

Diversity and Antimicrobial and Plant-Growth-Promoting Activities of Endophytic Fungi in *Dendrobium loddigesii* Rolfe

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Abstract Endophytic fungi are ubiquitously distributed in orchids and have a great impact on the host plant. The diversity of endophytic fungi in the medicinal orchid *Dendrobium loddigesii* Rolfe was investigated and their bioactivities in microbe and plant growth were explored here. Endophytic fungi were identified by using morphological and molecular biological methods. Antimicrobial activity was determined by a standard disk assay. Activity in promoting plant growth was confirmed by root inoculation of endophytic fungi in seedling tray and pot experiments. Overall, 48 isolates were isolated from *D. loddigesii* and identified to belong to 18 genera, with *Fusarium* and *Acremonium* being the most dominant populations. A total of 17 isolates belonging to 9 genera were screened for their antimicrobial activity, and *Fusarium* spp., 8 of the 17 isolates, was also the dominant population. In the seedling tray experiment, two isolates, one of *Fusarium* named DL26 and the other of *Pyrenochaeta* named DL351, were shown to enhance plant growth in alder bark–humus medium, and the latter displayed weak activity against *Bacillus subtilis* (As 1.308) and *Aspergillus fumigatus* (As 3.2910). In the pot experiment, after inoculation of DL26 and DL351, five out of seven media were fit for plant-endophyte symbionts. Medium #1 of red brick fragments and sphagna was optimal in accelerating plant

growth. In conclusion, a great diversity of endophytic fungi in *D. loddigesii* was first confirmed in a considerable proportion of antimicrobial isolates. Furthermore, two endophytes exhibited the ability to enhance plant growth although their activities were influenced by the growth media.

Keywords *Dendrobium loddigesii* · Endophytic fungi · Diversity · Antimicrobial activity · Plant growth promotion

Introduction

Petrini (1991) defined endophytes as “all organisms inhabiting plant organs that at some time in their life can colonize internal plant tissue without causing apparent harm to the host”. Up to now, endophytic fungi, divided into clavicipitaceous and nonclavicipitaceous groups (Rodriguez and others 2009), have been found to ubiquitously colonize plants and have a profound impact on plant communities (Arnold and others 2003; Sanchez Marquez and others 2007). The latter group in particular has had increasing attention in recent years because of its taxonomic diversity, that is, numerous endophytic species can be associated with a particular plant species (Stone and others 2004; Khan and others 2008); its multiple functions including host growth acceleration, fitness, and stress tolerance (Tudzynski and Sharon 2002; Tanaka and others 2006; Baltruschat and others 2008); and the abundant occurrence of plant-endophyte symbiosis in both above- and below-ground plant tissues (Faeth and Fagan 2002; Arnold and others 2007). Furthermore, as a potential source of bioactive substances (Campanile and others 2007; Tejesvi and others 2007), endophytic fungi have potential for development of new drugs and biocontrol agents.

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As a large genus of Orchidaceae, *Dendrobium* comprises over 900 species, and 63 species have already been identified in China (Li and others 2005), many of which, including *D. loddigesii* Rolfe, can be used as a traditional Chinese medicine named *Caulis Dendrobii* (also called *Shihu* in Chinese). As a perennial epiphytic herbaceous plant, *D. loddigesii* is highly susceptible to changes in habitat (Qian and others 2008); this leads to difficulties in natural regeneration to meet the medicinal demand. With respect to artificial reproduction, aseptic seed germination and in vitro rapid propagation of seedlings have been combined extensively to propagate *D. loddigesii*. However, because of its low tolerance to environmental change, it is often difficult for the delicate sterile plantlets to survive after transplant. It is thought that the lack of a fungi-plant symbiont in sterile seedlings might be one of the reasons.

In view of the universal occurrence of endophytic fungi and endophyte-conferred benefits of fitness, detection of endophytic fungi in *D. loddigesii* would not be surprising and could play an important role in plant growth. Yuan and others (2009) systematically investigated the diversity of fungal endophytes from different tissues in *D. nobile* and reported 172 endophytic isolates belonging to 33 morphospecies and 14 genera. Endophytic fungi have been shown to improve plantlet growth of *D. nobile* and *D. huoshanense* and increase the polysaccharide content of *D. nobile* (Chen and Guo 2005; Wang and others 2007). This information led us to carry out investigations on endophytic fungi in *D. loddigesii* to understand the biological characteristics of endophytic fungi, explore their bioactivities, and discover, if possible, endophytes with the potential for utilization in artificial propagation of *D. loddigesii*.

