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菌根真菌促进金钗石斛的生长及氮利用

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摘要: 菌根在兰科的生命周期和进化史上起着关键作用。兰科中大多数是附生兰, 但它们的菌根研究相对缺乏。为了探讨菌根对附生兰的影响, 本研究用金钗石斛 (*Dendrobium nobile*) 与通过形态学特征和分子生物学鉴定的分属于瘤菌根菌属 (*Epulorhiza*) 的 S1 和胶膜菌属 (*Tulasnella*) 的 S3 真菌共培养。共培养结果表明, S1 和 S3 与金钗石斛形成了共生关系, 且不同程度地促进了其生长。^{15}N 稳定同位素标记实验证实, S1 菌株显著促进了金钗石斛对有机氮的利用, 而 S3 菌株没有显著的促进作用。同时, S1 和 S3 真菌均能提高金钗石斛中石斛碱的含量。研究结果表明, 菌根真菌能促进附生兰幼苗的生长、有机氮的利用和次生代谢产物的积累。

关键词: 金钗石斛; 菌根真菌; 生长; ^{15}N; 石斛碱
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Mycorrhizal Fungi Promote Growth and Nitrogen Utilization by *Dendrobium nobile* (Orchidaceae)

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Abstract: Mycorrhizal associations play a key role in the life cycle and evolutionary history of orchids. Although most orchid species are tropical and epiphytic, their mycorrhizae are poorly understood compared with those of temperate, terrestrial orchids. To investigate the influences of such fungi on photosynthetic, epiphytic orchids, we inoculated seedlings of *Dendrobium nobile* with *Epulorhiza* sp. (S1) or *Tulasnella* sp. (S3). These fungi had been identified based on their morphological and molecular characters. Both S1 and S3 formed symbiotic associations with our seedlings, promoting their growth and development to various degrees. Results from signature experiments with the ^{15}N stable isotope suggested that the utilization of organic nitrogen by orchid seedlings was significantly improved by S1, but not by S3. Dendrobine contents were significantly higher in all inoculated seedlings. Our findings demonstrate that these mycorrhizal fungi enhance plant growth, their utilization of organic nitrogen, and the accumulation of secondary metabolites in this epiphytic orchid species.

Key words: *Dendrobium nobile*; Mycorrhizal fungi; Growth; ^{15}N; Dendrobine

Orchidaceae, the largest plant family in the world, is estimated to comprise more than 25 000 species.

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Because orchid seeds are minute and contain few stored food reserves, colonization by a compatible fungus is essential for germination and/or early growth and development (protocorm stage) in the substrate (Dearnaley, 2007). Bayman *et al.* (2002) have suggested that the development of mycorrhizal associations was a crucial event in the evolution of this family. Even though most orchid species are tropical and epiphytic, their mycorrhizae are poorly understood compared with those of temperate, terrestrial orchids.

Mycorrhizal infections resulted in significantly higher N contents in individuals of the green-leaved terrestrial orchid *Goodyera repens* beyond the protocorm stage (Cameron *et al.*, 2006). However, the influence that those fungi have on N metabolism in epiphytic orchids remains unclear. Such orchids live on trees or rocks with mosses, liverworts, and ferns (Suárez *et al.*, 2006), sites where organic N is abundant. This nutrient is a major constituent of secondary metabolites, proteins, and nucleic acids. Organic and inorganic N can affect various plant processes, from growth and development to metabolism (Scheible *et al.*, 2004). Because alkaloids are synthesized mostly from amino acids (Haslam, 1986), the higher the level of available N, the greater the production of alkaloids.

When devising new strategies of the conservation and artificial propagation of orchids, researchers must evaluate the possibly strong impact of these fungal symbionts (Liu *et al.*, 2010). One of the most popular orchids within the *Dendrobium* genus, *D. nobile* Lindl. is an epiphytic and tropical plant (Mohanty *et al.*, 2012). This species is well known in the pharmaceutical industry, primarily for the formation of alkaloid compounds such as dendrobine (Zha *et al.*, 2007). Therefore, improving our understanding of the mycorrhizal relationships in these plants would be beneficial for the commercial production of dendrobine. Our study objectives were to 1) detect whether endophytic fungi from *D. officinale* can form successful mycorrhizal associations with the roots of *D. nobile* seedlings and improve growth of the host plants, and 2) investigate whether organic N utilization and dendrobine contents in *D. nobile* seedlings can be increased via fungal symbiosis.

