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Effects of a Dark-Septate Endophytic Isolate LBF-2 on the Medicinal Plant *Lycium barbarum* L.

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Dark septate endophytes (DSE) are ubiquitous root associated fungi; however, our understanding of their ecological function remains unclear. Here, we investigated the positive effect of a DSE fungus on its host plant *Lycium barbarum* L. A DSE isolate, LBF-2, isolated from the roots of *L. barbarum*, was inoculated onto the roots of plants, which were grown under greenhouse conditions for five weeks. The result of molecular analyses of internal transcribed spacer regions indicated that LBF-2 was 96% similar to *Paraphoma chrysanthemicola*. Melanized septate hyphae were observed in the root cortical cells of *L. barbarum* using a light microscope. Inoculation with LBF-2 increased the total biomass by 39.2% and also enhanced chlorophyll fluorescence. Inoculation increased the concentration of total chlorophyll by 22.8% and of chlorophyll *a* by 21.3%, relative to uninoculated controls. These data indicate that the LBF-2 isolate might be used to facilitate the cultivation of *L. barbarum*, which has medicinal applications.

***Keywords*:** chlorophyll fluorescence, dark septate endophyte, *Lycium barbarum*, medicinal plant

Introduction

In natural ecosystems, the roots of herbaceous plants almost host arbuscular mycorrhizal (AM) and dark septate endophytic (DSE) fungi (Jumpponen and Trappe, 1998; Mandyam and Jumpponen, 2005). DSE are ascomycetes with melanized septate hyphae that are found in a wide range of ecosystems such as alpine, polar, desert, and temperate habitats (Jumpponen and Trappe, 1998). DSE are actually a "form taxon" that includes many different species such as *Phialocephala fortinii*, *Heteroconium chaetospira*, *Leptodontidium orchidicola* that are often only loosely related (Schmidt *et al.*, 2008; Porras-Alfaro *et al.*, 2008; Newsham *et al.*, 2009; Wu *et al.*, 2010; Yuan *et al.*, 2010). Compared with the massive literature on AM fungi, research on DSE symbioses is limited. The effects of DSE fungi on plants are much less understood and are currently the subject of much debate in the scientific community (Jumpponen and Trappe, 1998; Mandyam and Jumpponen, 2005; Grünig *et al.*, 2008; Newsham, 2011).

Previous publications either have recorded positive, neutral or negative responses to inoculation with DSE fungi (Jumpponen, 2001; Mandyam and Jumpponen, 2005; Wu and Guo, 2008; Alberton *et al.*, 2010). While DSE fungi were found to negatively affect survival and biomass of *Pinus* spp., *Betula alleghaniensis*, and *Menziesia ferruginea* (e.g. Wilcox and Wang, 1987; Stoyke and Currah, 1993), DSE had positive effects on the height and biomass of *Carex* spp. (Haselwandter and Read, 1982), *Vulpia ciliata* ssp. *ambigua* (Newsham, 1999), *Rhododendron* cv. Azurro (Vohník *et al.*, 2005), and *Saussurea involucrata* Kar. et Kir (Wu and Guo, 2008), the rutin content of *S. involucrata* (Wu *et al.*, 2010), and the fruit yield and quality of *Solanum lycopersicum* L. (Andrade-inares *et al.*, 2011). In order to reach a consensus for how plants respond to inoculation with DSE, a meta-analysis was conducted on 18 independent studies. This analysis indicated that, on average, DSE inoculation increases total, shoot and root biomass, and shoots nitrogen, and phosphorus contents of plants by 26–103%, relative to uninoculated control plants (Newsham, 2011). Recently, the results of Li *et al.* (2011) also indicated that DSE isolate H93 can promote the growth of maize (*Zea mays* L.) exposed to stressful heavy metalconditions.

