* sp. (KS0012) and *P. macrospinosa* (KS0019, KS0058, KS0060, KS0093 and KS0100) were symptom free during 6 wk incubation. The sterile green fungus (KS0001) produced extensive superficial hyphae around the roots with no visible inter- or intracellular

colonization. Isolates of *P. macrospinosa* colonized the leek roots with typical DSE structures. Melanized hyphae were rarely seen in the roots. However melanized microsclerotia were found abundantly in the cortex with lipids staining red with Sudan IV. Hyaline vesicles occurred frequently in the cortex. Hyaline vesicles are likely the initial stages of microsclerotia (Mandyam and Jumpponen 2008); a variety of stages ranging from hyaline vesicles to partially melanized microsclerotia were observed. *Periconia* conidiophores and conidia were observed frequently on colonized leek roots. *Microdochium* sp. formed inter- and intracellular melanized chlamydospores in the cortical cells. Similar to the case of *P. macrospinosa*, *Microdochium* sp. did not produce melanized hyphae in the leek roots.

Select DSE isolates (*Microdochium* KS0012; *Periconia* KS0019, KS0045 and KS0100) also were inoculated into *A. gerardii* in a resynthesis system. All these isolates produced typical DSE morphologies similar to those observed in leek. However hyaline vesicles were not seen in *A. gerardii*, although they were relatively common in leek roots.

Host growth responses.—Overall leek was unresponsive to the endophyte inoculation (FIG. 1A). None of the tested fungi with the exception of *Periconia* isolate KS0100 affected leek biomass. Compared to the control *Periconia* isolate KS0100 decreased the total leek biomass. The total biomass of leek inoculated with *Microdochium* sp. (KS0012) and green fungus (KS0001) did not differ from the control.

In contrast to leek resynthesis none of the tested isolates had a negative effect on *A. gerardii* total dry weights (FIG. 1B). Compared to the control, *Periconia* isolates KS0045 and KS0100 and the *Microdochium* isolate increased the total biomass while *Periconia* isolate KS0019 had a neutral effect.

Enzymatic capabilities.—Results of enzyme hydrolysis are provided (TABLE I). Most isolates tested positive for all tested extracellular enzymes. The intensity of enzyme hydrolysis varied within and among taxa. All fungi hydrolyzed starch and cellulose. Laccase and tyrosinase were produced by all isolates except one, *Periconia* isolate (KS0060) and *Microdochium* (KS0012). Laccases oxidize a variety of organic compounds including diphenols, polyphenols and aromatic amines (Thurston 1994). Gelatinase was produced by all isolates except one *Periconia* isolate (KS0093).

Nitrogen use.—All tested nitrogen sources were used to some degree by *Periconia* isolates. Only one *Microdochium* isolate (KS0014) was tested. It performed poorly in liquid media, and its ability to use