1 Materials and methods

1.1 Isolation of endophytic fungi

Wild plants of *Dendrobium officinale* were collected in April 2009 from a subtropical forest at Xishuangbanna (21.7° N, 100.8° E), Yunnan Province, China. Healthy roots were selected, rinsed with tap water, and washed again in sterile distilled water. Once segmented, they were surface-sterilized by consecutive immersions for 8 to 10 min in 0.1% $HgCl_2$, and then rinsed five times with sterile distilled water. After surface-drying, the root segments were aseptically cut into approximately 0.5 to 1 cm sections, and transferred to 9 cm Petri dishes containing potato dextrose agar (PDA: 20% potato, 2% glucose, and 1.5% agar). The dishes were incubated in the dark at 25 ℃ until fungal hyphae emerged from inside the roots. Pure cultures were obtained by transferring the hyphae onto fresh PDA and storing them in PDA slant tubes at 4 ℃. From all of the isolates that were naturally present, we determined that two in particular—S1 and S3—stimulated growth of tissue-cultured seedlings of *D. nobile* through artificial inoculation. Therefore, they were selected for further study.

1.2 Identification of fungi

The S1 and S3 isolates were cultured on a PDA medium. Micro-morphology features of their colonies were observed with a light microscope (OLYMPUS CX31), and images were made with a video camera (JVC TK-C721EG). Because both S1 and S3 are sterile *in vitro*, we used the internal transcribed region (ITS) of the 5.8S rDNA and the large subunit gene of mitochondrial rDNA (mtLSU), respectively, to identify them. Briefly, DNA was extracted from 30-day-old PDA-cultured colonies according to the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). Universal fungal

primer combinations ITS1/ITS4 (Ma *et al.*, 2003) and ML5/ML6 (Bruns *et al.*, 1998) were selected for ITS and mtLSU amplification. PCR reactions (25 μL) were performed using 2.5 μL of 10 × buffer (with Mg^{2+}), 2 μL of 2.5 mmol · L^{-1} dNTP, 0.5 μL of TaqE (2.5 U), 2 μL of 5 μmol · L^{-1} of each primer, 2 μL of undiluted DNA template and 14 μL of ddH_2O. The cycle parameters included denaturation at 95 ℃ for 3 min; then 35 cycles of denaturation at 94 ℃ for 1 min, annealing at 53 ℃ for 50 s, and elongation at 72 ℃ for 1 min; followed by a final extension at 72 ℃ for 7 min. The PCR products were purified and directly sequenced in an ABI Prism 3730 Sequencer (Applied Biosystems, Foster City, CA, USA) at the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Their sequences were aligned by ContigExpress and adjusted manually. The BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) was used for comparing their sequence homology with other fungi. If the sequence to be identified and the vouchered sequence had >95% similarity, they were assigned to the same genus (Altschul *et al.*, 1990).

1.3 Inocula of fungi

The S1 and S3 fungi were transferred to 9 cm Petri dishes containing PDA media, where they were incubated in the dark at 25 ℃ for three weeks.

1.4 Inoculation experiment

Ripe capsules of *Dendrobium nobile* were collected in September 2010 from a cultivation base in Puer (22.78° N, 100.97° E), Yunnan Province, China. Intact capsules were washed with tap water and surface-sterilized with 75% ethanol. They were then soaked in a 0.1% $HgCl_2$ solution for 10 min, and rinsed three times with sterile distilled water. Afterward, they were blotted with sterile filter paper and split. The seeds were sown into culture bottles (8 cm diam) containing 100 mL of a Harvais medium (autoclaved beforehand at 121 ℃ for 30 min) and incubated in the greenhouse (12-h photoperiod, 50 μmol · m^{-2} · s^{-1}, 26±1℃). Germinated seedlings were aseptically transplanted into new medium and continually differentiated new seedlings.

Eight uniform seedlings of *D. nobile* were implanted into each new culture bottle (8 cm diam), which contained 100 mL of 1/2 MS media supplemented with 0.75% sucrose and 0.75% agar after weighing (Hou and Guo, 2009). For the inoculation treatment, one mycelial plug (6 mm diam), cut from the margin of a fungal colony (either S1 or S3), was placed in the middle of each bottle. As our control treatment, the same agar plugs were used, but without the fungal addition. Each treatment comprised 25 replicates, which were kept in the greenhouse for two months.

