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ORIGINAL PAPER

The promoting role of an isolate of dark-septate fungus on its host plant *Saussurea involucrata* Kar. et Kir.

Li-qin Wu · Ya-li Lv · Zhi-xia Meng · Juan Chen · Shun-Xing Guo

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Abstract A dark-septate endophytic (DSE) fungus EF-37 was isolated from the roots of *Saussurea involucrata* Kar. et Kir., an endangered Chinese medicinal plant. The molecular identification of the fungus was based on internal transcribed spacer regions and the result showed that EF-37 was congeneric to *Mycocentrospora*. This study was conducted to clarify the influence of the root endophyte EF-37 on the host plant *S. involucrata* using material grown in a sterile culture bottle. After cultivation for 40 days, fungal hyphae were found to be branching repeatedly and forming "hyphae nets" in the epidermal layers. Significant differences were detected between the study groups in plant dry weight, plant height, root dry weight, shoot dry weight, and the number of hair root tips. There was a positive effect of endophyte EF-37 on plant root development, with results showing that cortical cells dissolved and formed aerate structures. There was a positive effect of endophyte EF-37 on plant growth, but chlorophyll fluorescence analysis showed that there were no significant differences between the study groups. In addition, analysis of the chemical composition of seedlings showed that the level of rutin was higher in plants cultivated with the EF-37 fungus compared to the controls. This study helps to establish a basis for germplasm conservation and for further investigation of the interaction between dark-septate fungi and this alpine plant.

Keywords Dark-septate endophytic (DSE) fungus · *Saussurea involucrata* · Molecular identification · Microscope observation · Chlorophyll fluorescence analysis · HPLC analysis

Introduction

Saussurea involucrata Kar. et Kir., popularly known as snow lotus, grows in wild, rocky habitats within the alpine zone of the Tianshan, A'er Tai, and Kunlun mountain ranges of China at elevations of 2,600 m or higher. *S. involucrata* is an endangered medicinal plant and was listed as a second-grade national protected wild plant in China (Fu 1992). *S. involucrata* is a perennial herbaceous plant of Asteraceae and is used to treat rheumatoid arthritis, cough due to lung infection, impotence, and other ailments (Li and Zhao 1989; Guo et al. 2007). *S. involucrata* is now becoming a new variety of traditional Chinese medicine for use in anti-inflammatory analgesia, antiaging, and termination of early pregnancy (Li and Cai 1998). These medical applications are most likely attributable to its secondary metabolites, flavonoids, particularly 4′,5,7-trihydroxy-3′,6-dimethoxy flavone (jaceosidin) and 4′,5,7-trihydroxy-6-methoxy flavone (hispidulin; Zhao et al. 1998; Fu et al. 2005). These compounds also have anticancer (Liu et al. 1985; Woerdenbag et al. 1994) and

Li-qin Wu and Ya-li Lv contributed equally to this work.

L.-q. Wu · Y.-l. Lv · Z.-x. Meng · J. Chen · S.-X. Guo (✉)
Institute of Medicinal Plant Development,
Chinese Academy of Medical Sciences and Peking Union Medical College,
No. 151, Malianwa North Road, Haidian District,
Beijing 100193, People's Republic of China
e-mail: sxguo2006@yahoo.com.cn

L.-q. Wu
e-mail: wulq1999@yahoo.com.cn

Y.-l. Lv
e-mail: lvyali1978@sina.com

anti-inflammatory activities (Tan et al. 1999; Zhao et al. 2003). Therefore, it is one of the highest priority species for conservation.

Dark-septate root endophytic (DSE) fungi are conidial or sterile ascomycetous fungi that colonize living plant roots without causing apparent negative effects such as tissue disorganization (Jumpponen and Trappe 1998a). The fungi that live in the intracellular or extracellular parts of the root have darkly pigmented and septate hyphae and are also defined as DSE fungus (Jumpponen 2001). They are interrelated with many plants ranging from the tropics to the arctic and are particularly common in habitats under stress such as alpine environments (Read and Haselwandter 1981). Although the function of DSE fungi remains unclear (Jumpponen and Trappe 1998a), some have been found to be beneficial to host growth and development (Fernando and Currah 1996; Shivanna et al. 1994; Jumpponen et al. 1998; Wu and Guo 2008).

