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Interaction between an isolate of dark-septate fungi and its host plant *Saussurea involucrata*

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Abstract A dark-septate endophytic (DSE) fungus EF-M was isolated from the roots of an alpine plant *Saussurea involucrata* Kar. et Kir. ex Maxim. The fungus was identified by sequencing the PCR-amplified rDNA 5.8S gene and ITS regions. The sequence was compared with similar sequences in the GenBank, and results showed that EF-M was congeneric to *Leptodontidium*. Resynthesis study was conducted to clarify the relationship between the root endophyte EF-M and the host plant *S. involucrata* using the material grown in sterile culture bottle. In roots recovered 6 weeks after inoculation, epidermal cells were colonized by intercellular and intracellular hyphae and “microsclerotia” formed within individual cells in the epidermis layers. However, hyphae did not invade the cortex and the stele. There were no profound effects of endophyte EF-M on plant root development, but significant differences were detected in plant height and shoot dry weight between the treatments. The present study is the first report hitherto on DSE fungi in *S. involucrata*.

Keywords Dark-septate endophytic (DSE) fungi · ITS and 5.8S rDNA · Molecular identification · Plant growth · *Saussurea involucrata* Kar. et Kir. ex Maxim

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Introduction

Dark-septate root endophytes (DSE) are an artificial assemblage of fungi that have darkly pigmented, septate hyphae and are frequent or distinctive intracellular root associates of apparently healthy plants (Piercey et al. 2004). Although they have been found to be associated with plants of many families from the tropics to the arctic and are particularly common in stressful habitats such as the arctic (Jumpponen and Trappe 1998) or alpine environments (Read and Haselwandter 1981), the function of DSE remains controversial (Jumpponen and Trappe 1998; Jumpponen 2001). However, their widespread occurrence and abundance suggest not only ubiquitous presence and lack of host specificity but also a role of importance in natural ecosystems (Jumpponen and Trappe 1998). Some DSE have been found to enhance host mineral nutrition and growth (Fernando and Currah 1996; Shivanna et al. 1994; Jumpponen et al. 1998).

In the investigation of the fungal endophyte diversity in roots of wild *Saussurea involucrata* Kar. et Kir. ex Maxim (Asteraceae), we isolated 43 endophytic strains from surface-sterilized roots; 11 were characterized by darkly pigmented, separate hyphae. In the screening experiment, in vitro grown *S. involucrate* seedling displayed enhanced vegetative growth when infected by the isolate EF-M. *S. involucrata*, known as snow lotus, has limited distributions on rocky habitats 2,600 m in elevation or higher in the Tianshan and A'er Tai areas in China. In folk medicine, it is used for the treatment of rheumatoid arthritis, impotence, irregular menses, expelling placenta after birth, and altitude sickness (Li and Cai 1998). *S. involucrata* grows very slowly, and in recent years, wild sources of *S. involucrata* have decreased dramatically because of exhaustive collection for use in pharmaceutical preparations. To conserve the

natural sources of *S. involucrata*, cultivars of *S. involucrata* are being developed which might serve as potential substitutes for wild *S. involucrate* in the pharmaceutical industry.

Our objectives in this study were to measure the morphological responses of *S. involucrate* to inoculation of the endophyte and to better understand the taxonomy and biology of the endophyte in order to highlight some of the principal aspects of this mutualism. Results may enable future biotechnological applications of the endophytic mycelium in cultivating *S. involucrata*.

Materials and methods

Sampling and isolation of strains

Three wild plants of *S. involucrata* Kar. et Kir. ex Maxim were collected from a site around 3,000 m above sea level on Tianshan Mountain, Xinjiang Uygur Autonomous Region, P.R. China. Roots were washed in de-ionized water and separated into two subsamples, the first of which was placed in formalin–acetic acid–alcohol (FAA) and stained using a modification of the method of Phillips and Hayman (1970) as described by Mullen and Schmidt (1993). The second subsample was surface sterilized in 0.1% $HgCl_2$ for 7 min and placed on 90-mm Petri dishes containing potato dextrose agar (PDA). Petri dishes were sealed with a sealing film and incubated at 25°C. Mycelia growing out of roots were subcultured and maintained on PDA. Colony morphology and growth of the isolates were studied on PDA. Growth and spore formation were also examined on PDA, cornmeal agar, and 2% malt extract agar. When no sporulation was obtained, plates were incubated under cool white fluorescent light for a photoperiod of 12:12 h ratio of light to dark or maintained at low temperatures (5–6°C) over prolonged periods (6–7 months). Representative strains were deposited at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College. One isolate of DSE, EF-M, was chosen for further investigation.