1.5 Visualization of fungal infection in roots

The fungal hyphae were stained with a chitin-specific dye, i.e., the wheat germ agglutinin-alexa fluor (WGA-AF) 488 conjugate (Molecular Probes, Karlsruhe, Germany). Fungal infections were observed as a previously described (Doehlemann *et al.*, 2008) with some modifications to the technique. Briefly, at two months post-inoculation, the host roots were incubated in the staining solution for 60 min. During this period, the solution was vacuum-infiltrated three times (3 min each) at 25 mm of Hg. After the samples were rinsed three times in 1×PBS (phosphate buffer saline; pH 7.4), they were transferred into a Propidium Iodide (PI) solution (20 μg · mL^{-1}) for 3 min, then rinsed three times in 1×PBS (pH 7.4). Finally, the samples were mounted on glass slides.

Images were recorded from a laser scanning confocal microscope (LSCM; OLYMPUS FV1000). The WGA-AF 488 was excited with a 488-nm laser and detected at 500 to 540 nm. The PI was excited with a 559 nm laser and detected at 580 to 619 nm.

1.6 Experimental design for ^{15}N stable isotope signature in a microcosm

Our experimental microcosms were lidded culture bottles. Each microcosm contained 100 mL of a 1/2 MS medium supplemented with 5 mg of ^{15}N-labelled glycine, 0.75% sucrose, and 0.75% agar. Before the experiments began, the seedlings were

trimmed to provide uniformly sized materials. Three inoculation treatments were established (S1, S3, and control), with each applied as described for our inoculation experiment. A mycelia plug was placed near the roots of each seedling, so that suitable infections would occur as soon as possible. Replicate treatments ($n=6$) were performed to compensate for any contamination. The experiments were carried out in the greenhouse (12 h photoperiod, 50 μmol·m^{-2}·s^{-1}, 26±1 ℃) for two months.

1.7 Analysis of ^{15}N stable isotope

After two months, the plants were harvested from each bottle and dried at 80 ℃ for 48 h. They were then ground to a fine powder to obtain a representative subsample of tissue for further analysis. Using the protocol of Liebel and Gebauer (2011), we measured the relative abundances of the N isotope with an isotope ratio mass spectrometer (Delta V Advantage; Thermo Fisher Scientific, Inc., USA) at the Institute of Desertification Studies, China Academy of Forestry. Three test substances of varying sample weight were routinely analyzed within each treatment. The maximum variation in δ^{15}N was always below 0.2‰.

1.8 Determination of dendrobine percentages

We extracted and quantified the levels of dendrobine according to the method of Li *et al*. (2009), with some modifications. Briefly, approximately 0.3-g dry samples of *D. nobile* from each treatment were soaked for 30 min in 2 mL of ammonium hydroxide. Afterward, 50 mL of chloroform was added. The samples were weighed and then extracted refluently for 3 h. An appropriate amount of chloroform was added to each sample to compensate for any weight loss. The filtrate was evaporated under vacuum. After the residue was dissolved with methanol, it was transferred to a 1 mL volumetric flask and diluted to constant volume with methanol. The liquid was passed through a 0.45 μm microfiltration membrane, and the dendrobine content of the filtrate was determined via liquid chromatography-mass spectrometry (LC-MS). The LC separation was conducted on a Zorbax SB column (4.6 mm×250 mm i.d., 5.0 μm). The elution comprised 25% acetonitrile and 75% water with 0.2% formic acid at a flow rate of 1.0 mL min^{-1}. In addition to an injector volume of 10 μL, the LC-MS conditions included capillary voltage, 2 500 V; cone voltage, 30 V; temperatures of desolvation, 350 ℃; and gas flow rate, 400 L h^{-1}. Peak retention times were compared with that of the dendrobine standard. For all treatments, we used regression equations to calculate the dendrobine percentages in terms of their peak areas. Each treatment was repeated three times.

1.9 Data collection and statistical analysis

Two months after inoculation, the plants were harvested to record their heights, main stem lengths and diameters, internodal lengths and numbers of nodes. For each culture bottle, fresh weights were obtained for all eight plants, and the numbers of new roots and tillers were tallied. Those plants were then dried for 48 h at 80 ℃ before determining their dry weights. The increment in fresh weight per bottle was calculated as the difference in plant weight before and after inoculation. All data analyses were performed with the statistical package SPSS 17.0. Differences among treatments and control groups were tested with a one-way analysis of variance (ANOVA), followed by tests for least significant differences (LSD). Values were presented as means ± standard errors (SE).