In our present work, among 28 endophytic strains isolated from surface-sterilized roots, nine were found to be DSE fungi. We investigated if the DSE fungus EF-37 was able to promote growth of *S. involucrata*.

The objective of this research study was to measure the influence of the endophyte on *S. involucrata*, including morphological responses, growth effect, and chemical compositions. Results from this study will help refine the basis for germplasm conservation and for further investigation of the interaction between DSE fungi and alpine plants.

Materials and methods

Surface sterilization and isolation of endophyte

S. involucrata Kar. et Kir. was collected from Tianshan Mountain, Xinjiang Uygur Autonomous Region, People's Republic of China. Roots were washed in deionized water, sterilized in 70% ethanol for 50 s, 0.1% $HgCl_2$ for 7 min, rinsed three times in deionized water for surface sterilization, and placed in 90-mm Petri dishes containing potato dextrose agar (PDA). The Petri dishes were sealed with a sealing film and incubated at 25°C. Different mycelia growing out of the roots were subcultured and maintained on PDA individually. Colony morphology and growth of the isolates were studied on PDA. Growth and spore formation were also examined on PDA, oatmeal agar, and cornmeal agar. The isolate strains were deposited at the Institute of Medical Science and Peking Union Medical College where they were maintained at low temperatures (4–5°C). The DSE fungus EF-37 was chosen for further investigation.

Molecular identification of DSE fungus EF-37

DNA was extracted using an epiphyte DNA extracting kit (Sangon). The internal transcribed spacer (ITS) region was amplified using primers ITS1 and ITS4 (White et al. 1990). Polymerase chain reaction (PCR) was performed in 50 μL reaction volumes containing 2 μL genomic DNA, 0.3 μM of each primer, 200 μM of each deoxynucleotide triphosphate, 5 μL 10× PCR buffer (containing $MgCl_2$), and 2 U of *Taq* DNA polymerase (Sangon). PCR was performed in a Minicycler™ (MJ Research, Reno, NV, USA) with the following program: 3 min initial denaturation at 95°C, followed by 35 cycles of 1 min denaturation at 94°C, 50 s primers annealing at 52°C, 1 min extension at 72°C, and a final 7 min extension at 72°C. PCR products were analyzed in 1% agarose gels (mixed with goldview) by electrophoresis and visualized under UV light. After purification and sequencing, the sequence was compiled and deposited in the GenBank with the accession number FJ843591. The ITS sequences were used to retrieve similar sequences from the GenBank using the NCBI BLAST program (Altschul et al. 1997). The sequences alignments and maximum parsimony analysis were carried out using Clustal X (1.83). Bootstrap percentages used to assess the support for the branching topologies were calculated using PAUP* 4.0b8a (Swofford 1998). Bootstrap analysis (Felsenstein 1985) was performed with 1,000 replications. The maximum parsimony tree was drawn using Treeview (Page 1996).

Plant material and cultivation

Seeds of *S. involucrata* were surface sterilized in 70% ethanol for 50 s, 0.1% $HgCl_2$ for 7 min, and rinsed three times in deionized water. The seeds of *S. involucrata* were