Plant material

Seeds of *S. involucrata* were surface sterilized with 0.1% $HgCl_2$ for 7 min under agitation and aseptically planted onto MS medium (Murashige and Shoog 1962) supplemented with 0.7% (*w*/*v*) agar in culture bottles. Seedlings were grown alone for 3 weeks before fungi inoculation. All cultures were carried out in a growth chamber at 25°C in the day and 18°C in the night with a photoperiod of 12 h, 300 μmol m^{-2} s^{-1}.

Plant inoculation

In experiments for in vitro plant inoculation, 21-day-old seedlings grown in culture bottles were used. Seedlings were aseptically transplanted into the culture bottles filled with a mixture of 40 mL vermiculite–litter (10:1) and 12 mL MS liquid medium, covered with the gas-permeable plastic film, and autoclaved for 30 min. Inoculum of EF-M was added as two 5-mm plugs excised from an edge of an actively growing colony on PDA. Controls were mock inoculated with plugs excised from PDA plate without fungus. For each experiment, 30 inoculated and 30 non-inoculated seedlings were processed, and each experiment was run in triplicate.

Growth response of *S. involucrate*

Plants were harvested 6 weeks after being planted into the culture bottles, and the shoot and the root were carefully separated with a scalpel. Plant height and total number of hair root tips were measured for each plant. The shoots of 30 plants and roots of 20 plants from each treatment were oven-dried at 105°C and weighed. Roots of the remaining 10 plants were fixed in FAA.

Light microscopy studies

Roots fixed in FAA were dehydrated in a graded ethanol series and embedded in paraffin. For routine light microscope observations, histological sections were stained with safranine and fast green following the protocol of Feder and O'Brien (1968). Preparations were observed and photographed on an Olympus microscope.

DNA extraction, PCR amplification, and sequencing

DNA was extracted from a 15-day-old axenic culture of EF-M by the CTAB method (Doyle and Doyle 1987). The internal transcribed spacer (ITS) region was amplified using primers ITS 1 and ITS 4 (White et al. 1990). Polymerase chain reactions (PCRs) were carried out on a Minicycler™ (MJ Research, Reno, NV, USA) using 50-μL reaction volumes which contained PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl, pH 8.8, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.3 μM of each primer, 2-μL template DNA, and 2 units of Taq DNA polymerase (Fermentas). The thermal cycling program was as follows: 3-min initial denaturation at 94°C, followed by 35 cycles of 1-min denaturation at 94°C, 45-s primer annealing at 55°C, 2-min extension at 72°C, and a final 8-min extension at 72°C. Products were electrophoresed in 1% agarose gels, stained with ethidium bromide, and visualized under UV light. After purification with mini-

Table 1 Taxa and GenBank accession numbers used in the study

GenBank accession no.	Taxa
DQ122928	*Phialocephala* sp.
AF486126	*Phialocephala scopiformis*
AY606284	*Phialocephala fortinii*
AF486133	*Leptodontidium orchidicola*
AY371510	*Cadophora luteo-olivacea*
U66731	*Phialophora gregata*
AF083202	*Phialophora malorum*
AY266144	*Helgardia anguioides*
AY266169	*Oculimacula yallundae*
AY266145	*Helgardia aestiva*
AY259134	*Mollisia dextrinospora*
AY249065	*Graphium silanum*
AY078151	*Acephala applanata*
AY266146	*Oculimacula acuformis*
AJ305236	*Pyrenopeziza brassicae*
AF168783	Dark-septate endophyte DS16B
AF281397	*Neofabraea perennans*
DQ008242	*Mollisia minutella*
AY259138	*Mollisia fusca*
AJ430229	*Tapesia fusca*
AJ430228	*Tapesia cinerella*
DQ148411	EF-M
AF011283	*Blumeria graminis*
Z73799	*Sclerotinia sclerotiorum*

columns (Sangon), purified DNAs were directly sequenced in the ABI PRISM 377 DNA sequencer (Applied Biosystems). Primers were the same as those of the initial PCR and used singly in forward and reverse reactions. The sequence was compiled and deposited in the GenBank with the accession number DQ148411.