2 Results

2.1 Morphological characterization of mycorrhizal fungi

Colonies of isolate S1 were light-yellow with concentric rings, lacked aerial hyphae, and assumed a leathery texture within one month (Fig. 1A). The hyphae were septate, and 2.5 to 5.5 μm in diameter, with nearly right-angled branches. The bases of those branches were constricted (Fig. 1B). Ellipsoidal monilioid cells were 4.0 to 10.5 μm by 9.0 to 12.0 μm (Fig. 1C). By contrast, S3 colonies were white and presented a cottony texture with age.

White aerial hyphae were well developed along the submerged margins on PDA medium (Fig. 1D). The hyphae were hyaline, septate, 2.0 to 5.0 μm in diameter, with nearly right-angled branches. Those branch bases were also constricted (Fig. 1E). Monilioid cells (Fig. 1F) were obovoid, fusiform or irregularly ellipsoidal (6－10 μm×8－22 μm). Because of their cultural and morphological characteristics (Nontachaiyapoom *et al.*, 2010), both isolates were identified as being *Rhizoctonia*-like fungi.

2.2 Molecular identifications of isolates

We amplified and sequenced the rDNA ITS region of S1 and the mtLSU region of S3. BLAST searches revealed that the ITS sequence of S1 shared high identity (96%; 602/625) with that of *Epulorhiza* sp. (Table 1). The mtLSU sequence of S3 shared 98% identity (316/321) with uncultured *Tulasnella* sp. and 96% identity (317/330; 314/327) with *Tulasnella* sp. (Table 2). Based on their morphological and molecular data, we identified S1 and S3 as *Epulorhiza* sp. and *Tulasnella* sp., respectively.

2.3 Visualization of fungal infection

After two months of inoculation, the S1 and S3 hyphae had spread over the root surfaces, invaded the cortical cells, and colonized the root intracellular

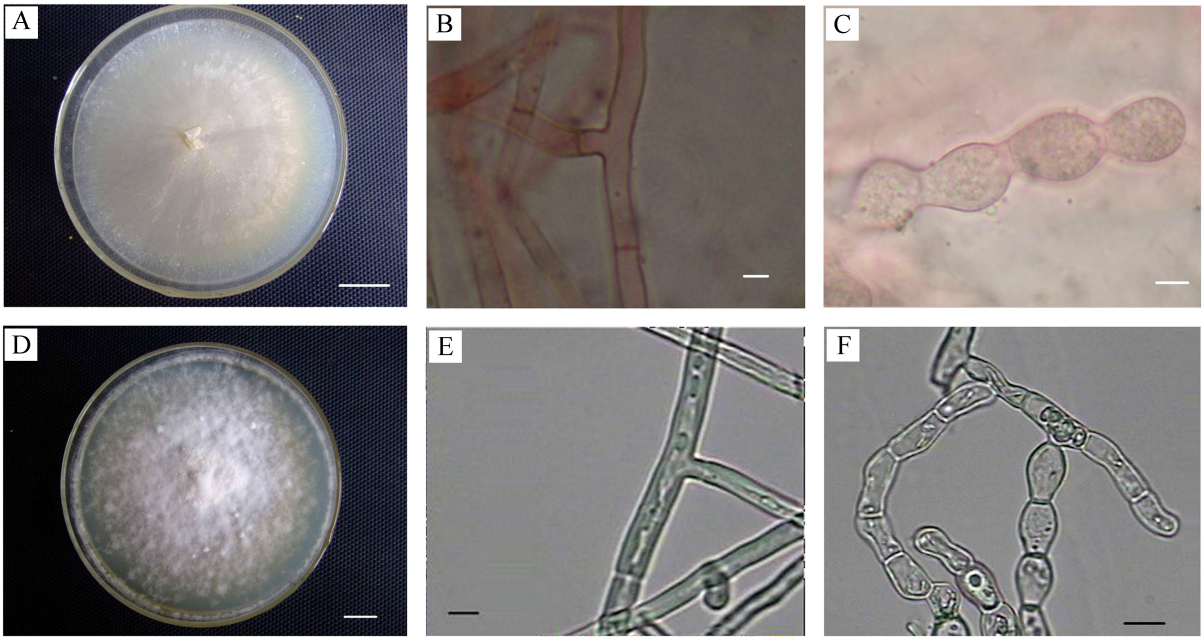

Fig. 1　Cultural and morphological characteristics of mycorrhizal fungi on PDA media. Isolate S1: A, Colony (Scale bar=1 cm); B, hyphae (Scale bar=2 μm); C, ellipsoidal monilioid cells (Scale bar=2 μm) Isolate S3: D, Colony (Scale bar=1 cm); E, hyphae (Scale bar=4 μm); F, monilioid cells (Scale bar=10 μm)