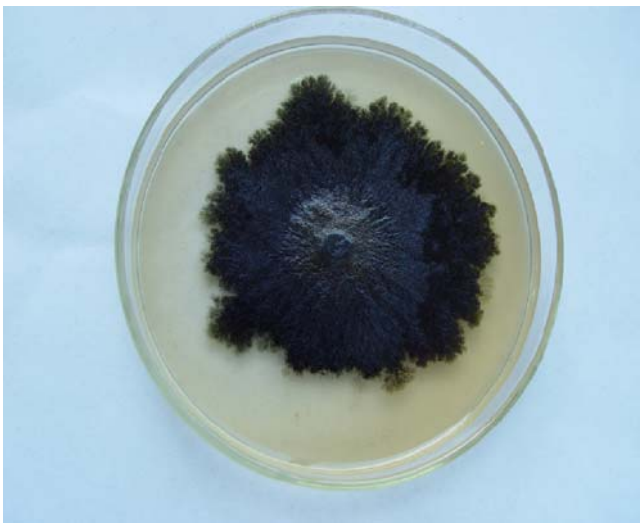

Fig. 1 One-week-old colonies of fungal endophyte EF-37 on PDA

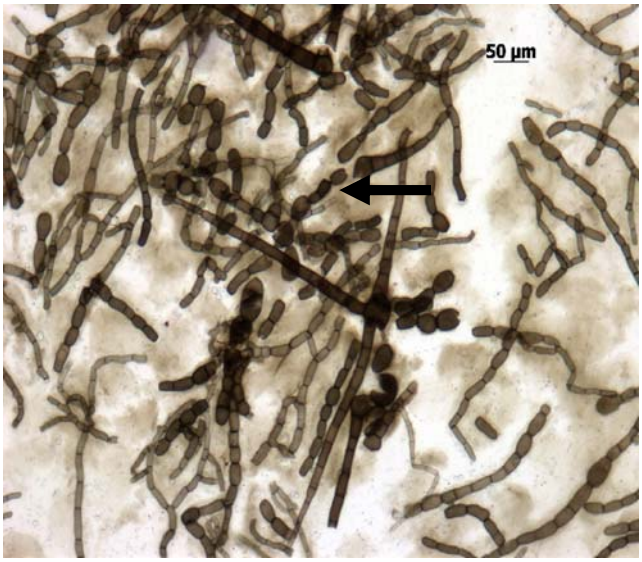

Fig. 2 Chlamydospores (*arrow*) of fungal endophyte EF-37 in light micrograph

then aseptically planted onto Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 0.8% (*w*/*v*) agar in culture bottles. Seedlings were grown for 3 weeks before fungi inoculation. Then the seedlings were aseptically transplanted into the sterile chambers containing 50 ml vermiculite–robur leaves (10:1, *v*/*v*) and 10 ml MS liquid medium. Inoculum of EF-37 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. All cultures were carried out in a growth chamber at 25°C with a 12-h photoperiod.

Interaction studies

After cultivation for 40 days, the plants were harvested, the holder rinsed, and air dried. Plant height and total number of hair root tips were measured for each plant, and then the shoots of 50 plants and roots of 20 plants from each treatment were parched at 50°C and weighed. Fresh roots of ten plants were fixed in formalin–acetic acid–alcohol (FAA). After