Sequence alignment and phylogenetic analysis

The ITS and 5.8S rDNA sequences were used to retrieve similar sequences from the GenBank using the NCBI BLAST program (Altschul et al. 1997). The ITS data included closely related *Phialocephala* spp., *Leptodontidium orchidicola*, and *Phialophora*; sequences for species in the genera *Oculimacula*, *Helgardia*, *Mollisia*, *Pyrenopeziza*, *Tapesia*, and *Neofabraea* (Dermateaceae); sequences for species in the mitosporic Helotiales genera *Acephala*, *Cadophora*, and *Graphium*; and unidentified endophyte DS16B. *Blumeria graminis* and *Sclerotinia sclerotiorum* were chosen as outgroup taxa. GenBank accession numbers are given in Table 1. The complex ITS region sequences were aligned with Clustal X (1.83), and the results adjusted manually, where necessary, to maximize alignment. The alignments were subsequently subjected to parsimony analysis using the heuristic search option with the random addition sequence (1,000 replications) and the branch swapping (tree bisection–reconnection) option of PAUP* 4.0b8a (Swofford 1998). All characters were unordered and given equal weight during the analysis. Gaps were treated as missing data. Bootstrap percentages used to assess the support for the branching topologies were calculated using PAUP* 4.0b8a. Bootstrapping was performed with 1,000 replications. Alignments are available from Treebase under pin number 10499.

Statistics

Data were statistically analyzed with standard ANOVA. Significant differences between treatments were determined using the post hoc test with Tukey–Kramer HSD simultaneous pairwise main comparison.

Results

Dark separate mycelia and "microsclerotia" were observed in cleared and stained root of wild *S. involucrata* Kar. et Kir. ex Maxim (Fig. 1a,b). Our isolates were morphologically similar to typical DSE observed by other researchers (Jumpponen and Trappe 1998). The isolate EF-M, chosen to inoculate the plant, grew moderately on PDA and MEA (about 10-mm diameter in 2 weeks) as gray colonies with a

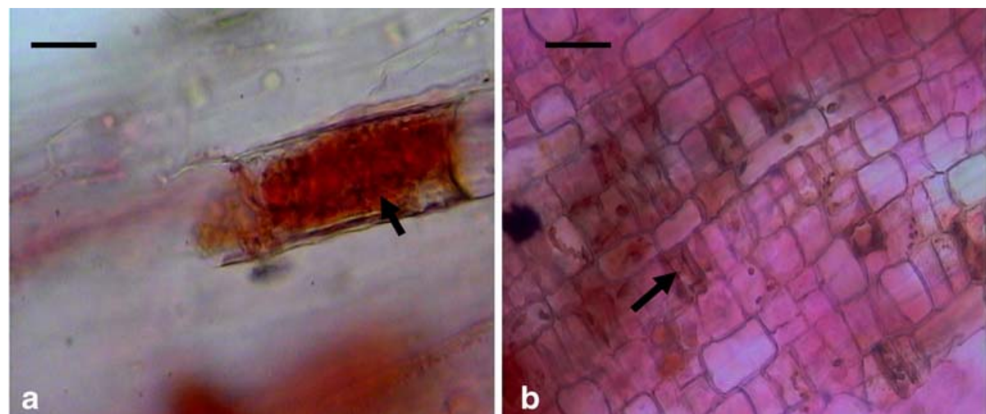

Fig. 1 Dark-septate fungal colonization of root cells of wild *S. involucrata* plants (*bars*; 5 μm). **a** Squash mounts of roots showing microsclerotia characteristic of DSE fungi in root cell. **b** Squash mounts of roots showing darkly pigmented hyphae in root cells