Table 1　Blast results for the S1 isolate based on its ITS region

Strain	Percent identity	Gap	Reference
FJ594913 *Epulorhiza* sp.	96% (602/625)	2% (10/625)	Li *et al.*, unpublished
FJ594914 *Epulorhiza* sp.	96% (601/624)	2% (10/624)	Li *et al.*, unpublished
FJ594919 *Epulorhiza* sp.	96% (600/623)	2% (10/623)	Li *et al.*, unpublished
FJ594918 *Epulorhiza* sp.	96% (598/621)	2% (10/621)	Li *et al.*, unpublished
FJ594916 *Epulorhiza* sp.	96% (599/624)	2% (10/624)	Li *et al.*, unpublished
FJ594915 *Epulorhiza* sp.	96% (594/617)	2% (10/617)	Li *et al.*, unpublished
FJ594917 *Epulorhiza* sp.	96% (595/619)	2% (12/619)	Li *et al.*, unpublished
FJ594912 *Epulorhiza* sp.	96% (594/618)	2% (11/618)	Li *et al.*, unpublished
GQ241863 uncultured Tulasnellaceae	96% (597/620)	1% (8/620)	Yuan *et al.*, 2010

spaces of inoculated seedlings (Fig. 2A, B). In the cortical region, the hyphae penetrated through the cell walls and entered next to the cortical cells (Fig. 2C). Under the LSCM, no fungal hyphae were observed within root cells from the control (non-inoculated) seedlings (Fig. 2D).

Table 2 Blast results for the S3 isolate based on its mtLSU region

Strain	Percent identity	Gap	Reference
AY192522 uncultured *Tulasnella* sp.	98% (316/321)	0 (0/321)	Bidartondo *et al.*, 2003
AY192520 uncultured *Tulasnella* sp.	98% (316/321)	0 (0/321)	Bidartondo *et al.*, 2003
AY192514 uncultured *Tulasnella* sp.	98% (316/321)	0 (0/321)	Bidartondo *et al.*, 2003
AY192512 uncultured *Tulasnella* sp.	98% (316/321)	0 (0/321)	Bidartondo *et al.*, 2003
AY192511 uncultured *Tulasnella* sp.	98% (316/321)	0 (0/321)	Bidartondo *et al.*, 2003
AY382811 *Tulasnella pruinosa*	98% (318/326)	0 (1/326)	McCormick *et al*, 2004
AF345560 *Tulasnella irregularis*	98% (312/320)	0 (1/320)	Kristiansen *et al.*, 2001
AY382794 *Tulasnella* sp.	96% (317/330)	1% (2/330)	McCormick *et al.*, 2004
DQ834411 *Tulasnella* sp.	96% (314/327)	1% (2/327)	Porras-Alfaro and Bayman, 2007