Here, we examined the functional relationship between the DSE and *Lycium barbarum* L. (Solanaceae), a deciduous perennial frequently used in northeast Asia, southeastern Europe and the Mediterranean basin for its significant biological activities, such as the inhibition of cancer cell proliferation and enhancement of immune responses (Lu and Wang, 2003; Zhang *et al.*, 2005; Yin and Dang, 2008). The objective of the present study was, therefore, to evaluate the interaction between the DSE isolate LBF-2 and *L. barbarum*. Specifically, we aimed (1) to detect the distribution of LBF-2 in the tissues of *L. barbarum* seedlings (2) to evaluate the effects of LBF-2 isolate on the growth, chlorophyll concentration, and chlorophyll fluorescence of *L. barbarum* seedlings, thus providing an experimental evaluation of the practical application of DSE fungi in cultivation of *L. barbarum* for medicinal purposes.

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Materials and Methods

Plant and fungal materials

L. barbarum cv Ningqi No.1 is a cultivated variety of *L. barbarum* with particularly strong drought and salt resistance, and thus it is widely distributed in arid and semi-arid regions of Northwest China, primarily in the Ningxia Hui Autonomous Region (Gu *et al.*, 2007; Zheng *et al.*, 2010). Following the method of Silvani *et al.* (2008) with slight modification, we initiated the isolation process for obtaining endophytic fungi from the roots within 48 h of their collection. We maintained the isolates on potato dextrose agar (PDA) medium in the dark at *c.* 25°C and deposited the representative isolates in a microbial laboratory at the College of Forestry, Northwest A&F University, China. A preliminary study confirmed that the LBF-2 isolate had a positive influence on *L. barbarum* development, and it was therefore chosen for the following investigations.

Morphological and molecular identification of LBF-2

The LBF-2 isolate was identified through a combination of morphological and molecular sequencing techniques. We placed the isolate on Modified Melin Norkrans (MMN) agar medium (Marx, 1969) and incubated it at *c.* 25°C in the dark for two weeks, after which the morphology of hyphae was observed under a light microscope (Olympus BX51, Japan) and by scanning electron microscopy (JSM-6360LV, JSM, Japan). For molecular identification of LBF-2, we extracted DNA from 1.0 g fresh mycelium of LBF-2 using the CTAB method (Doyle and Doyle, 1987) and amplified the internal transcribed spacer (ITS) region of the nuclear rDNA repeat using the ITS-1/ITS-4 primer set (White *et al.*, 1990). Next, we purified the PCR product using a DP 209 kit (Tiangen, China) and immediately cloned it using a DH5α cloning kit with a pGM-T vector (Tiangen). Reconfirmed clones were sequenced by a DNA Sequence Analyzer (3730 XL, ABI, USA) at Sangon Biotech Co., Ltd. (China). We then compared the obtained sequence to the available sequences in the database of the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool. We deposited the sequence in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/LAST; accession number: HM242215). We aligned the rDNA ITS region sequences using Megalign program of the Lasergene Package (Version 4, DNA Star Inc., USA), and constructed a phylogenetic tree based on ITS sequences of LBF-2 and other ITS data using the neighbour-joining method in PAUP*4.0 b10. Bootstrapping was performed with 1000 replications (Thompson *et al.*, 1994).

Inoculation with LBF-2

To more fully explore the effects of the DSE isolate LBF-2 on the performance of *L. barbarum*, we inoculated seedlings of the host plants with the isolate LBF-2. We collected seeds of *L. barbarum* cv Ningqi No.1 in October 2009 from the Ningxia Academy of Agriculture and Forestry Sciences and sterilized their surfaces with 10% (v/v) H_2O_2 for 10 min, followed by a gentle wash with deionized water at room temperature, and put them on a sterilized moist filter paper to trigger germination. The well-growing seedlings were transferred to pots.

Using the method described by Wu and Guo (2008), with modification, we added 4.5 mm diameter disks containing the mycelium obtained from the MMN medium plates onto the rhizosphere of selected seedlings. Controls consisted of uninoculated MMN medium added to the rhizosphere of seedlings. The experiment consisted of a randomized design with twice three replicates for the control and for the inoculum treatment. Each pot contained five seedlings (n=30, control and inoculated treatment) growing in autoclave-sterilized nursery substrate: vermiculite (10:1, v/v), with a pH of 6.5, and organic carbon, with available nitrogen and phosphorus concentrations of 108 g/kg, 191 mg/kg and 190 mg/kg, respectively. Seedlings were grown under greenhouse conditions with 12 h of light per day at 28°C, and substrate moisture was constantly kept at 60–70% (w/w) by irrigating each pot with 50 ml sterile Hoagland's nutrient solution every two weeks (Hoagland and Arnon, 1950).