Materials and Methods

Isolation of Endophytic Fungi from *D. loddigesii*

Five individual plants of *D. loddigesii* were collected from the Nature Reserve of Pogang, Xingyi city, Guizhou province, People's Republic of China (25°13'N, 104°95'E) in June 2007. Samples were washed with tap water to remove soil and other debris. Healthy roots, stems, and leaves were surface-sterilized by consecutive immersions for 1 min in 75% ethanol, 5 min in 5% sodium hypochlorite, and then 1 min in sterile distilled water; the immersion in sterile distilled water was repeated three times. The materials were then surface-dried with sterile paper (Guo and others 2000). Roots and stems were cut into 0.5-cm sections and leaves were cut into 0.3-cm² pieces (totaling 120 samples) in a laminar flow hood, cultured on potato dextrose agar (PDA, 2%) plates, and incubated at 25°C until emergence of fungal

hypha from inside the samples (Bayman and others 1997). The isolated pure cultures were stored in PDA slant tubes at 4°C. The effectiveness of the surface sterilization was confirmed by randomly imprinting sterilized sections on PDA plates. The surface was considered sterile when no microbial growth was observed on the imprinted plates after 3–5 days of incubation.

All isolates were incubated on PDA and corn meal agar (CMA, 2%) plates under the conditions of (1) at 25°C in darkness for 4 weeks, (2) 25°C in darkness for 2 weeks and then at 4°C in darkness for 4 weeks, and (3) 25°C with a light regime of 12 h light/12 h dark for 4 weeks to induce sporulation.

Identification of Endophytic Fungi

Specimens were mounted in 10% KOH and observed with a ZEISS Axio Imager A1 microscope for morphological characteristics. Isolates failing to sporulate were subjected to molecular analysis of the internal transcribed region (ITS) of the 5.8S rDNA, using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGTTATTGATATGC-3') (White and others 1990). DNA was extracted from 14-day PDA-cultured colonies using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987). PCR reactions (50 µl) were performed on a Mastercycler[®] Gradient (Eppendorf AG, Germany) using 2.5 U *Taq* and 6 µl 10 × GC buffer, 200 µM each dNTP, 0.3 µM of each primer, 1.5 mM MgCl₂ and 2 µl undiluted DNA template. An initial 3 min at 95°C was followed by 35 cycles of 95°C for 2 min, 53°C for 25 s, and 72°C for 2 min, with a final cycle at 72°C for 10 min. PCR products were purified with minicolumns (Sangon, Shanghai, China) and directly sequenced in an ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA, USA) at Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. Sequences were aligned using Clustal X and adjusted manually. The BLAST search program (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to look for nucleotide sequence homology for the 5.8S ITS region for fungi. The phylogenetic relationship was constructed using PAUP 4.0. Sequences were compared to other fungal 5.8S rDNA sequences in GenBank, and those with greater than 95% similarity to a vouchered sequence were assigned to a genus. Phylotypes were considered the same species when sequences were greater than 99% similar (Altschul and others 1990).

Screening for Antimicrobial Fungi

Six human pathogens, including bacteria *Escherichia coli* As1.355, *Staphylococcus aureus* As 1.72, and *Bacillus*

subtilis As 1.308, yeasts *Candida albicans* As 2.538 and *Cryptococcus neoformans* As 2.1490, and filamentous fungus *Aspergillus fumigatus* As 3.2910, were used as antimicrobial test strains. Bacteria, yeasts, and filamentous fungus were grown on Mueller-Hinton agar (MHA), Sabouraud dextrose agar (SDA), and Czapek agar, respectively.

Each fungus was grown in 500-ml Erlenmeyer flasks, each containing 200 ml wheat bran broth (WBB) medium (2.0% glucose, 0.3% KH_2PO_4 , 0.15% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 3.0% wheat bran, that was boiled for 30 min and strained), with stirring at 120 rpm at 25°C for 8 days. The culture filtrate of isolate was extracted with equal volumes of EtOAc two times. After vacuum concentration to remove organic solvent, the EtOAc extract was diluted with sterile distilled water at 10 mg/ml. The solution was aseptically filtered using a 0.22- μm Millipore filter.

Antimicrobial bioassays were conducted according to a procedure in the literature (Vicente and others 2001). The final colony-forming units (cfu) of bacteria in MHA and yeasts and *A. fumigatus* in SDA were 10^6 , 10^5 , and 10^4 cfu ml^{-1} , respectively. In standard disk assays, the EtOAc extract was absorbed onto individual paper disks (6-mm diameter) at 100 μg disk^{-1} and placed on the surface of MHA and SDA, respectively. The antibacterial inhibitor ampicillin sodium (10 μg disk^{-1}) and the antifungal inhibitor fluconazole (25 μg disk^{-1}) were used as positive controls. Assay plates were incubated at 37°C for 24 h for bacteria and at 28°C for 48 h for fungus, and the diameters (mm) of the inhibition zones were measured. The experiment was replicated three times.