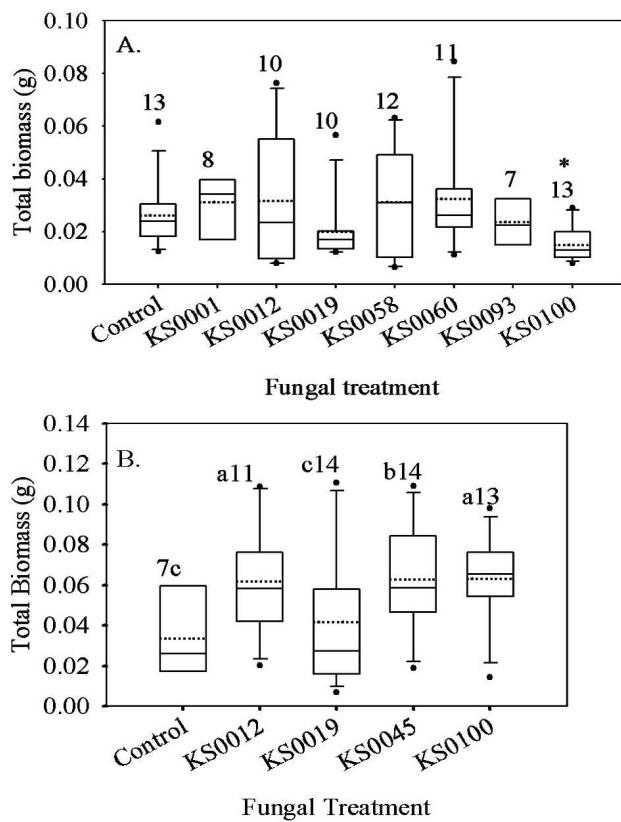


FIG. 1. Leek and *Andropogon* growth responses to DSE fungi. A. Leek total dry weight. Nonparametric median test was used for pairwise comparison of treatment differences with control at $\alpha = 0.05$. * Treatment significantly different from control. B. *Andropogon* total dry weight. Nonparametric median test was used for all pairwise comparisons at $\alpha = 0.05$. Treatments sharing a letter are not significantly different from each other. The boxes indicate 75th and 25th percentile. Bars above and below the box indicate respectively 90th and 10th percentile. Median and mean are indicated respectively by solid and dotted lines. Outliers are indicated by closed black circles. Numbers above box plots indicate the sample size per treatment. Fungal treatments include uninoculated control, sterile green fungus (KS0001), *Microdochium* sp. (KS0012), and *P. macrospinosa* (KS0019–KS0100).

the tested N sources remains uncertain. Biomass after 3 wk incubation varied substantially among isolates (FIG. 2). *Periconia* isolates did not show any specific trend in N use, but all isolates were able to use both organic and inorganic N. Results are missing for arginine, ammonium and nitrate treatments for some isolates because those substrates were prone to bacterial contamination and were omitted from analyses.

DISCUSSION

Root endophytes.—Field-collected roots from a native tallgrass prairie are commonly colonized by charac-

teristic DSE structures (Mandyam and Jumpponen 2008). Our isolation experiment from roots of mixed plant communities at Konza yielded some frequently encountered soil fungi including *Acremonium* sp., *Aspergillus* sp., *Cladosporium* sp., *Curvularia* sp. and *Fusarium* sp. In leek resynthesis these fungi did not produce typical DSE structures in the roots. In addition we isolated two interesting endophytic fungal taxa that formed typical DSE structures both in leek and *A. gerardii*. Among DSE isolates *Microdochium* sp. was infrequent whereas *P. macrospinosa* was the most frequently isolated. It is possible some additional endophyte taxa might have escaped detection.

Ellis (1971) and Ellis (1976) described several *Periconia* (anamorphic Microascales) species based on spore size, morphology and colony characteristics. *Periconia* spp. have been isolated from a wide variety of environments ranging from temperate to tropical and from arable to native grassland ecosystems (Ellis 1971, Domsch et al. 1980). Most *Periconia* spp. have been considered saprobic (Dunkle 1992), although a few are known pathogens (Ellis 1971). *Periconia macrospinosa*, although not usually considered pathogenic to graminoids (Domsch et al. 1980, Dunkle 1992), can be pathogenic to wheat under experimental conditions (Carter et al. 1999). Our study indicated that *P. macrospinosa* is a common septate endophyte in tallgrass prairie. *Periconia macrospinosa* has been isolated repeatedly from native tallgrass species and consistently forms typical DSE morphologies in *A. gerardii* resynthesis (Kageyama et al. unpubl.).

While our *Periconia* isolates matched *P. macrospinosa* on the basis of morphology and ITS sequence homology, some did not produce chlamydospores in culture. The colony characteristics of *P. macrospinosa* isolates also were highly variable (SUPPLEMENTAL INFORMATION). One *Microdochium* isolate (KS0012) with 93% similarity to *Microdochium* sp. could not be confidently identified to species. *Microdochium* is a relatively poorly studied genus and its species concepts poorly defined (Kwaśna and Bateman 2007). Kwaśna and Bateman (2007) list the morphological characteristics of known *Microdochium* species, among which only four species produce both sporodochia and chlamydospores, as observed for our isolates, *Microdochium bolleyi* (R. Sprague) de Hoog and Herm.-Nijh., *Microdochium dimerum* (Penz.) Arx, *Microdochium lunatum* (Ellis and Everh.) Arx. and *Microdochium tainanense* (Ts. Watan.) de Hoog and Herm.-Nijh. Although our isolate resembles *M. bolleyi* morphologically its ITS sequence is only 93% similar to the *M. bolleyi* ITS sequence.