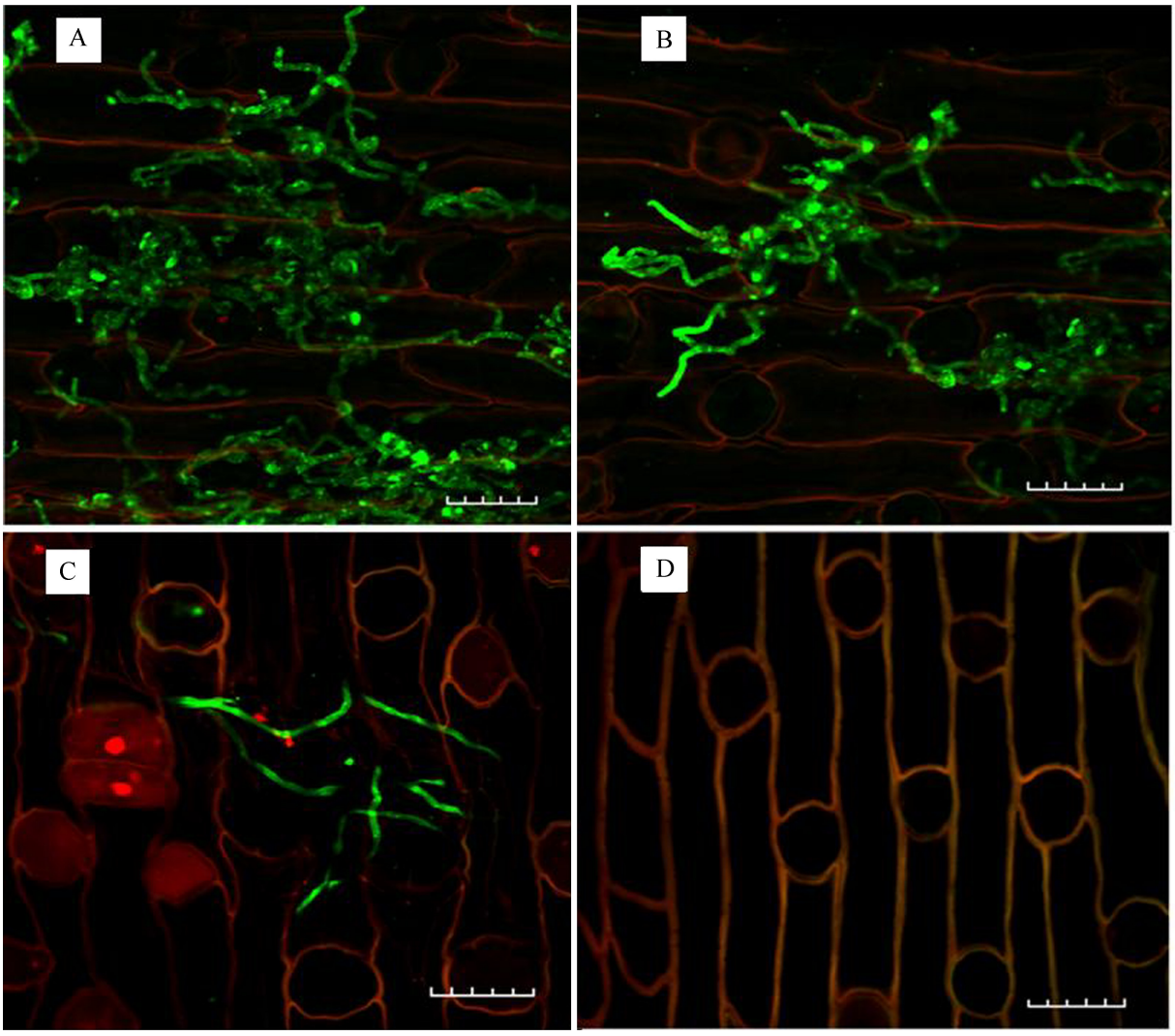

Fig. 2 Fungal infection in *Dendrobium nobile* seedlings. A. Inoculation with S1; hyphae and hyphal coils (green) in root cortical cells; B. Inoculation with S3; hyphae and hyphal coils (green) in root cortical cells; C. Inoculated with S1; hyphae (green) penetrating cell wall and invading neighboring cells in root; D. Root cortical cells from non-inoculated control seedlings. Scale bar = 50 μm

2.4 Growth responses by *D. nobile* to fungal inoculation

At two months after inoculation, growth was enhanced for the host plants (Table 3). Compared with the untreated control, increment of fresh weights and internodal lengths were significantly increased for seedlings inoculated with either S1 or S3 fungi. However, the numbers of nodes were not significantly different among control and treated plants. Dry weights, stem lengths and diameters and numbers of tillers were significantly higher for S1- inoculated seedlings than for the control. However, plant heights and numbers of new roots did not differ significantly between the two types. When the control and S3-inoculated seedlings were compared, no significant differences were found with dry weights, stem lengths and diameters, and numbers of tillers. Values for all growth characteristics, except plant heights and numbers of nodes, were significantly higher for S1- inoculated seedlings than for S3- inoculated seedlings.

The S1 and S3 fungi were re-isolated from the *D. nobile* roots at two months post-inoculation. Their cultural and morphological characteristics were the same as those recorded for the original isolates. We also sequenced their ITS-5.8S rDNA sequences and mtLSU sequences from the inoculated roots, and verified that the re-isolated fungi were the same as those used for the first inoculations.