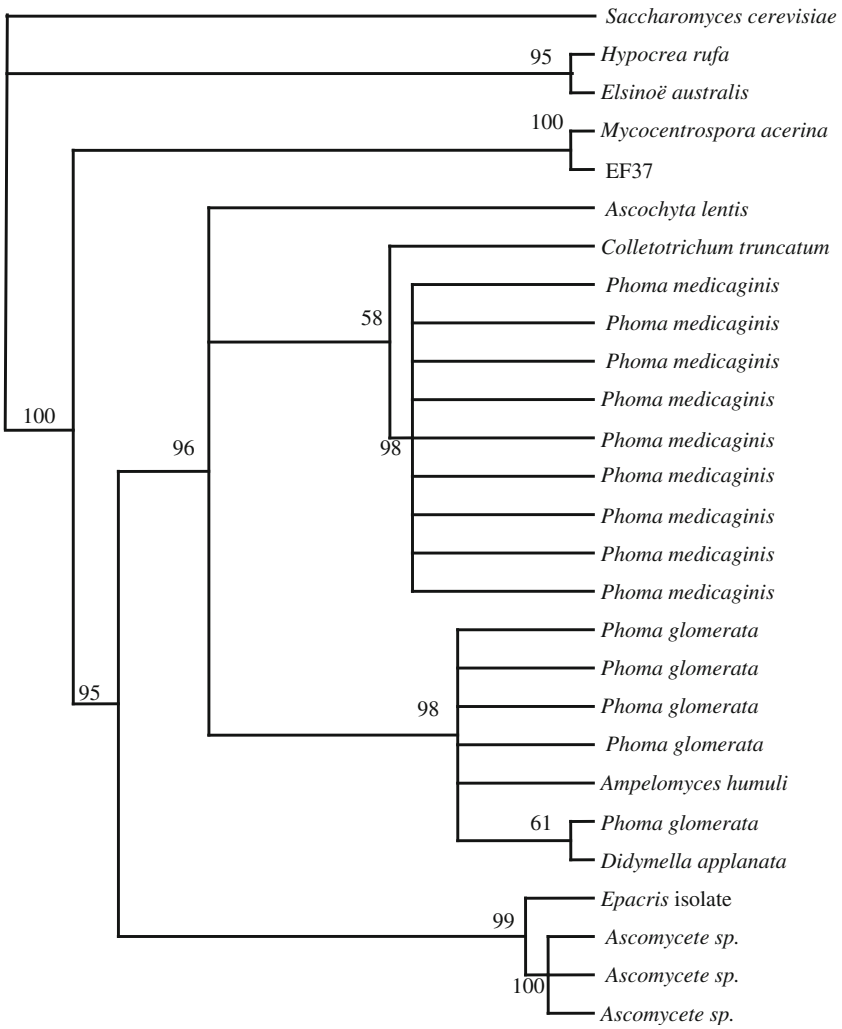

Fig. 3 Maximum parsimony tree generated from the 5.8S and ITS (ITS1 and ITS2) sequences of 27 taxa showing the relationships of EF-37 with reference taxa

Table 1 The influence of EF-37 on *S. involucrata* seedling growth

Parameter	Control	EF-37+	Significance level (*P* value)
Root dry weight (mg)	3.70±0.57	6.75±1.32	<0.05
Shoot dry weight (mg)	13.80±3.53	22.5±4.83	<0.05
Plant dry weight (mg)	16.32±3.50	29.8±5.11	<0.05
Plant height (cm)	6.83±0.76	12.67±0.58	<0.01
Number of hair root tips	2.00±0.89	5.00±1.41	<0.01

Values are presented as the mean ± SE

Control growth of *S. involucrata* under axenic conditions, *EF-37+ S. involucrata* cultivation with the fungal endophyte EF-37

being taken out of the FAA, the roots were dehydrated in a graded ethanol series, embedded in paraffin, stained with safranine and fast green, and sealed by Canada gum (Feder and O'Brien 1968) for routine light microscope observations (Tan et al. 2006). Roots of ten plants were fixed in 3% glutaric dialdehyde for 4–6 h, rinsed six times in phosphate buffer (pH 6.8), with each time interval being 30 min. They were then dehydrated in graded acetone, put in pure acetate isoamyl alcohol twice, each time for 30 min, then dried, fixed, and metallic membrane plated at the CO_2 critical point for scanning electronic microscope (SEM) observations. The remainder of the roots were fixed in 1% osmic acid at 4°C and then dehydrated in graded ethanol series, each time for 30 min. They were then embedded in LR white gum and polymerized for 2 days at 60°C in preparation for transmission electron microscope observations (Guo and Xu 1990a, b).

Chlorophyll fluorescence was measured on fully expanded leaves near those used for photosynthesis measurement using a PAM-2100 fluorometer (WALZ, Germany). F_0 (minimal fluorescence), F_m (maximal fluorescence), F_v ($F_v=F_m-F_0$; variable fluorescence), F_v/F_m (maximal photochemical efficiency of PS2), F_v/F_0 (potential activity of PS2), q_P (photochemical), and q_N (nonphotochemical quenching) were measured shortly after keeping the leaves in darkness for 30 min (Dau 1994; Demming and Winter 1987; Maxwell and Johnson 2000; Larson and Whitham 1991; Huang et al. 2004).