DSE colonization

Root samples were cleared and stained by the methods described by Barrow and Aaltonen (2001) and modified for microscopic observation. Six plants per treatment were randomly selected and samples from different parts of the root system were examined. Thirty five root fragments per sample were mounted on glass slides and observed under a microscope with a digital sensor (Olympus). Picture showing of melanized septate hyphae was managed by Image-Pro Express 6.0 analyses software (Olympus).

Plant growth responses to LBF-2 inoculation

To evaluate the effects of LBF-2 on the growth performance of *L. barbarum*, we carefully removed the seedlings from their substrate 35 d after inoculation. Six plants per treatment were randomly selected for assessment. Roots were washed with tap water and then distilled water. The numbers of leaves and root tips, root length and shoot length were then measured. Seedlings from the six pots in each treatments were subsequently dried at 70°C for 48 h to measure dry weight (AUW220, Shimadzu, Japan).

Chlorophyll fluorescence and chlorophyll concentration

To determine the response of the photosynthetic system to inoculation with LBF-2, we measured chlorophyll fluorescence using a portable Chlorophyll Fluorometer (Mini PAM, Germany). We used standard instrument settings (saturating pulse of 12,000 mmol m^{-2} sec^{-1} for 0.8 sec) with additional far red light with a wavelength of 735 nm to enable estimation of ground state fluorescence (Walz, 1993). We measured chlorophyll fluorescence on four fully expanded leaves of six plants per treatments, still attached to the plants, after putting them in darkness for 30 min (Sheng *et al.*, 2008). We measured non-photochemical fluorescence quenching (NPQ), ground fluorescence (F0'), non-photochemical quenching (qN), photochemical quenching (qP) and Y(II), because these parameters are highly sensitive to the presence of AMF and DSE endophytes (Sheng *et al.*, 2008; Wu *et al.*, 2010). For chlorophyll concentration quan-

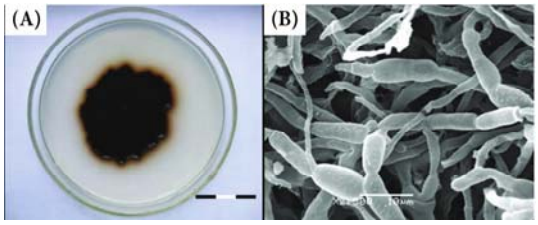

Fig. 1. (A) Growth form of LBF-2 on MMN medium at two weeks. Diameter of Petri dish is 90 mm. (B) Morphology of hyphae viewed under a scanning electron microscopy. Bars in (A) and (B) are 30 mm and 10 μm, respectively.

tification, we added leaf tissue (0.2 g fwt) to 5 ml of a 1:1 mixture of acetone to ethanol and incubated the extracts at 4°C in the dark until the leaves had lost their pigmentation. We measured chlorophyll *a* and *b* concentrations as functions of absorbance at 663 and 645 nm wavelengths with a spectrophotometer (UVmini-1240, Japan) using the equations given by Porra (2002).

Statistical analyses

Biomass, the number of leaves and root tips, height, and chlorophyll concentrations, chlorophyll fluorescence parameters were compared between inoculated and uninoculated seedlings using Student's *t*-test. Statistical analyses were carried out with JMP 9.0 (SAS Institute Inc., USA).

Results

Morphological and molecular identification of LBF-2

LBF-2 grew at *c*. 20 mm week^{-1} on MMN medium. The colony was dark (Fig. 1A). Scanning electron microscopy showed LBF-2 to have trabeculate hyphae with diameter about 4μm (Fig. 1B). The light microscopy showed the LBF-2 to have melanized septate hyphae (Fig. 2A). LBF-2 did not produce pycnidia. Sequencing of the ITS regions of rDNA indicated that LBF-2 is a member of the phylum Ascomycota and that the isolate had 96% similarity to the GenBank sequence of *Paraphoma chrysanthemicola* FJ426984, FJ426985 (Fig. 3).