2.5 Nitrogen utilization by different treatments

The ^{15}N derived from ^{15}N-labelled glycine was readily detectable in the inoculated and non-inoculated seedlings (Fig. 3). Although the $\delta^{15}N$ value in the S1- inoculated seedlings was significantly higher than that in the control and S3-inoculated seedlings, that value did not differ significantly between the control and S3- inoculated seedlings.

2.6 Dendrobine contents

Infection with mycorrhizal fungi considerably increased the levels of dendrobine by 60 d post-inoculation (Fig. 4). Contents were about 25- (S1) and 2- fold (S3) higher than in the control seedlings. In addition, the percentage of dendrobine was significantly higher in S1-inoculated seedlings than in those exposed to the S3 isolate.

3 Discussion

Both S1 and S3 isolates were identified as being *Rhizoctonia*-like fungi. Because mycelia from that type do not yield many distinguishing characters, identification is generally not possible below the generic level (Rasmussen, 2002). Thus, we used two genes to aid in our examination. Based on the sequences in the NCBI database and effective primers (Bruns *et al.*, 1998; Ma *et al.*, 2003) for their ITS-5.8S rDNA and the mtLSU rDNA sequences, we were able to identify S1 and S3 as being species within *Epulorhiza* and *Tulasnella*, respectively. These have previously been reported as mycorrhizal fungi of orchid plants (Ma *et al.*, 2003; Nontachaiyapoom *et al.*, 2010).

Table 3　Influence of S1 and S3 fungal isolates on the growth of *Dendrobium nobile* seedlings ($n=25$)

Parameter	S1	S3	Control
Increment of fresh weight/g	0.88 ± 0.06b	0.72 ± 0.05c	0.50 ± 0.03a
Dry weight/g	0.08 ± 0.00b	0.04 ± 0.00a	0.03 ± 0.00a
Plant height/cm	2.37 ± 0.06ab	2.10 ± 0.08b	2.54 ± 0.14a
Stem length/cm	1.29 ± 0.04b	1.08 ± 0.05a	1.03 ± 0.03a
Stem diameter/cm	0.21 ± 0.01b	0.18 ± 0.01a	0.16 ± 0.01a
Number of nodes	3.33 ± 0.12a	3.16 ± 0.13a	3.23 ± 0.09a
Internodal length/cm	0.36 ± 0.01b	0.32 ± 0.01c	0.28 ± 0.01a
Number of tillers	17.28 ± 0.79b	12.65 ± 0.76a	11.00 ± 0.81a
Number of new roots	22.84 ± 1.60a	17.15 ± 1.05b	22.80 ± 1.48a

Different letters within the same row indicate that values (mean±standard error) are significantly different among treatments at $P<0.05$, based on LSD tests. Control, non-inoculated seedlings

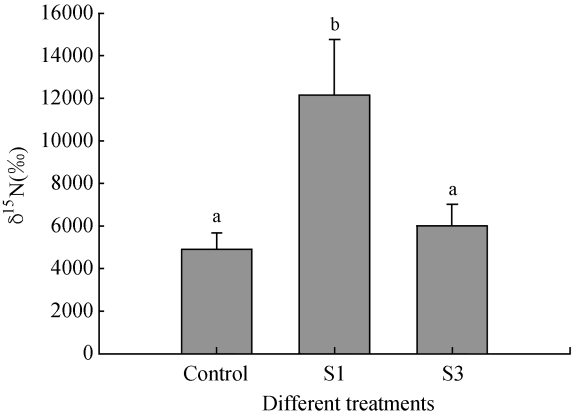

Fig. 3 $\delta^{15}N$ calculated from inoculated and non-inoculated (control) seedlings of *Dendrobium nobile* ($n=3$). Values (mean ± standard error) not followed by the same letter are significantly different at $P<0.05$, based on LSD tests

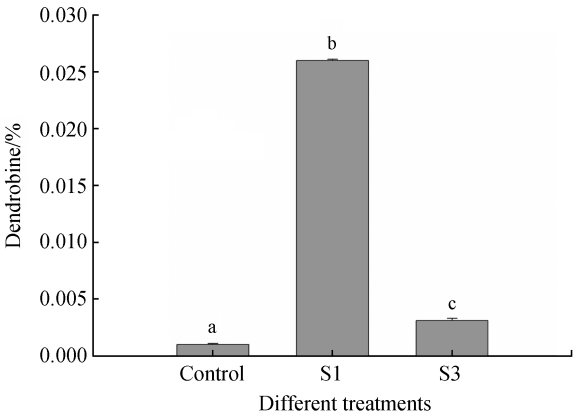

Fig. 4 Effects of mycorrhizal fungi treatment on dendrobine contents in *Dendrobium nobile* seedlings ($n=3$) Values (mean ± standard error) not followed by the same letter are significantly different at $P<0.05$, based on LSD tests. Control, non-inoculated seedlings