DSE fungi and resynthesis.—While a number of studies have implied that *Periconia* and *Microdochium*

TABLE I. Hydrolytic capabilities of select *Periconia macrospinosa* (KS0019-KS0100) and *Microdochium* sp. (KS0012 and KS0014)

Enzymes	<i>Periconia macrospinosa</i>								<i>Microdochium</i> sp.	
	KS0019	KS0025	KS0045	KS0054	KS0060	KS0082	KS0093	KS0100	KS0012	KS0014
Amylase ^a	+	++	++	++	+++	++	+	+++	++	++
Cellulase ^b	+	+	+	++	+	+	++	+++	++	+
Laccase ^c	++	+	+++	+++	-	+	+++	++	-	++
Tyrosinase ^d	+	+	+	+	-	+	+	+	-	+
Gelatinase ^e	+	++	+	+++	++	+	-	++++	++++	+++

^a Amylase test: -, absence of clearing around fungal mat, negative for amylase; +, clearing 1–3 cm diam; ++, clearing 3–6 cm diam; +++, clearing >6 cm diam.

^b Cellulase test: -, absence of clearing, negative for cellulose; +, clearing <2 cm diam; ++, clearing about 2 cm diam; +++, clearing >2 cm diam.

^c Laccase test: -, absence of brown under or around fungal mat, negative for Laccase; +, dark brown under fungal mat at the center, visible only on the underside of the plate; ++, dark brown formed under most of mat but not extending to margin, seen from under side of the plate; +++, dark brown extending beyond margin of fungal colony and visible from the topside of the plate.

^d Tyrosinase test: -, absence of orange-brown, negative for cresolase; +, presence of orange-brown, positive for cresolase.

^e Gelatinase test: -, absence of liquefaction at 4 C, negative for gelatinase; +, liquefaction <25% medium; ++, liquefaction 26–50% medium; +++, liquefaction 51–75% medium; +++, liquefaction 76–100% medium.

are associated with plant roots (Kirk and Deacon 1987, Opperman and Wehner 1994, Caretta et al. 1999, White and Backhouse 2007) our resynthesis experiments with nonnative and native hosts show that these fungi produce structures characteristic of DSE symbiosis. *Microdochium* sp. produced inter- and intracellular chlamydospores without microsclerotia, whereas *P. macrospinosa* formed typical microsclerotia in leek and *A. gerardii* and hyaline vesicles in leek. Melanized hyphae or hyaline hyphae were not seen in either host. These observed differences may be attributed to various factors affecting fungal morphology and development: (i) choice of host plant and/or fungal isolate; (ii) incubation conditions (resynthesis did not mimic field conditions); and (iii) time of incubation (6 wk might be insufficient). Although our resynthesis system was artificial it was adequate to address our main objectives—to identify and confirm root endophytes in a tallgrass prairie ecosystem.

Many DSE fungi have been observed to induce variable host responses in inoculation studies. These responses have been attributed to choices of host species, endophyte taxa or strains and experimental conditions (Kageyama et al. 2008). Similarly the growth responses of native and nonnative plants to DSE inoculation were variable in our study; *Periconia* isolates KS0045 and KS0100 and *Microdochium* KS0012 elicited positive responses in *A. gerardii*, while responses were neutral in leek, except for KS0100, which negatively affected leek. *Periconia* isolate KS0019 was neutral to both *A. gerardii* and leek.

Enzymatic capabilities.—Mandyam and Jumpponen (2005) reviewed observations of enzymatic capabil-

ities of DSE fungi. The reported activities included amylase, cellulases, lipase, pectinase, polyphenol oxidases (laccase and tyrosinase), protease and xylanase. Similarly our studies show that *Periconia* and *Microdochium* isolates produce enzymes capable of metabolizing complex substances, suggesting saprobic capabilities.

The hydrolytic capabilities of these endophytes are interesting. These enzymes can break down detritus or may aid in penetration into plant tissues. Unlike mycorrhizal fungi, no experimental evidence supports the transfer of nutrients released by hydrolysis to the host plant (Addy et al. 2005). In our study most isolates produced laccase. Burke and Cairney (2002) discuss many functions for laccase in nonmycorrhizal fungi including lignin degradation, a role in mycelial growth and hyphal cross-linking, fruiting body differentiation, detoxification of phenolics and melanin production. A role in melanin production seems appropriate given that DSE fungi often produce abundant melanized tissues. A role in lignin degradation must be confirmed via lignin-degradation assays. We did not test for lignolytic activity, but we expect it to be highly variable as determined for other assayed enzymatic activities. Ultimately the importance of these observed enzymatic activities must be confirmed in planta to assess their relevance for plant nutrient acquisition.