Media and Growth Conditions of Sterile Seedlings and Solid Cultured Fungi

The 1/2 MS medium supplemented with potato extract, 3% sucrose, 0.1% active carbon, and 0.8% agar was used in *D. loddigesii* seedling propagation. The seedlings were incubated under fluorescent light at 1500 lux with a 16-h photoperiod at 22–24°C for 3 months.

Endophytic fungi were solid cultured in wheat bran and rice hull (WBRH) medium (the mixture of equal weight of WB and RH that watered solution containing 2.0% glucose, 0.3% KH_2PO_4 , and 0.15% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ to water content at 65–70%) at 25°C in darkness for 4 weeks for seedling tray and pot experiments.

Seedling Tray Experiment

Seven isolates used in this experiment were selected from all recovered endophytes through a preliminary investigation in which fungi were inoculated near seedlings growing on 1/2 MS medium supplemented with 0.75% sucrose and

0.8% agar and cocultured with plants for 8 weeks. The specifications of the seedling tray were as follows: tray size = 540 × 280 mm; cell count = 4 × 8; size of upper cell = 58 mm; size of lower cell = 43 mm; depth of cell = 52 mm. Three to four seedlings, each with five to seven leaves and 4–6 cm high (from above the root to the top tip of stem), were planted in each tray cell filled with alder bark and humus medium (mixture of two substances in 1:1 v/v and autoclaved at 121°C for 1.5 h). For endophyte inoculation, 1 g WBRH culture was put under the roots of seedlings per tray cell while planting. The control group was inoculated with 1 g of autoclaved WBRH medium. A completely randomized block design with 12 replicates of every treatment was used. Plants were maintained in the greenhouse at 22–24°C, 70–80% relative humidity (RH), and with a 12-h photoperiod for 2 months.

Pot Experiment

The two isolates DL26 and DL351, which were confirmed to be capable of accelerating plant growth in the seedling tray experiment, and seven plant growth media numbered 1–7 were used to evaluate the adaptability of endophytes in different artificial fungus–plant symbionts. The growth media were as follows: (1) red brick fragment and sphagna (about 6 cm of brick pieces in the bottom of a pot covered with 2 cm of sphagna), (2) alder bark and humus, (3) pine bark and humus, (4) alder bark and red brick fragment, (5) pine bark and red brick fragment, (6) alder bark and caly ball, and (7) pine bark and caly ball. For media 2–7, the two substances were mixed 1:1 v/v. The sizes of bark and brick fragments were 0.5–1.5 × 0.5–1.5 cm and 1–2.5 cm^3 , respectively. Humus was sieved through a 5-mesh sifter. The diameter of the caly ball was 5–8 mm. The specifications for the pot were as follows: size of the top of the pot = 100 mm, size of the bottom of the pot = 68 mm, and depth of the pot = 80 mm. Three to four seedlings, each with eight to ten leaves and 8–10 cm high, were planted in each pot filled with varied media. Root inoculation of two isolates and corresponding control groups in the seven media were treated as described above, except for an increase in the quantity of inoculation to 3 g. A completely randomized block design with ten replicates for every treatment was used. Plants were maintained in the greenhouse at 22–24°C, 70–80% RH, and a 12-h photoperiod for 3 months.

Calculation, Data Collection, and Statistical Analysis

Colonization rate (CR), expressed in percentage, was used to demonstrate the degree of infection using the following formula: $\text{CR} = A/C$. Isolation rate (IR) was used to demonstrate the richness of endophytic fungi and the degree of

multiple colonization via the following formula: $IR = B/C$, where A is the total number of samples yielding at least one isolate, B is the total number of isolates yielded in a given trial, and C is the total number of samples in a trial (Li and others 2007).

In the seedling tray and the pot experiments, data were collected from each tray cell or pot. The height of one randomly selected seedling was measured from the end to the tip of the stem before being planted and after harvest. The increase in seedling height (ISH) was the difference in seedling height between harvesting and planting. After cultivation, the number of buds was recorded and the seedlings' dry weight (DW) was determined after fresh seedlings were placed in a paper bag and kept at 55°C for 5 days. In the pot experiment, effects of varied growth media on the growth of the plant inoculated with 3 g of WBRH medium were first estimated. Then, effects of CK and the two endophyte treatments for each growth media were assessed. Because growth media were tested to significantly affect plant growth, no data analysis was done to compare the influence of each fungus on plant growth with different media.

The statistical package SPSS 11.5 for Windows was used for the analyses. Homogeneity of variances was tested by Hartley's or Cochran's test. Square root transformation was used when violations were detected. The data were analyzed by a one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test ($p = 0.05$).