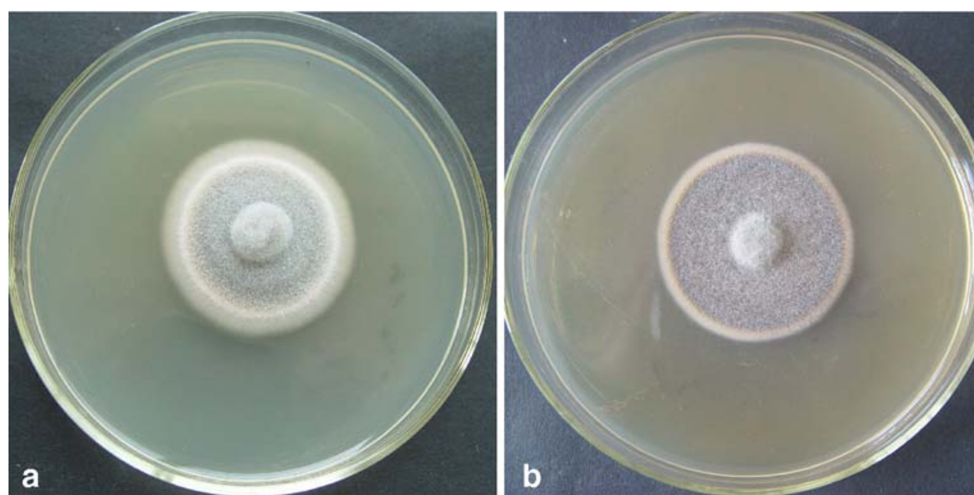

Fig. 2 Two-week-old colonies of fungal endophyte EF-M on PDA (**a**) and MEA (**b**)

submerged lighter-colored margin (Fig. 2a) and light brown on MEA, becoming dark gray with a smooth velvety surface (Fig. 2b). Vegetative hyphae are dark, septate, and about 7.5 μm wide. No conidia formed on any media, only chlamydospores in short chains (Fig. 3). Culturing the isolate over prolonged periods on all media at 5°C in the dark or at 25°C under the light was ineffective for the development of spores.

The inoculated endophyte grew over and into the medium and around the shoot bases and roots, but did not grow up or around the aerial shoots (Fig. 4). After 6 weeks of incubation, EF-M+ plants appeared more vigorous than control plants. Plant height and shoot dry weight increased by approximately 22.7% and 31.25% in the EF-M+ plants compared to controls, but there were no significant effects on plant root development (Table 2 and Fig. 4).

Endophyte EF-M hyphae were hyaline in association with the roots, making them difficult to see in squash mounts of roots. Light microscopy of cross sections of noninoculated *S. involucrata* roots showed that the control plant remained uninfected during the experiment (Fig. 5a). Six weeks after inoculation with EF-M, hyphae grew on the surface of the host roots and penetrated the epidermal cells, but did not advance into the cortex or vascular cylinder (Fig. 5b,c). Occasionally, hyphae formed masses of closely packed, deeply pigmented fungal cells within the regions of the epidermis. Many of these developed into microsclerotia in the epidermal cells, but no structures indicative of a typical mycorrhizal symbiosis were developed (Fig. 5d).

The ITS data set used in this study were selected from sequences previously accessioned for teleomorphic genera or asexually reproducing fungi and also alignable to the sequence for EF-M, except for the unidentified fungus DS16B. The alignments comprised 24 taxa and 437 total characters, of which 273 were parsimony informative. A maximum-parsimony tree of 543 steps was generated, with CI, RI, and RC indices of 0.8048, 0.9211, and 0.7413, respectively (Fig. 6).

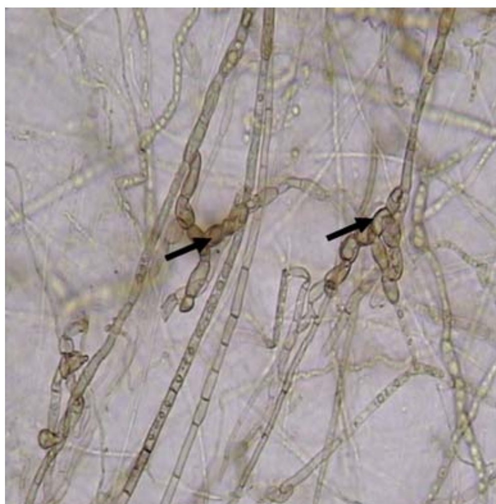

Fig. 3 Light micrograph of fungal endophyte EF-M showing short chains of chlamydospores (*arrows*). ×400