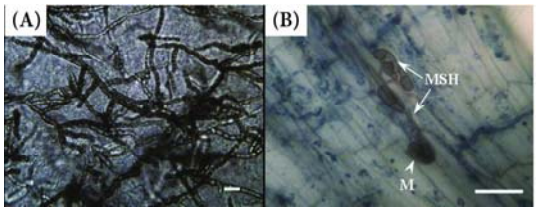

Fig. 2. (A) Morphology of LBF-2 hyphae viewed under a light microscope. (B) Light micrograph of LBF-2 colonized in the root of *L. barbarum* Ningqi No.1 seedlings. MSH and M represented melanized septate hyphae and microsclerotia, respectively. Bars in (A) and (B) are 20 μm and 50 μm, respectively.

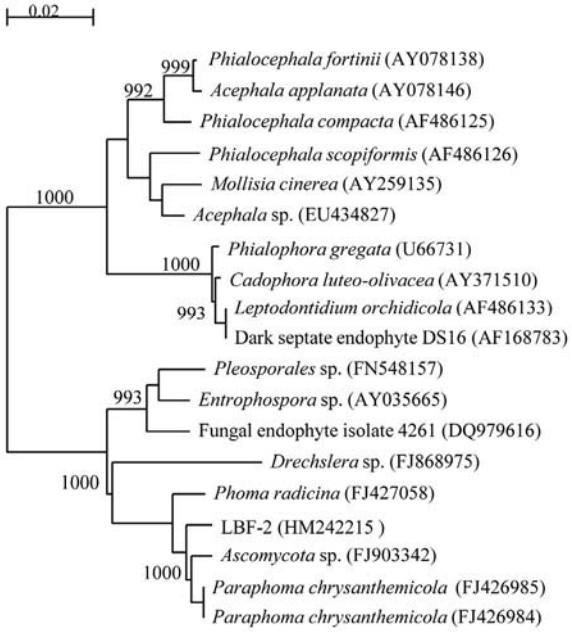

Fig. 3. Phylogenetic tree based on ITS sequences of LBF-2 and other DSE isolates, as assessed by the neighbour-joining method. Numbers at nodes indicate bootstrap values. Scale bar denotes genetic distance.

Colonization and distribution of LBF-2 in the tissue of *L. barbarum*

LBF-2 produced melanized septate hyphae (MSH) and microsclerotia (M) in root cortical cells of inoculated *L. barbarum* seedlings, and the percent root length colonization was 81.2% (Fig. 2B). The uninoculated control seedlings were not infected.

Effect of DSE isolate on the growth performance

We found that inoculation with LBF-2 had beneficial effects on *L. barbarum* seedling performance. Inoculated *L. barbarum* seedlings were 39.2% heavier than uninoculated seedlings ($P<0.05$; Fig. 4A). Inoculation with LBF-2 had no effect on root length ($P>0.05$), but increased plant height by 43.7% ($P<0.001$; Fig. 4B). Inoculation with LBF-2 also increased the number of root tips by 1.2 times ($P<0.001$) and the number of leaves by 21.5% ($P<0.05$; Fig. 4C).

Effect of DSE isolate on photosynthesis

Inoculation with LBF-2 had beneficial effects on the photosynthesis of *L. barbarum* Ningqi No.1 seedlings. We recorded 21.3% and 22.8% increases in chlorophyll *a* ($P<0.001$) and chlorophyll *a*+*b* ($P<0.05$) concentrations in inoculated plants, compared with uninoculated controls (Fig. 5A). No effects were found on chlorophyll *b* ($P>0.05$) concentrations or chlorophyll *a*/*b* ratio (data not shown). There were no significant differences in the chlorophyll fluorescence kinetics parameters F0', qN and NPQ between the control and the LBF-2-inoculated seedlings (all $P>0.05$), but Y(II) and qP were both significantly higher in inoculated seedlings than in uninoculated controls (both $P<0.001$; Fig. 5B).