Results

Overall, 48 fungal cultures were isolated from 120 healthy samples of *D. loddigesii* and grouped into 18 identified genera, of which *Fusarium* and *Acremonium* represented 21 (43.8%) of the total isolates and were the dominant genera in the plants. *Chaetomella*, *Cladosporium*, *Nigrospora*, *Pyrenochaeta*, *Sirodesmium*, and *Thielavia* were found in *Dendrobium* for the first time (Table 1). Compared to stems and leaves, more endophytes were found in roots (Table 2), implying that roots might provide a better niche for endophyte colonization. In addition, organ specificity of fungal endophytes was observed. For instance, *F. solani*, *F. venfricosum*, and *Acremonium* spp. preferred to colonize roots, whereas *Colletotrichum* spp. inhabited only leaves.

A total of 17 (35.4%) isolates, all obtained from roots, showed antimicrobial activity against one or more of the five human pathogenic microbes (Table 3). None exhibited inhibitory activity against *E. coli*. Except for *F. solani*2 and *Bionectria* sp2., the most active fungi displayed weak activities against the tested pathogens. Of the eight active fungi that inhibited more than one pathogen, six could

restrict both bacterial and fungal pathogens, which validated the extensive antimicrobial spectrum of those endophytes in *D. loddigesii*. Accounting for almost half of the positive isolates, *Fusarium* was the predominant genus in antimicrobial isolates.

In the seedling tray experiment, endophytic fungi significantly affected the dry weight (DW) ($p < 0.01$; $F_{7,88} = 109.50$) (Fig. 1a) and the increase of seedling height (ISH) ($p < 0.01$; $F_{7,88} = 28.29$) (Fig. 1b) but not the number of buds ($p = 0.11$; $F_{7,88} = 1.76$) (Table 4) of *D. loddigesii* plantlets. Compared to controls (CK), DL26 (*Fusarium* sp1.) distinctly increased the DW and the seedling height by 142.7 and 84.5% to 102.9 mg and 21.4 mm, respectively, demonstrating its capability in promoting plant growth. DL351 (*Pyrenochaeta* sp.) increased the DW by 88.9% to 80.1 mg compared with CK; besides, although the differences were not significant, the number of buds was the largest with DL351 inoculation, implying its favorable effect on lateral bud differentiation. Because of these results, DL26 and DL351 were selected for further investigations in pots.

In the pot experiment, growth media influenced the DW of seedlings inoculated with 3 g of WBRH medium ($p < 0.01$; $F_{6,63} = 6.94$) but not the number of buds ($p = 0.14$; $F_{6,63} = 1.68$) or the increase of seedling height ($p = 0.27$; $F_{6,63} = 1.31$) (Table 5). Inoculation with DL26 and DL351 in every medium could not influence the seedling height (data not shown). Except for media 2 and 5, DL26 and/or DL351 significantly affected DW (Fig. 2) in the media used. Medium 1 was the second best of the seven media in terms of the DW and the best for two fungi in accelerating DW ($p < 0.01$; $F_{2,27} = 114.10$). In comparison to CK in medium 1, DL26 and DL351 remarkably increased DW by 73.6 and 123.9% to 214.0 and 276.0 mg, respectively, which were the largest and the second largest values in the experiment, suggesting that medium 1 was the optimal medium for plant-fungus symbionts. DL26 also significantly increased the DW in medium 3 ($p < 0.01$; $F_{2,27} = 27.97$) and medium 6 ($p < 0.01$; $F_{2,27} = 18.82$) by 62.9 and 70.7% to 183.8 and 169.5 mg, respectively, compared with the relevant CK, suggesting a more extensive applicability of DL26 than that of DL351.

Endophytes remarkably affected number of buds only in media 2 and 4 (Table 6). In medium 2, DL351 increased the number of buds ($p < 0.01$; $F_{2,27} = 9.93$) by 3.25-fold compared with CK, giving a maximum value of 1.7 per pot in the experiment. The materials of medium 2 were the same as those used in the seedling tray experiment but were not autoclaved. Medium 4 was the only one that showed positive effects of DL351 inoculation on both the DW ($p < 0.01$; $F_{2,27} = 8.51$) and the number of buds ($p < 0.05$; $F_{2,27} = 3.96$), implying the fitness of the fungus-plant symbiont to this media. Furthermore, it seemed