Fig. 4 *S. involucrata* plants at the end of the incubation period. The growth response induced by the endophyte is clearly depicted by the EF-M+ plants (*right*) having the same age of the controls (*left*)

Table 2 Growth of *S. involucrate* under axenic conditions (CK) and in the presence of fungal endophyte EF-M (EF-M+)

Parameter	CK	EF-M+	pH test
Root dry weight (mg)	3.75±0.95	4.50±1.29	NS
Shoot dry weight (mg)	12.00±1.41	15.75±0.96	$P \leq 0.05$
Plant height (cm)	7.33±0.58	9.00±1.00	$P \leq 0.05$
Number of hair root tips	7.00±1.00	8.67±2.08	NS

NS Not significant

The fungi sampled in this study formed a clade comprising three main subclades. In subclade A, *Acephala applanata* and two species of *Tapesia* formed a monophyletic clade with 98% bootstrap support, and in subclade C, species of *Phialocephala* and *Mollisia* formed a strongly supported clade (100%). EF-M formed subclade B with *Pyrenopeziza brassicae*, *Cadophora luteo-olivacea*, two species of *Phialophora*, *Graphium silanum*, *L. orchidicola*, DS16B, two species of *Oculimacula*, and two species of *Helgardia* with a 98% bootstrap support. Within the subclade, EF-M, *L. orchidicola*, and DS16B formed a terminal cluster with moderate support (73%). In particular, EF-M sequences matched the entire ITS region of *L. orchidicola* and DS16B (98–99% identity over 540-bp character alignment). There were only two nucleotide differences in the entire ITS region between DS16B and *L. orchidicola*, and five nucleotide differences between EF-M and *L. orchidicola*.

Discussion

Dark-septate endophytes are common in the roots of arctic and alpine plants over broad host and geographical ranges (Jumpponen and Trappe 1998). Similarly, we observed abundant DSE hyphae on roots of the alpine plant *S. involucrata*. Our isolate had colony morphologies and hyphal structures similar to that of the DSE fungi described by Jumpponen and Trappe (1998). The fungus produced no conidia or other reproductive structures in the culture media