Nitrogen use.—The *Microdochium* isolate we tested was unable to grow in liquid medium; its ability to use organic and inorganic N sources could not be tested. *Periconia macrospinosa* isolates used both organic (amino acids) and inorganic N sources. In addition

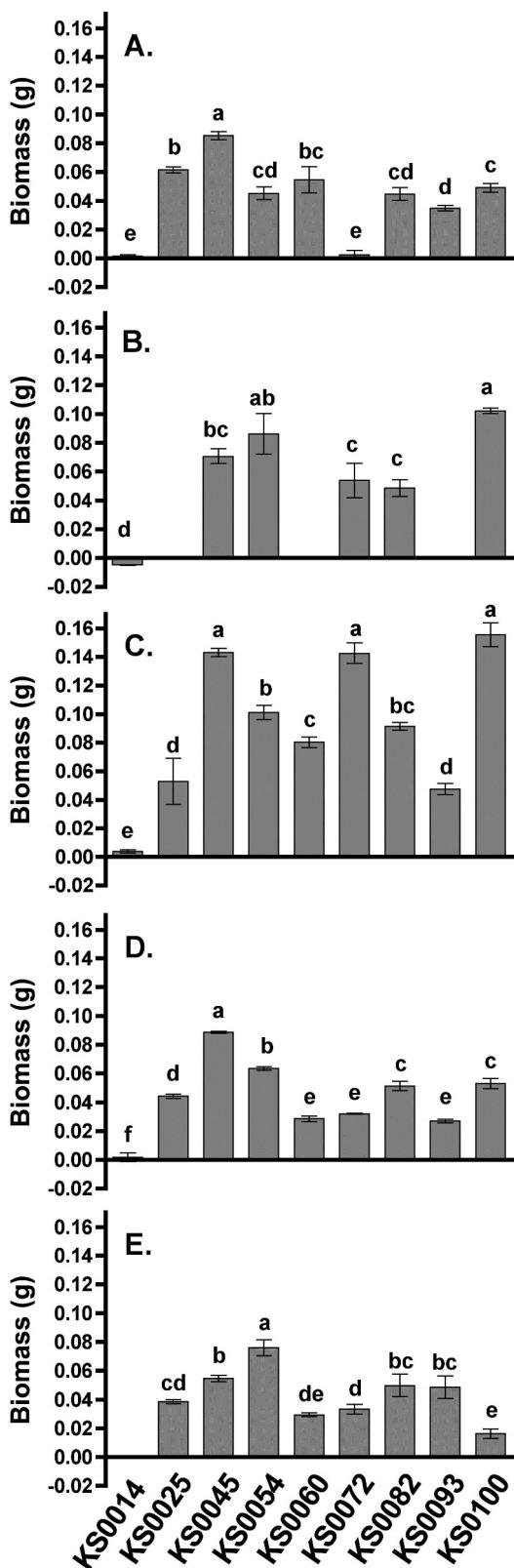


FIG. 2. Variation in growth among DSE isolates after 20 d incubation on media containing different organic and inorganic nitrogen sources. A. Alanine. B. Arginine. C. Glycine. D. Ammonium. E. Nitrate. Control biomass was

tested isolates produced a proteolytic enzyme, gelatinase. Pure cultures of some fungal isolates characterized as septate endophytes are known to use heterocyclic N sources; guanine and uric acid establish their N use in vitro (Caldwell and Jumpponen 2003a). Experimental verification of the uptake during symbiosis and its relevance to plant nutrient uptake are lacking. Our results confirm that one common DSE species from a tallgrass prairie can use both organic and simple inorganic N compounds. Mullen et al. (1998) hypothesized that root endophytic fungi could be important in N uptake in an alpine environment during snowmelt. Green et al. (2008) provide indirect evidence in support of C and N exchange between a patch mosaic of grasses and biological crust dominated by a fungal network of symbiotic DSE fungi in a semi-arid ecosystem. Mandyam and Jumpponen (2008) also suggest a role of DSE fungi in N uptake based on greater DSE colonization in early spring compared to mycorrhizal fungi at Konza Prairie. However no adequate data are available to confirm DSE aid in N uptake. Further empirical evidence must be collected to test the hypotheses on the endophyte role in host nutrition.

In conclusion *P. macrospinosa* and *Microdochium* sp. that are commonly associated with roots of grasses are common DSE fungi in a tallgrass prairie ecosystem. To our surprise we did not detect any *Phialocephala* sp., a DSE fungus frequently isolated from various ecosystems. This suggests that DSE fungal constituents might vary among biomes or ecosystems and that many DSE fungi might remain undetected. Our results from this and Mandyam and Jumpponen (2008) establish that not only does a native tallgrass prairie have a great abundance of DSE fungi it also hosts a distinct assembly of fungi that represent root-associated endophytes. Given the abundance and the distinct communities of DSE fungi in various ecosystems, further studies are vital to further our understanding of their roles in ecosystem functioning.