Table 1 Distribution and identification of endophytic fungi in *D. loddigesii*

Taxa	Strain number	Organs			GenBank accession number
		Leaves	Stems	Roots	
Dothideomycetes					
<i>Lasiodiplodia</i> sp.	DL339	–	–	1	FJ 450045
<i>Cladosporium</i> sp1.	DL319	–	–	1	– ^a
<i>Cladosporium</i> sp2.	DL12	1	–	–	FJ 450037
<i>Alternaria</i> sp1.	DL36	–	–	1	–
<i>Alternaria</i> sp2.	DL111	1	–	–	FJ 450015
<i>Alternaria</i> sp3.	DL35	–	–	1	FJ 450016
<i>Ampelomyces</i> sp.	DL110	1	–	–	FJ 450034
<i>Davidiella</i> sp.	DL324	–	–	1	FJ 450038
<i>Paraconiothyrium</i> sp.	DL316	–	–	1	FJ 450022
<i>Pyrenochaeta</i> sp.	DL351	–	–	1	GQ895137
Eurotiomycetes					
<i>Sirodesmium</i> sp1.	DL18	1	–	–	–
<i>Sirodesmium</i> sp2.	DL19	1	–	–	–
<i>Sirodesmium</i> sp3.	DL337	–	–	1	–
Leotiomycetes					
<i>Chaetomella</i> sp.	DL350	–	–	1	–
Sordariomycetes					
<i>Thielavia californica</i>	DL317	–	–	1	–
<i>Xylaria</i> sp1.	DL23	–	1	–	FJ 449994
<i>Xylaria</i> sp2.	DL330	–	–	1	FJ 449995
<i>Nigrospora</i> sp.	DL334	–	–	1	FJ 449963
<i>Colletotrichum</i> sp1.	DL11	1	–	–	FJ 449930
<i>Colletotrichum</i> sp2.	DL113	1	–	–	FJ 449931
<i>Acremonium nepalense</i>	DL342	–	–	1	–
<i>Acremonium stromaticum</i>	DL39	–	–	1	–
<i>Acremonium</i> sp1.	DL331	–	–	1	–
<i>Acremonium</i> sp2.	DL332	–	–	1	–
<i>Acremonium</i> sp3.	DL310	–	–	1	–
<i>Acremonium</i> sp4.	DL311	–	–	1	–
<i>Acremonium</i> sp5.	DL312	–	–	1	–
<i>Acremonium</i> sp6.	DL320	–	–	1	–
<i>Acremonium</i> sp7.	DL338	–	–	1	–
<i>Verticillium</i> sp.	DL345	–	–	1	FJ 449912
<i>Fusarium dimerum</i>	DL315	–	–	1	–
<i>Fusarium solani</i> 1	DL326	–	–	1	–
<i>Fusarium solani</i> 2	DL327	–	–	1	–
<i>Fusarium stoveri</i>	DL313	–	–	1	–
<i>Fusarium udum</i>	DL32	–	–	1	–
<i>Fusarium venfricosum</i> 1	DL341	–	–	1	–
<i>Fusarium venfricosum</i> 2	DL343	–	–	1	–
<i>Fusarium</i> sp1.	DL26	–	1	–	–
<i>Fusarium</i> sp2.	DL314	–	–	1	–
<i>Fusarium</i> sp3.	DL336	–	–	1	–
<i>Fusarium</i> sp4.	DL353	–	–	1	–
<i>Fusarium</i> sp5.	DL352	–	–	1	FJ 449962
<i>Bionectria</i> sp1.	DL21	–	1	–	FJ 449976

Table 1 continued

Taxa	Strain number	Organs			GenBank accession number
		Leaves	Stems	Roots	
<i>Bionectria</i> sp2.	DL333	–	–	1	FJ 449977
<i>Bionectria</i> sp3.	DL335	–	–	1	FJ 449978
<i>Bionectria</i> sp4.	DL329	–	–	1	FJ 449979
<i>Bionectria</i> sp5.	DL349	–	–	1	FJ 449980
<i>Cercophora</i> sp.	DL24	–	1	–	FJ 449910

^a Identified based on morphological characteristics

Table 2 Colonization and isolation rates of endophytic fungi in organs of *D. loddegisii*

Plant organs	Leaves	Stems	Roots
Amount of samples	40	40	40
Amount of samples with isolates	7	4	31
Amount of isolates recovered	7	4	37
Colonization rate	18%	10%	78%
Isolation rate	0.18	0.10	1.19

that DL351 played a clearer role in lateral bud differentiation promotion than DL26 in media 2 and 4. Interestingly, although medium 7 yielded maximum DW values, inoculation of endophytes in it exhibited a negative effect on the

DW ($p < 0.01$; $F_{2,27} = 7.90$). In contrast with medium 7, the DW in medium 5 was significantly lower than that in any others; however, there was a positive effect on the DW ($p < 0.05$; $F_{2,27} = 5.03$) with fungi inoculation. The results revealed that growth media can significantly affect the interactions between plant and fungus and suggested that the role of medium in the fungus-plant symbiont deserves intensive study.