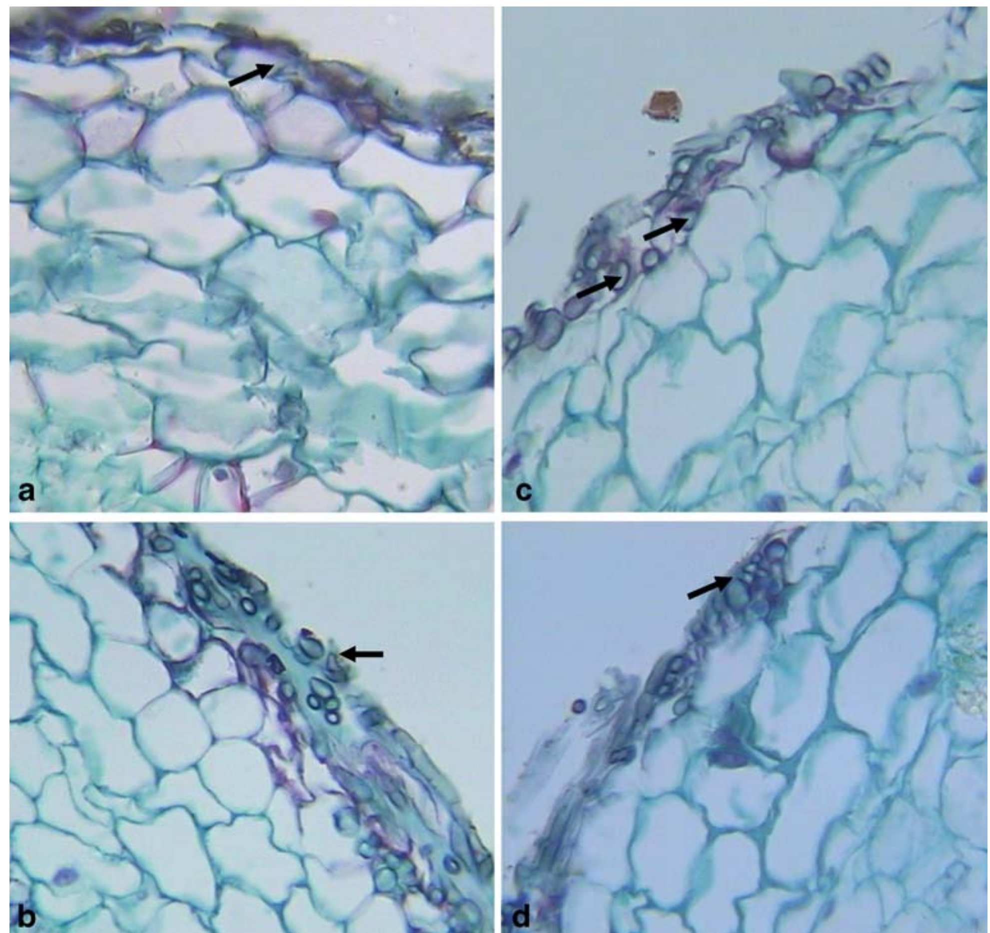

Fig. 5 Light micrographs of interactions between root of *S. involucrata* and EF-M. **a** Transverse section of *S. involucrata* root (noninoculated control) from the axenic cultures showing epidermal cell (*arrow*). **b** Transverse section showing epidermal cells with a layer of hyphae (*arrow*) developing on the surface. **c** Transverse section showing EF-M hyphal penetration of the epidermal cells (*arrow*), and fungal colonization was restricted to the epidermis. **d** Transverse section showing microsclerotia forming within the cells of the epidermis (*arrow*). ×800

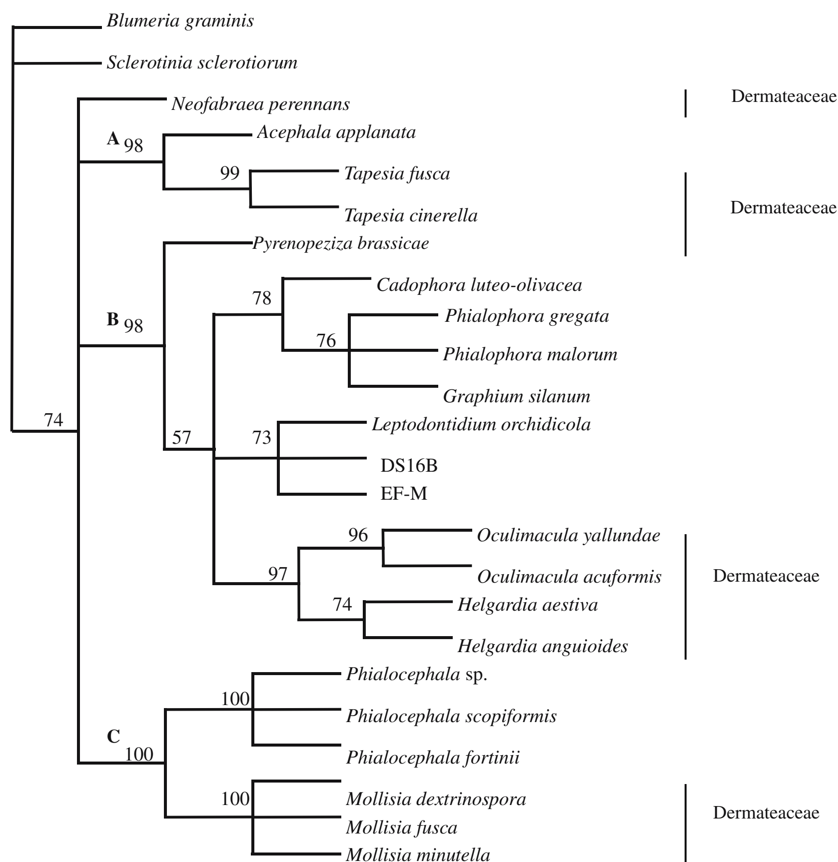

Fig. 6 Maximum-parsimony tree generated from the 5.8S and ITS (ITS1 and ITS2) sequences of 24 taxa showing the relationships of EF-M with reference taxa. The tree was rooted with *B. graminis* and *S. sclerotiorum* (tree length=543, CI=0.8048, RI=0.9211, RC=0.7413). The numbers at branches indicate the percentages of trees from 1,000 bootstrap replications in which the branch occurs