For the quantitative analysis of rutin, 200 mg of each portion of powdered plant material was first mixed with 10 ml of 80% methanol, extracted 20 min by ultrasonic sound, marinated for one night, followed again by 20 min of ultrasonic extraction, and then filtered. The supernatant was centrifuged at 5,000×*g* for 5 min and filtered through a 0.45-μm membrane before being injected into a high-performance liquid chromatography (HPLC) valve (Waters HPLC System). The injection volume was 20 μl. Sample analyses were performed on an Agilent Zorbax SB-C18 (150×4.6 mm I.D., 5 μm; Li et al. 2007). The elution composition of the mobile phase (acetonitrile–water, *v*/*v*) was altered from 20:80 to 100:0 in 60 min at a flow rate of 1 ml·min^{-1} and the UV detection wavelength was 270 nm. The reference standard of rutin with purity no less than 98% was supplied by Prof. Chun-lan Wang, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College.

Statistical analysis

All experiments were carried out in a complete randomized block design and each treatment consisted of 50 explants. All of the experiments were repeated three times and data were statistically analyzed using one-way analysis of variance. Significant differences between treatment groups were determined using the post hoc test with Tukey–Kramer honestly significant difference simultaneous pairwise main comparison. Data were presented using average ± standard error (SE).

Fig. 4 *S. involucrata* plants after 40 days of inoculation