Discussion

In this study we have demonstrated the great diversity of endophytic fungi, with a considerable number of

Table 3 Antimicrobial activities of endophytic fungi in *D. loddigesii*^a

Taxa	Diameter of inhibition zone on assay plate (mm)				
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>Can. albicans</i>	<i>Cryp. neoformans</i>	<i>A. fumigatus</i>
<i>Lasioidiplodia</i> sp.	– ^b	9.8 ± 0.2	–	–	–
<i>Cladosporium</i> sp1.	–	–	–	–	9.0 ± 0.2
<i>Alternaria</i> sp1.	–	8.0 ± 0.2	–	–	–
<i>Alternaria</i> sp3.	7.6 ± 0.4	14.3 ± 0.2	10.2 ± 0.3	–	9.1 ± 0.2
<i>Pyrenochaeta</i> sp.	–	10.5 ± 0.3	–	–	12.3 ± 0.1
<i>Chaetomella</i> sp.	11.8 ± 0.9	8.7 ± 0.4	–	–	–
<i>T. californica</i>	13.3 ± 0.6	16.3 ± 0.3	–	–	–
<i>Nigrospora</i> sp.	9.1 ± 0.5	–	10.3 ± 0.3	–	–
<i>F. dimerum</i>	16.2 ± 0.4	–	–	11.3 ± 0.3	–
<i>F. solani</i> 1	7.3 ± 0.1	–	–	–	–
<i>F. solani</i> 2	22.7 ± 0.7	–	–	–	–
<i>F. venfricosum</i> 1	16.7 ± 0.7	–	16.3 ± 0.3	–	–
<i>F. venfricosum</i> 2	19.3 ± 0.4	–	–	–	–
<i>Fusarium</i> sp3.	–	–	8.4 ± 0.3	–	–
<i>Fusarium</i> sp4.	–	13.3 ± 0.5	–	–	–
<i>Fusarium</i> sp5.	–	14.3 ± 0.2	–	–	–
<i>Bionectria</i> sp2.	24.0 ± 0.6	–	20.3 ± 0.6	–	–

Values are mean (±SE) of three replications

^a All isolates failed to show antimicrobial activity to *E. coli*

^b No antimicrobial activity

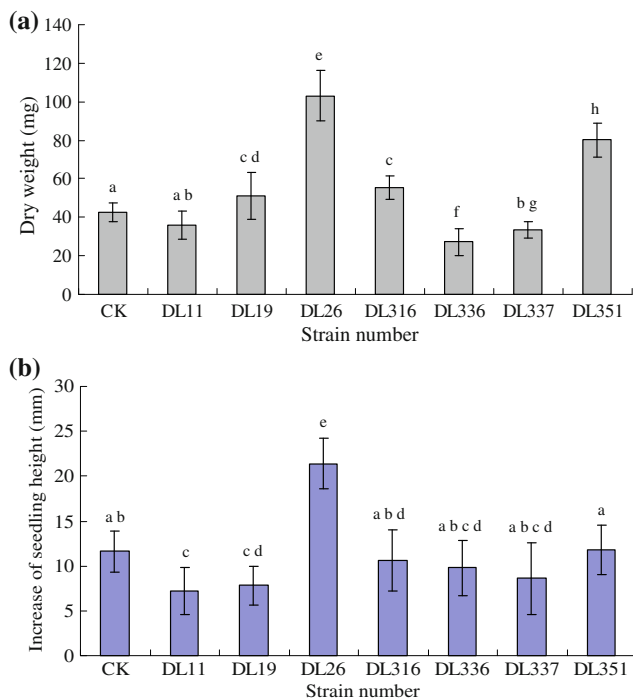


Fig. 1 Effects of endophytic fungi on the dry weight (a) and the increase of seedling height (b) of *D. loddigesii* in the seedling tray experiment. Different letters denote significant differences according to Duncan's multiple-range test at the $p < 0.05$ level. Each data point represents the mean (\pm SE) of 12 replications

antimicrobial strains in *D. loddigesii*, and confirmed that two endophytes were capable of enhancing the growth of *D. loddigesii* plantlets by root inoculation. However, the level of growth was influenced by the growth medium. Among the media used in the pot experiment, media 1, 2, 4,

and 6 were first proven to be favorable to the growth of *D. loddigesii* sterile seedlings by a preliminary investigation; this also implied that alder bark was an important constituent of the media. Considering that pine bark is more common and economical in most of China's rural areas, media 3, 5, and 7 were then designed to replace alder bark with pine bark. The results showed that root inoculation managed to improve the plant growth in all the media used except 5 and 7. However, growth media seemed likely to influence the effects of endophytes on plant growth, especially with respect to dry weight accumulation and lateral bud differentiation.

The activities of the two fungi in the three media containing alder bark were more stable and credible than in those containing pine bark. It has been suggested that the outcomes of plant-endophyte interactions depend on the genotypes of the plant and the fungus, together with the environmental context (Yuan and others 2010). Here we just confirmed the influence of media on the effects of endophytes, but the mechanism of the influence remains unknown. Only one report (Tefera and Vidal 2009) demonstrated that plant growth medium could apparently influence colonization rates of sorghum by the entomopathogenic fungus *Beauveria bassiana*. It suggested that environmental factors should be given more attention in investigations of plant-endophyte interactions.