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subtracted from each treatment biomass. The tested isolates include *Microdochium* sp. (KS0014) and *P. macrospinosa* (KS0025-KS0100). Treatments sharing a letter are not significantly different (Bonferroni pairwise comparisons; $P < 0.05$). Some treatments are missing biomass values due to bacterial contamination.

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LITERATURE CITED

- Addy HD, Piercy MM, Currah RS. 2005. Microfungal endophytes in roots. *Can J Bot* 83:1–13.
- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.
- Barrow JR, Aaltonen RE. 2001. Evaluation of the internal colonization of *Atriplex canescens* (Prush) Nutt. roots by dark septate fungi and the influence of host physiological activity. *Mycorrhiza* 11:99–205.
- Blair JM, Seastedt TR, Rice CW, Ramundo RA. 1998. Terrestrial nutrient cycling in tallgrass prairie. In: Knapp AK, Briggs JM, Hartnett DC, Collins SL, eds. *Grassland dynamics: long-term ecological research in tallgrass prairie*. New York: Oxford Univ. Press. p 222–1257.
- Burke RM, Cairney JWG. 2002. Laccases and other polyphenol oxidases in ecto- and ericoid mycorrhizal fungi. *Mycorrhiza* 12:105–116.
- Caldwell BA, Castellano MA, Griffiths RP. 1991. Fatty acid esterase production by ectomycorrhizal fungi. *Mycologia* 83:233–236.
- , Jumpponen A, Trappe JM. 2000. Utilization of major detrital substrates by dark septate root endophytes. *Mycologia* 92:230–232.
- , —. 2003a. Utilization of heterocyclic organic nitrogen by mycorrhizal fungi. Abstract 276: 4th International Conference on Mycorrhizae (ICOM4), 10–15 Aug 2003. Montreal, Canada.
- , —. 2003b. Arylsulfatase production by mycorrhizal fungi. Abstract 277: 4th International Conference on Mycorrhizae, 10–15 Aug 2003. Montreal, Canada.
- Caretta G, Piontelli E, Picco AM, del Frate G. 1999. Some filamentous fungi on grassland vegetation from Kenya. *Mycopathologia* 145:155–169.
- Carter JP, Spink J, Cannon PF, Daniels MJ, Osbourn AE. 1999. Isolation, characterization and avenacin sensitivity of a diverse collection of cereal-root-colonizing fungi. *Appl Environ Microbiol* 65:3364–3372.
- Currah RS, Tsuneda A. 1993. Vegetative and reproductive morphology of *Phialocephala fortinii* (Hyphomycetes, *Mycelium radicans atrovirens*) in culture. *Trans Mycol Soc Jap* 34:345–356.
- Domsch KH, Gams W, Anderson T. 1980. *Compendium of soil fungi*. Vol. 2. New York: Academic Press. 860 p.
- Dunkle LD. 1992. *Periconia*. In: Singleton LL, Mihail JD, Rush CM, eds. *Methods for research on soilborne phytopathogenic fungi*. St Paul: American Phytopathological Society. p 137–141.
- Ellis MB. 1971. *Dematiaceous Hyphomycetes*. Kew, Surrey, UK: Commonwealth Mycological Institute. 608 p.
- . 1976. *More dematiaceous Hyphomycetes*. Kew, Surrey, UK: Commonwealth Mycological Institute. 507 p.
- Fernando AA, Currah RS. 1996. A comparative study of the effects of the root endophytes *Leptodontidium orchidicola* and *Phialocephala fortinii* (Fungi Imperfici) on the growth of some subalpine plants in culture. *Can J Bot* 74:1071–1078.
- Finlay RD, Frostegård Å, Sonnerfeldt A-M. 1992. Utilization of organic and inorganic nitrogen sources by ectomycorrhizal fungi in pure culture and in symbiosis with *Pinus contorta* Dougl. ex Loud. *New Phytol* 120:105–115.
- Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorrhizae and rusts. *Mol Ecol* 2:113–118.
- , —. 1996. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and belowground views. *Can J Bot* 74:1572–1583.
- Gramss G, Günther TH, Fritsche W. 1998. Spot tests for oxidative enzymes in ectomycorrhizal, wood- and litter-decaying fungi. *Mycol Res* 102:67–72.
- Green LE, Porras-Alfaro A, Sinsabaugh RL. 2008. Translocation of nitrogen and carbon integrates biotic crust and grass production in desert grassland. *J Ecol* 98:1076–1085.
- Hettmansperger TP, McKean JW. 1998. *Robust nonparametric statistical methods*. London and New York: Arnold/Wiley & Co. 484 p.
- Higgins JJ. 2003. *Introduction to modern non-parametric statistics*. North Scituate, Massachusetts: Duxbury Press. 500 p.
- Hsu JC. 1996. *Multiple comparisons: theory and methods*. Boca Raton: Chapman & Hall/CRC Press. p 72–73.
- Hutchinson LJ. 1990. Studies on the systematics of ectomycorrhizal fungi in axenic culture III. Patterns of polyphenol oxidase activity. *Mycologia* 82:424–435.
- Jumpponen A. 2001. Dark septate endophytes—are they mycorrhizal? *Mycorrhiza* 11:207–211.
- , Trappe JM. 1998. Dark septate endophytes: a review of facultative biotrophic root colonizing fungi. *New Phytol* 140:295–310.
- Kirk JJ, Deacon JW. 1987. Invasion of naturally senescent root cortices of cereal and grass seedlings by *Microdochium bolleyi*. *Plant Soil* 98:239–246.
- Kovács GM, Szigetvári C. 2002. Mycorrhizae and other root-associated fungal structures of the plants of a sandy grassland on the great Hungarian Plain. *Phyton* 42: 211–223.
- Kwaśna H, Bateman GL. 2007. *Microdochium triticicola* sp. nov. from the roots of *Triticum aestivum* in the United Kingdom. *Mycologia* 99:765–776.
- Leukel RW. 1948. *Periconia circinata* and its relation to milo disease. *J Agric Res* 77:201–222.
- , Johnson AG. 1948. *Periconia circinata*, the cause of milo disease. *Science* 107:93–94.
- Mandyam K, Jumpponen A. 2005. Abundance and possible