even under different conditions to promote sporulation. Colonies generally became black with age, which is similar to the descriptions of most anamorph species of *Leptodontidium* (de Hoog 1977). Minor differences in morphology between the isolate EF-M and species in *Leptodontidium* were detectable at the colony margins. The cultural variant may reflect an adaptation to the abiotic factors in the different ecosystems where host plants thrive or represent a different taxon (Hambleton and Currah 1997).

Only the epidermal cells of inoculated seedlings were colonized by EF-M, both inter- and intra-cellularly. Host tissue was left largely undamaged. Occasionally, hyphae formed masses of closely packed, deeply pigmented fungal cells within regions of the epidermis, many developing into microsclerotia. Currah and Tsuneda (1993) suggested that sloughed-off cortical cells filled with intracellular-melanized hyphal masses may act as dispersal propagules for these fungi. Such somatic features could at least partly compensate the lack of reproductive structures for dispersal and survival in these fungi.

Seedlings of *S. involucrata*, inoculated with EF-M, appeared healthy with no external symptoms of disease, which could be an indication of compatibility in the association. The dry weights of inoculated *S. involucrata* seedlings increased significantly compared to controls. It is possible that DSE fungi possess a wide range of enzymes that allow for efficient utilization of organic matter (Currah and Tsuneda 1993; Caldwell et al. 2000), thereby making available nutrients that promote plant growth. Kohara et al. (1993) obtained a significant correlation between the cellulose- and starch-degrading activity of certain sterile PGPF isolates and plant-growth promotion. Alternatively, production of a diffusible growth-regulating substance may be taking place. IAA has been identified in culture filtrates from *H. ericae* strains (Gay and Debaud 1986) and in a *Phialophora* sp. (Rommert et al. 2002). Rommert et al. (2002) reported a positive effect on the growth of *Larix decidua* plants and an increase in root system branching when either an extract of *Phialophora* sp. was applied or the fungus colonized the roots.

Based on the phylogenetic analysis and sequence similarity comparison of the ITS regions, a high level of similarity in the ITS and 5.8S sequences between EF-M, *L. orchidicola*, and DS16B was observed. These results suggest that isolate EF-M was congeneric to the genus *Leptodontidium*, and the fungal taxa examined appeared to be affiliated with the Dermateaceae, with findings consistent with those of Piercey et al. (2004). *L. orchidicola* is one of at least four taxa known among fungi comprising the *Mycelium radicis atrovirens* (Melin 1921) complex. This

dematiaceous hyphomycete was isolated from the roots of terrestrial orchids (Currah et al. 1987, 1990) and other plants growing in alpine and subalpine habitats with humus-rich soils (Fernando and Currah 1995). In axenic culture, *L. orchidicola* formed associations with roots of common alpine–subalpine plants without causing any inhibition of root elongation or having apparent effect on seedling growth and survival (Fernando and Currah 1995). No indication of pathogenicity has been observed with orchids in nature (Currah et al. 1990). However, in axenic culture with *Salix glauca* seedlings, *L. orchidicola* increased root length but also invaded the stele, causing extensive cellular lysis. In pot inoculation with *Potentilla fruticosa*, *Dryas octopetala*, *S. glauca*, and *Picea glauca* seedlings, the effects of four *L. orchidicola* strains on host dry weight were strain and host specific. *L. orchidicola* formed a range of symbiotic associations that could be considered mycorrhizal to parasitic. Resynthesis experiments by Fernando and Currah (1996) demonstrated that the effects of *L. orchidicola* varied according to cultural conditions. Under our resynthesis conditions, colonization of epidermal cells by *S. involucrata* led to a significant increase in shoot height and biomass, thus suggesting a beneficial association.

The associations between host plants and fungal endophytes can vary from mutualistic to pathogenic even within the same host species depending on experimental conditions (Jumpponen and Trappe 1998). To confirm the nature of associations between *S. involucrata* and EF-M, further resynthesis work is necessary under different experimental conditions.

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