Although in orchid biological research most studies focus on orchid root-associated endophytic fungi and relatively little is known about the endophytes in the above-ground tissues and their role in the establishment and growth of the orchid, some investigations of other plant families revealed that foliar-associated endophytic fungi

Table 4 Effect of endophytic fungi on the number of buds of *D. loddigesii* in the seedling tray experiment

Strain number	CK	DL11	DL19	DL26	DL316	DL336	DL337	DL351
Number of buds per tray cell	0.7 \pm 0.7a	1.3 \pm 1.1a	0.9 \pm 1.2a	0.8 \pm 0.5a	1.2 \pm 0.7a	0.8 \pm 0.9a	1.0 \pm 1.3a	1.8 \pm 0.9a

Values are mean (\pm SE) of 12 replications. Means (\pm SE) followed by the same letter are not significantly different at $p < 0.05$

Table 5 Effect of different growth media on the dry weight (DW), the number of buds (NB), and the increase of seedling height (ISH) of *D. loddigesii* in the pot experiment

No. medium	Medium name	DW (mg)	NB	ISH (mm)
1	Red brick fragment–sphagna	123.3 \pm 14.7ab	0.7 \pm 1.5a	12.0 \pm 3.3a
2	Alder bark–humus	112.8 \pm 29.1abcd	0.4 \pm 0.5a	14.9 \pm 4.3a
3	Pine bark–humus	112.8 \pm 20.5abcde	0.8 \pm 0.9a	15.4 \pm 6.6a
4	Alder bark–red brick fragment	116.1 \pm 34.1abd	0.3 \pm 0.5a	16.3 \pm 6.1a
5	Pine bark–red brick fragment	68.5 \pm 26.1f	0.7 \pm 0.7a	17.1 \pm 4.7a
6	Alder bark–caly ball	99.3 \pm 19.1acdeg	0.1 \pm 0.4a	12.5 \pm 7.6a
7	Pine bark–caly ball	131.0 \pm 22.6b	0 \pm 0a	12.4 \pm 5.4a

Values are mean (\pm SE) of ten replications. Different letters following means (\pm SE) within a column denote significant differences according to Duncan's multiple-range test at the $p < 0.05$ level

Fig. 2 Effects of DL26 and DL351 inoculated in different growth media on the dry weight of *D. loddigesii* seedlings in the pot experiment. *Stripe bars* indicate controls, *white bars* indicate DL26 inoculation, and *gray bars* indicate DL351 inoculation. Different letters denote significant differences according to Duncan’s multiple test at the $p < 0.05$ level. Each data point represents the mean (\pm SE) of ten replications

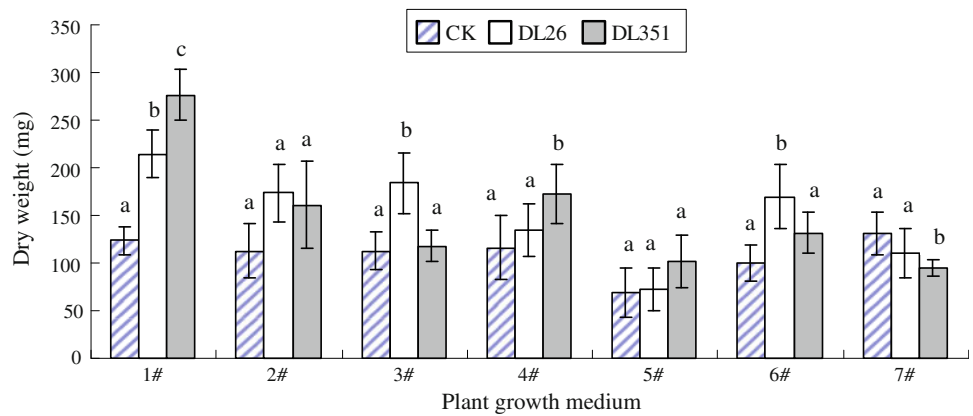


Table 6 Effect of DL26 and DL351 inoculated in different growth media on the number of buds per pot of *D. loddigesii* in the pot experiment

Medium number	Medium name	CK	DL26	DL351
1	Red brick fragment–sphagna	0.7 \pm 1.5a	0.5 \pm 0.7a	0.6 \pm 0.7a
2	Alder bark–humus	0.4 \pm 0.5a	0.7 \pm 0.8a	1.7 \pm 0.7b
3	Pine bark–humus	0.8 \pm 0.9 a	0.7 \pm 0.9a	0 \pm 0b
4	Alder bark–red brick fragment	0.3 \pm 0.5a	1.2 \pm 0.9b	1.1 \pm 0.9b
5	Pine bark–red brick fragment	0.7 \pm 0.7a	0 \pm 0b	0 \pm 0b
6	Alder bark–caly ball	0.1 \pm 0.3a	0.9 \pm 0.7a	0.4 \pm 0.5a
7	Pine bark–caly ball	0 \pm 0a	0 \pm 0a	0.5 \pm 0.7b