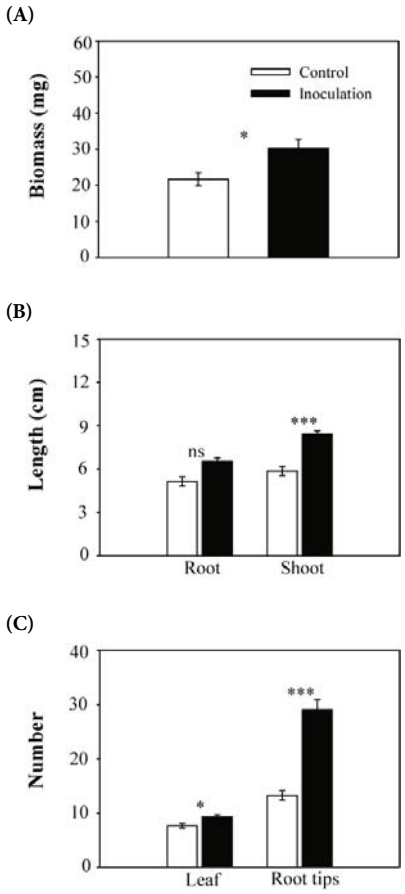

Fig. 4. The effects of inoculation with LBF-2 on (A) biomass, (B) root length and shoot height, and (C) numbers of leaves and root tips of *L. barbarum* Ningqi No.1 seedlings. Values are means (n=6) and vertical bars are standard errors (S.E). Values for inoculated plants that are significantly different from control values are denoted by **P*<0.05 and ****P*<0.001.

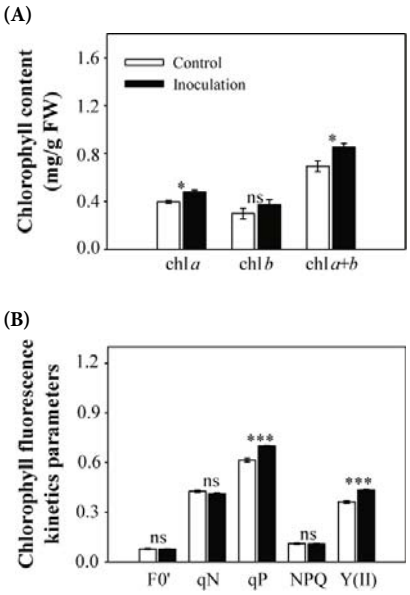

Fig. 5. The effects of inoculation with LBF-2 on (A) the chlorophyll concentration and (B) chlorophyll fluorescence kinetic parameters of *L. barbarum* Ningqi No.1 seedlings. FW represented fresh weight. Values are means (n=6) and vertical bars are standard errors (S.E). Values for inoculated plants that are significantly different from control values are denoted by **P*<0.05 and ****P*<0.001, ns represents no significant difference.

Discussion

Most medicinal plants host dark septate endophytic (DSE) and mutualistic arbuscular mycorrhizal (AM) fungi (Muthukumar *et al.*, 2006; Zubek and Błaszkowski, 2009; Zhang *et al.*, 2010), both of which have significant effects on plant growth performance (Smith and Read, 2008; Newsham, 2011). Whereas AM fungi are known to have beneficial effects on the photosynthetic capacity and secondary metabolite production of medicinal plants (Abu-Zeyad *et al.*, 1999; Copetta *et al.*, 2006), knowledge of how these plants respond to DSE fungi is presently limited. In the present study, we explored the effects of a DSE fungal isolate LBF-2 on the growth of its host medicinal plant *L. barbarum* under controlled conditions.