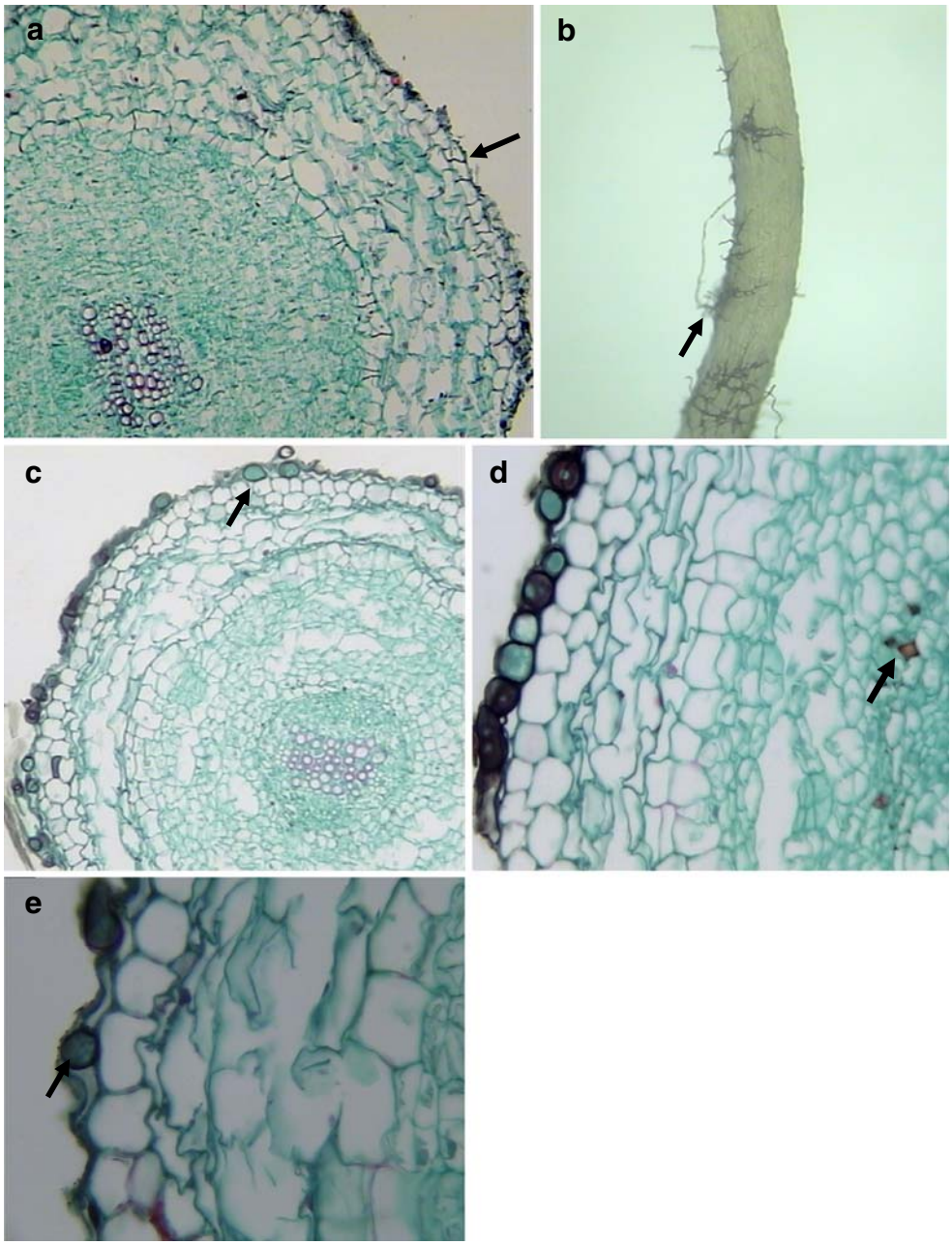

Fig. 5 **a**–**e** Light micrographs of interactions between roots of *S. involucrata* and EF-37

Results

Morphological identification of EF-37

The fungal endophyte EF-37 grew rapidly on PDA. After 1 week, colonies were 5–5.5 cm, dark green to black, usually with leafy shallow margins, flattened with low, felty, slightly lanose, or radiating aerial mycelium (Fig. 1). Vegetative mycelium with swollen, dark, and septated hyphae formed short chains of chlamydospores (Fig. 2). EF-37 fungus produced no conidia or other reproductive structures in our experiment. Our molecular analysis of the ITS nuclear rDNA region (FJ843591) showed that it closely resembles *Mycocentrospora acerina* (95% similarity to the GenBank sequence from *M. acerina* EU346864) and sequence alignment showed that EF-37 and *M. acerina* formed a cluster of 100% (Fig. 3).

Effect of EF-37 on the growth and development of *S. involucrata*

After 40 days of cultivation, EF-37+ plants appeared more vigorous than controls. Root dry weight, shoot dry weight, plant dry weight, and plant height of the EF-37+ plants

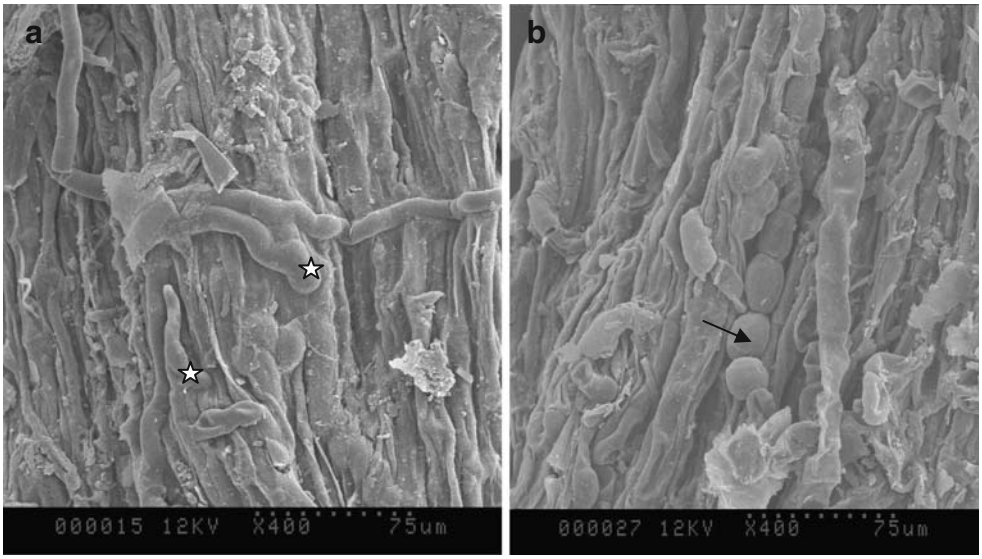

Fig. 6 **a**, **b** Root surface scanning structure of *S. involucrata* interaction with EF-37

increased 82.43%, 63.04%, 82.6%, and 85.5%, respectively, more than the controls. Moreover, the EF-37+ plants had significantly more effects on plant root development (Table 1 and Fig. 4).