Values are mean (\pm SE) of ten replications. *Different letters* following means (\pm SE) within a row denote significant differences according to Duncan’s multiple-range test at the $p < 0.05$ level

conferred underappreciated benefits to the host plant (Herre and others 2007). Our results showed that DL26 isolated from stems of *D. loddigesii* was compatible with the root and sufficiently promoted plant growth in a variety of media. As far as we know, this is the first study to show that cauline-associated endophytic fungi of *Dendrobium* is compatible with the root of the host plant and confers benefits to the host. Therefore, non-root-associated endophytes should receive more attention in orchid biological research.

It is well-known that fungus-plant symbionts, involving a diversity of fungal species, are distributed in all plants in natural ecosystems and play an important role in the host’s life cycle (Rodriguez and others 2009). Our study agreed with research that detected a rich endophyte assemblage in the roots of *Lepanthes* (Orchidaceae) (Bayman and others 1997) and wild rice (*Oryza granulata*) (Yuan and others 2010). However, these studies were different from recent literature reporting a more uniform distribution of endophytes in three organs of *D. nobile* (Yuan and others 2009). In terms of fungal species diversity of *Dendrobium* spp., it was reported that the common fungal genera in the roots of wild *Dendrobium* in Taiwan were *Acremonium* spp., *Fusarium* spp., *Trichoderma* spp., *Rhizoctonia* spp., and *Alternaria* spp. (Chang 2007). Our results revealed that

Fusarium spp. and *Acremonium* spp. were the dominant populations in the roots, whereas we did not discover *Trichoderma* spp. and *Rhizoctonia* spp. in whole plants, nor high frequencies of *Xylaria* spp., which is a common endophytic inhabitant of most tropical plants investigated. In regard to organ specificity of fungal endophytes in the orchid, the exclusive appearance of *F. solani* in the roots of *D. nobile* was also reported (Yuan and others 2009). Similarly, *X. cf. arbuscula* was isolated just from the roots of some *Lepanthes* spp. (Bayman and others 1997). However, based on all relative investigations, it was not clear whether the apparent organ specificity of the fungus was related to organ physiology or to environmental factors required by the orchid habitat. It has been shown that the distribution, diversity, and abundance of endophytes depend on the host species, host genotype, and environmental conditions. Although studies of endophytic fungi associated with the *Dendrobium* genus were insufficient, it seemed reasonable to believe that endophytic fungi constituted a fungal assemblage in *D. loddigesii*.

The genus *Fusarium* is a common endophytic inhabitant of many plants. More than 120 different formae speciales of *Fusarium* have been identified based on specificity to host species belonging to a wide range of plant families (Michielsse and Rep 2009). The endophytic *Fusarium* spp.

is capable of promoting host growth, performs activities against plant and clinical pathogens, and has different antibiotic coverage and potency among various strains (Perk and others 2003; Phongpaichit and others 2006; Vu and others 2006). Our results were consistent with these observations, showing that *Fusarium* was dominant among the antimicrobial isolates from *D. loddigesii* and that one isolate of *Fusarium* named DL26 accelerated plant growth. The former one was similar to that of endophytes from the medicinal plants *Dracaena cambodiana* and *Aquilaria sinensis* (Gong and Guo 2009). These studies suggest that endophytes affect plant communities by increasing fitness through biotic tolerance and increasing biomass. They also imply that the genus *Fusarium* has potential as a source of antibiotics and as a biological control agent.

Few articles on useful biological activities of the genus *Pyrenochaeta* are available except one about the metabolites of *P. terrestris* which showed antibiotic activity against fungi and bacteria (Sparace and others 1987). Three of the most common species, *P. lycopersici*, *P. romeroi*, and *P. terrestris*, turned out to be pathogens of plants, animals, and humans, respectively (Chen and Chen 2003; Desnos-Ollivier and others 2006; Fiume and Fiume 2008). In our antimicrobial assay, DL351 (*Pyrenochaeta* sp.) showed antimicrobial activities against clinical pathogens. We also showed for the first time, to our knowledge, the beneficial action of DL351 on host plants. Moreover, DL351 was the only strain to both reliably improve host growth and inhibit microbial activity in the present investigation. Because of the absence of a definite outcome, no specific *Dendrobium* pathogens were applied in the evaluation of the antimicrobial assay. This was a limitation of our study. The unknown biological nature of isolate DL351 responsible for the dual activity of microbial inhibition and plant growth promotion deserves further study.

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