Melanized septate hyphae, microsclerotia and hyaline hyphae were the typical structures of DSE (Barrow, 2003; Mandyam and Jumpponen, 2005). In this work, melanized septate hyphae were observed in the roots of *L. barbarum*. The increase in total biomass, height and numbers of root tips of *L. barbarum* caused by inoculation with LBF-2 is in accordance with previous studies finding plant biomass to increase following inoculation with DSE. For example, Wu and Guo (2008), Wu *et al.* (2010) found that the DSE isolate congeneric with *Mycocentrospora* had positive effects on the growth of the medicinal plant *Saussurea involucrata*. Inoculation with *Phialophora graminicola* and *Phoma fimeti* was similarly shown to enhance the root and shoot biomass, root length and tiller number of the winter annual grass *Vulpia ciliata* spp. *ambigua* (Newsham, 1994; 1999). Recently, Andrade- Linares *et al.* (2011) investigated the effects of three DSE isolates on the growth performance of *Solanum lycopersicum* and showed a doubling of fruit biomass, a 17% increase in fruit glucose content, and a reduced of the pathogen *Verticillium dahliae*.

At present, the positive growth responses of plants to DSE remain largely unexplained. Some researchers have suggested that DSE may, for example, enhance protection from soil pathogens, or increase the synthesis of phytohormones (Mandyam and Jumpponen, 2005; Oelmüller *et al.*, 2009; Andrade-Linares *et al.*, 2011). Others suggest that these fungi may synthesize proteolytic enzymes for mineralizing organic nitrogen (amino acids, peptides, and proteins) in the host rhizosphere, thus enhancing nitrogen (N) uptake. Upson *et al.* (2009) showed that positive growth responses to a range of DSE isolates were only observed when plants were grown in a substrate amended with organic nitrogen. More recently, a meta-analysis has similarly indicated that inoculation with DSE increases total, shoot and root biomass by 48–185% when plants are not supplied with inorganic N in nutrient solutions or growth media, or when all, or the majority, of N is supplied in organic form (Newsham, 2011). Further work is needed to explore the specific mechanisms underlying the beneficial effects of DSE fungi on plant

performance.

The increases in chlorophyll concentration and qP and Y(II) we found here suggest that inoculation with DSE increases the efficiency of PSII photochemistry. qP and Y(II) have been used as photochemical quenching parameters for monitoring the general trends in the energy dissipation adjustments in photosystem II (PSII) (Maxwell and Johnson, 2000; Sheng *et al*., 2008). The increase in the glucose content of fruits (Andrade-Linares *et al*., 2011) is also consistent with higher photosynthetic efficiency. However, in contrast to the data here, a previous study found that chlorophyll fluorescence parameters of *S. involucrata* were unresponsive to inoculation with EF-37, a DSE isolate (Wu *et al*., 2010). The most probable explanation is that different DSE fungal species and plant host species have different biological characteristics and responses.

The majority of DSE isolates belong to the Ascomycota (Jumpponen and Trappe, 1998; Newsham, 2011). In this work, LBF-2 did not produce any reproductive structures. According to the ITS sequence, LBF-2 belongs to the genus *Paraphoma*. DSEs belonging to *Paraphoma* are not widely reported in the literature; most belong to *Phialocephala*, *Cadophora*, *Cryptosporiopsis*, *Heteroconium*, and *Leptodontidium* (de Hoog, 1977; Jumpponen and Trappe, 1998; Rommert *et al*., 2002). Although several studies report *Phoma* sp. to be pathogens of plants (Garibaldi *et al*., 2007; Ni *et al*., 2010), *Phoma fimeti* has been found to form dark septate hyphae in the root cells of the winter annual grass *Vulpia ciliata* spp. *ambigua* (Newsham, 1994), but forming an apparent commensal relationship. Shivanna *et al*. (1996) also suggested that plant growth-promoting fungal isolates of *Phoma* spp. GS6-1 and GS7-4 from zoysia grass (*Zoysia* sp.) rhizosphere suppressed *Bipolaris sorokiniana*, a pathogenic fungus, in roots of wheat, due to the competitive root colonization in the greenhouse.

Our finding that LBF-2 has positive effects on *L. barbarum* is consistent with a similar study by Wu *et al*. (2010), who found that the DSE isolate EF-37 increased the growth of the endangered medicinal plant *Saussurea involucrata*. However, it is not yet known if the positive physiological response to LBF-2 inoculation observed in *L. barbarum* Ningqi No.1 also occurs in other cultivars of *L. barbarum*. Furthermore, although the responses of *L. barbarum* to inoculation with LBF-2 were examined in this work, field experiments are also worthy of further study.

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