Mycorrhizal structures

After 40 days of cultivation, light microscopy of transverse sections of the roots showed that the control plants remained uninfected (Fig. 5a). The fungal hyphae of EF-37+ plants branched repeatedly and formed incompact "hyphae nets" on the root surfaces (Fig. 5b). Transverse sections showed that hyphae were distributed on the surface of epidermal cells (Fig. 5c) and cortical cells dissolved and formed aerate structure (Fig. 5d). But the noninfected roots remained unaffected. Fungal hyphae adhered tightly on the epidermal cytoderm and did not penetrate the intracellular space (Fig. 5e). SEM also observed that the fungal hyphae of EF-37+ plants branched repeatedly and adhered tightly to the root surface. Moreover, hyphae swelled at sites of penetration of the root (Fig. 6a) and were distributed between the gaps of epidermal cells down the root axis direction and sometimes formed short chains of chlamydospores (Fig. 6b).

Chlorophyll fluorescence analysis

After 40 days of cultivation, the chlorophyll fluorescence detected that there were no significant differences of chlorophyll fluorescence kinetics parameters between the EF-37+ plants and controls (Fig. 7).

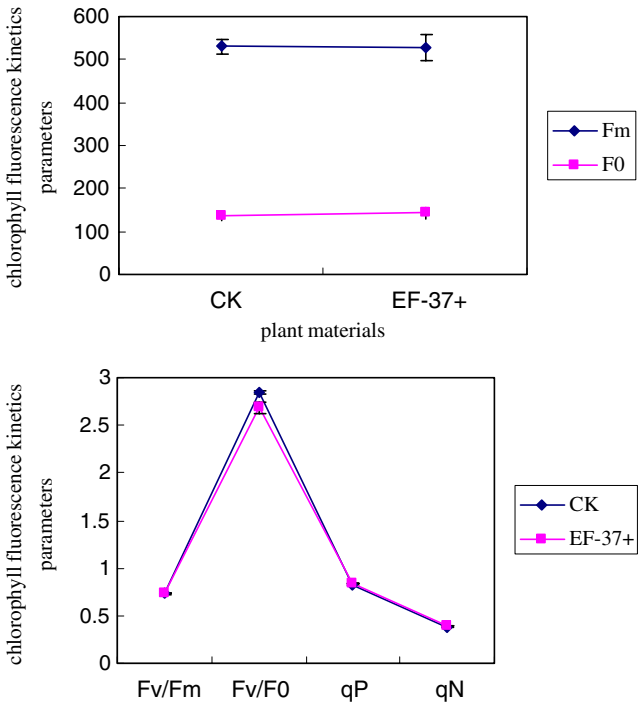

Fig. 7 Comparison of chlorophyll fluorescence kinetics parameters in control plants and EF-37+ plants of *S. involucrata*

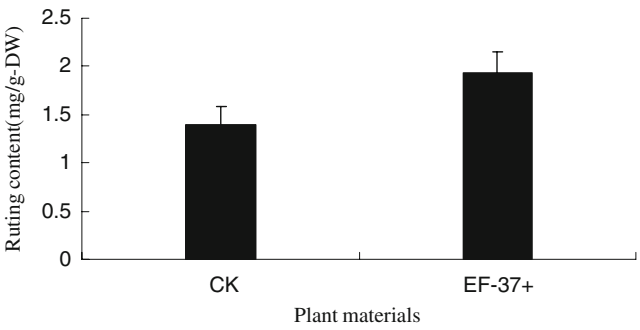

Fig. 8 Comparison of rutin content in control plants and EF-37+ plants of *S. involucrata*

Effect of EF-37 on *S. involucrata* chemical content

After 40 days of cultivation, all of the *S. involucrata* plants were collected and dried in an oven at 60°C for 24 h before HPLC analysis. The HPLC analysis showed that the content of rutin had increased in EF-37+ plants and reached 1.94 $mg \cdot g^{-1}$ dry weight in comparison with 1.40 $mg \cdot g^{-1}$ dry weight in control plants (Fig. 8).