- functions of the root-colonising dark septate endophytic fungi. *Stud Mycol* 53:173–190.
- , —. 2008. Seasonal and temporal variation of arbuscular mycorrhizal and dark septate endophytic fungi in a tallgrass prairie ecosystem. *Mycorrhiza* 18:145–155.
- Mullen RB, Schmidt SK, Jaeger III CH. 1998. Nitrogen uptake during snowmelt by the snow buttercup, *Ranunculus adoneus*. *Arc Alp Res* 30:121–132.
- Opperman L, Wehner FC. 1994. Survey of fungi associated with grass-roots in virgin soils on the Springbok Flats. *S Afr J Bot* 60:67–72.
- Schulz B, Boyle C. 2005. The endophytic continuum. *Mycol Res* 109:661–687.
- Teather RM, Wood PJ. 1982. Use of Congo red-polysaccharide interactions in the enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol* 43:777–780.
- Thurston C. 1994. The structure and function of fungal laccases. *Microbiol* 140:19–26.
- Towne EG. 2002. Vascular plants of Konza Prairie Biological Station: an annotated checklist of species in a Kansas tallgrass prairie. *Sida* 20:269–294.
- White IR, Backhouse D. 2007. Comparison of fungal endophyte communities in the invasive panicoid grass *Hyparrhenia hirta* and the native grass *Bothriochloa macra*. *Aust J Bot* 55:178–185.
- White TJ, Bruns T, Lee S, Taylor S. 1990. Analysis of phylogenetic relationships by amplification and direct sequencing of rRNA genes. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications*. New York: Academic Press. p 315–322.
- Wilczek AM, Roe JL, Knapp MC, Cooper MD, Lopez-Gallego C, Martin LJ, Muir CD, Sim S, Walker A, Anderson J, Egan JF, Moyers BT, Petipas R, Giakountis A, Charbit E, Coupland G, Welch SM, Schmitt J. 2009. Effects of genetic perturbation on seasonal life history plasticity. *Science* 323:930–934.
- Wilson BJ, Addy HD, Tsuneda A, Hambelton S, Currah RS. 2004. *Phialocephala sphaeroidea*, sp. nov., a new species among the dark septate endophytes (DSE) from a boreal wetland in Canada. *Can J Bot* 82:607–617.
- Yu T, Nassuth A, Peterson RL. 2001. Characterization of the interaction between the dark septate fungus *Phialocephala fortinii* and *Asparagus officinalis* roots. *Can J Micro* 47:741–753.