Although *Epulorhiza* sp. is known to stimulate biomass accumulations by plants of *D. nobile* (Song and Guo, 2001; Chen and Guo, 2005), the effects of *Tulasnella* sp. on *Dendrobium* have rarely been described (Nontachaiyapoom *et al.*, 2011). Some earlier studies have demonstrated that *Tulasnella* sp. promotes seed germination and growth by *Vanilla* sp. and *Pecteilis susannae* (Porras-Alfaro and Bayman, 2007; Chutima *et al.*, 2011). Our fingdings indicated that both S1 and S3 successfully colonized the roots of *D. nobile* and formed some mycorrhizal structures. Moreover, both fungi had positive influences on the growth of their host plants.

The S1- inoculated seedlings utilized significantly more organic N from amino acids in comparison with the control seedlings. However, the number of newly initiated roots was not significantly different between the two. Thus, most of the measured uptake of amino acids was probably by the fungi rather than the roots (Jones *et al.*, 2005). We also deduced here that more of the acquired organic N was transferred to the seedlings by the fungi. By contrast, $\delta^{15}N$ values did not differ significantly between the S3 -inoculated and control seedlings. This suggested that S1 and S3 fungi have different nutrient roles within the same host. Similarly, Midgley *et al.* (2006) have shown that the ability of a host plant to access nutrients from organic substrates depends upon the capacity of its associated fungi.

Both S1- and S3- inoculated seedlings performed better than the control, demonstrating that N-availability is of great importance. The supply of nitrogen has very marked effects on stem and leaf growth, and number of tillers. This is manifested by higher leaf-N contents and larger leaf areas. Up to 75% of the leaf N is found in the chloroplasts, with most of that being invested in the production of ribulose bisphosphate carboxylase alone (Cechin and Fumis, 2004). Consequently, increasing the supply of N improves chlorophyll contents and Rubisco activity, thereby enhancing the rates of photosynthesis and biomass accumulation. Our data showed that considerably more organic N was acquired by the S1-inoculated seedlings than by the control. Thus, the higher rate of photosynthesis was linked with the promotion of plant growth. An abundant N supply increases the number of meristems and the formation of shoots (Lawlor *et al.*, 1989). Here, we investigated only the acquisition of organic N. However, inorganic-N nutrition is also critical, being obtained by the plants directly or else transferred through symbiotic fungi (Gebauer and Meyer, 2003). Thus, we be-

lieve that the growth of our S3-inoculated seedlings was possibly improved because more non-isotopically labelled inorganic N was acquired.

Our results also indicated that dendrobine contents were significantly increased in seedlings inoculated with either the S1 or S3 isolates. Chen and Guo (2005) have also reported that the total alkaloid content can rise by 18.3% when *D. nobile* seedlings are co-cultured with *Mycena*. However, earlier investigations have not focused on dendrobine, a main bioactive component. Here, dendrobine contents were significantly elevated within infected seedlings when compared with the control. This suggested, therefore, that the quality of tissue-cultured seedlings might be elevated by inoculation with mycorrhizal fungi. Although all mineral nutrients can affect the composition of plant tissues to some extent, N is particularly influential. Its increased availability can lead to greater concentrations of nitrogen-containing compounds, such as alkaloids (Johnson *et al.*, 1987). Similarly, we could conclude that the rise in dendrobine contents in the inoculated seedlings was due to better acquisition of organic and/or inorganic N by the symbiotic fungi.

In summary, our data demonstrate that both S1 and S3 isolates form mycorrhizal associations with *D. nobile*. Moreover, seedling growth, organic-N acquisitions, and dendrobine accumulations can be promoted through these infections. Nevertheless, although those fungi have been proven beneficial to this photosynthetic, epiphytic orchid under laboratory conditions, further examinations are necessary to determine whether this symbiotic relationship can also influence plants of this species in the field. We must also continue to study the mechanism by which dendrobine contents are increased in inoculated plants.

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