Discussion

DSE fungi are distributed widely from the tropics to the North Pole and are found especially in the roots of arctic and alpine plants (Jumpponen and Trappe 1998a). The DSE fungi have dark hyphae which are made of certain components that can increase the hardness and reduce the permeability of the cytoderm, so that they can live in extreme conditions (Cousin 1996; Henson et al. 1999; Money et al. 1998). It is reported that DSE fungi have a polyphyletic origin (Jumpponen and Trappe 1998b; LoBuglio et al. 1996). In our study, EF-37 fungus could be congeneric to *Mycocentrospora*, which is a pathogenic fungus or saprophytic fungus of plants, but is regarded as endophytic fungi at a certain stage of its life (Schulz et al. 1999). EF-37 fungus clearly promoted the growth of seedlings and roots and formed a mutually symbiotic relationship with *S. involucrata*. These findings are similar to a report that concluded that DSE fungus could cause many types of growth response (Jumpponen and Trappe 1998a).

EF-37 fungus promoted the growth of *S. involucrata* roots, which is possible, because it dissolves the cortical cells and produces aerate structures. EF-37 fungus formed typical "incompact hyphae nets" on the root surfaces, and although fungal hyphae adhered tightly to the epidermal cytoderm, we did not find that it colonized intercellular or intracellular spaces. This has to do with the interaction time between the fungus and the seedlings. Wilcox and Wang (1987) believed that the morphology of DSE fungi that settle in the plant roots can change over time; it must have enough time to make the infection process complete. The roots we used were from seedlings which had been only inoculated with the EF-37 fungus for 40 days, which is the beginning period for mutual recognition of plant and fungus. In order to observe the interaction process between the fungus and the seedlings in detail, we would have to research the influence of fungi on *S. involucrata* seedlings over a longer period of time.

The mutual symbiosis of endophytic fungi and its host plant often promote host growth, but little has been known about the physiological basis of this effect (Marks and Clay 1996). Chlorophyll fluorescence analysis is frequently used to monitor the responses of photosynthetic apparatus to environmental stress (Krause and Weis 1991; Maxwell and Johnson 2000). Chlorophyll fluorescence of leaves was closely related to photosynthesis. Bacon (1993) found that the endophytic fungi *Acremonium* did not promote photosynthesis of its host. Marks and Clay (1996) also found that the effect of endophytic fungi on photosynthesis was related to the genotype of the host and surrounding temperature. Our results showed that there was no effect on the function of *S. involucrata* photosynthetic organs after inoculation with the EF-37 fungus. But how do endophytic fungi promote the growth of its host? Firstly, it is probably the hormone produced by the endophytic fungi that has a growth-promoting role on the host (Zhang et al. 1999). Secondly, it may be that endophytic fungi decompose photosynthate which can decrease or prevent feedback inhibition of photosynthate to photosynthesis. There are other reasons including increased uptake of phosphorus and other diffusion-limited nutrients, biological control of root pathogens, drought resistance, and enhanced biological N_2 fixation (Mada and Bagyaraj 1993). Thus, its host plants can grow significantly (Larson and Whitham 1991). At the same time, the fungi derive carbohydrates from the host plant (Fumiaki and Kazuhiko 2007). Based on these possibilities, further research should be carried out.

After infection by fungi, the host plant can enhance oxidase activity in vivo. And those oxidases are related closely with disease resistance of the host plant (Avdiushko 1993; Beaudoin-Eagan 1985). The process of colonization by fungi can cause a series of resistance reactions in host plants including a stimulative effect on secondary metabolism such as alkaloid, terpenoid, and so on (Brundrett 2002; Petrini 1991). In our study, EF-37 fungus promoted the content of flavonoids in *S. involucrata* seedlings.

The mechanism of interaction between endophytic fungi and its host plants is more complex (Jumpponen and Trappe 1998a), so a more comprehensive study to research the mechanism of mutual symbiosis between EF-37 and *S. involucrata* is necessary.

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