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Host-specificity of symbiotic mycorrhizal fungi for enhancing seed germination, protocorm formation and seedling development of over-collected medicinal orchid, *Dendrobium devonianum*

Hui Huang[1†], Xiao-Meng Zi[1†], Hua Lin[1], and Jiang-Yun Gao[2*]

[1]*Center for Integrative Conservation, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan 666303, P. R. China*
[2]*Laboratory of Ecology and Evolutionary Biology, State Key Laboratory for Conservation and Utilization of Bio-resources in Yunnan, Yunnan University, Kunming, Yunnan 650091, P. R. China*

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All orchids maintain an obligate relationship with mycorrhizal symbionts during seed germination. In most cases, germination-enhancing fungi have been isolated from roots of mature plants for conservation and cultivation purposes. To understand the germination biology of *Dendrobium devonianum*, an over-collected medicinal orchid, the seeds of *D. devonianum* were inoculated with a fungal strain (FDd1) isolated from naturally occurring protocorms of *D. devonianum* and two other germination-enhancing fungal strains (FDaI7 and FCb4) from *D. aphyllum* and *Cymbidium mannii*, respectively. The fungal strain was isolated from five protocorms of *D. devonianum* and identified as a species of the genus *Epulorhiza*. In germination trials, treatments with all of the three fungal strains showed a significant promoting effect on seed germination and protocorm formation, compared with the control treatment (no inoculation). However, FDd1 fungal strain showed the greatest effectiveness followed by FDaI7 and FCb4. For all inoculation and control treatments, seeds developed to protocorms regardless of the presence of illumination, whereas protocorms did not develop to seedlings unless illumination was provided. The results of our manipulative experiments confirmed the hypothesis that mycorrhizae associated with orchid seedlings are highly host-specific, and the degree of specificity may be life stage-specific under *in vitro* conditions. The specific mycorrhizal symbionts from protocorms can enhance restoration efforts and the conservation of orchids such as *D. devonianum*.

Keywords: *Dendrobium devonianum*, medicinal orchid, restoration-friendly cultivation, symbiotic seed germination

[†]These authors contributed equally to this work.
*For correspondence. E-mail: jiangyun.gao@ynu.edu.cn; Tel.: +86-871-65030650; Fax: +86-871-65030650
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Introduction

The Orchidaceae is one of the largest plant families with more than 25,000 species globally (Chase *et al.*, 2015), but is also among the most threatened of all flowering plants. Many genera are threatened, and almost all genera contain at least some species under threat from habitat loss and over-collection (Swarts and Dixon, 2009). China is an orchid-rich country with 1,447 recorded species, mainly from the tropical and subtropical regions in the south and southwest (Zhang *et al.*, 2015). In addition to recent habitat loss resulting from the country's rapid economic growth and rural development, over-collection is a particularly serious threat to wild orchids in China, especially in some "hotspot" areas (Liu *et al.*, 2014, 2015). This is principally because there is a long history of using orchid species in traditional Chinese medicine (TCM), and about 350 species (approximately 25%) of Chinese orchids are used in TCM, 97 of which are Chinese endemics (Luo *et al.*, 2003; Liu *et al.*, 2014). Among these species, many species of *Dendrobium* (Shi-Hu in Chinese) are very popular TCM herbs both in prescribed medicine and as a health food supplement. In consequence many populations of these species have been exploited to the point of local extirpation (Liu *et al.*, 2014).

Two methods have been suggested to reduce harvesting pressure of over-exploited wild species: increasing supply and reducing demand (Wilkie and Godoy, 2001). For medicinal *Dendrobium*, the Shi-Hu industry has developed rapidly in southern China since the 1990s as a result of massive commercial cultivation, but this has not alleviated pressure on wild populations. This reflects a consumer preference for wild-collected materials and the fact that wild collection maintains the livelihoods of local suppliers who do not have the capital to invest in rearing facilities. Preference for wild-collected medicines by Chinese consumers reflects beliefs that they are more effective and contaminant-free than cultivated materials (Gao *et al.*, 2014). The continuing demand for wild grown materials has led to the proposal of a restoration-friendly cultivation model for epiphytic medicinal orchids, especially for *Dendrobium* species, in which orchids are planted in natural settings (Liu *et al.*, 2014). This may facilitate the conservation of threatened species, encourage protection of natural forests, and benefit marginalized rural communities (Gao *et al.*, 2014; Liu *et al.*, 2014).

Germination of orchid seeds without fungal symbionts ('asymbiotic' cultivation) is the most straightforward way of producing seedlings in large quantities (Stewart and Kane, 2006), and has been successfully and widely applied in many

orchids for both commercial production and conservation (*e.g.*, Wright *et al.*, 2009; Decruse *et al.*, 2013; Chen *et al.*, 2015). In some cases, however, the slow growth and high mortality of transplanted asymbiotic seedlings have inhibited restoration-friendly cultivation (Zeng *et al.*, 2003). The main reason for this failure has been the absence of symbiotic relationships with mycorrhizal fungus in seedlings upon transplantation, or the inability to establish these associations after reintroduction (Smith *et al.*, 2015).

In principle, therefore, symbiotic seedlings (that is: seedlings carrying appropriate mycorrhizae internally) should be more suitable for reintroductions. They develop faster and have higher survival rates and are more likely to establish in the nature (Bustam *et al.*, 2014). Symbiotic seed germination has practical merit for horticulture and species recovery, and is considered as an effective conservation tool for orchids (Stewart *et al.*, 2003; Batty *et al.*, 2008; Otero *et al.*, 2013). The challenge, then, is to obtain compatible fungi for symbiotic seed germination. In most reported cases, the fungi have been isolated from roots of mature plants (*e.g.*, Zettler and Hofer, 1998; Stewart and Zettler, 2002; Massey and Zettler, 2007). There are, however, a high diversity of root-associated fungi and this method may not provide the suitable species that are important for the orchids' germination stage. Recent studies have suggested that orchids in the seed germination stage require more specific mycorrhizal association, and non-compatible fungi may stimulate germination per se, but not support subsequent seedling development (Bidartondo and Read, 2008; Zi *et al.*, 2014; Rasmussen *et al.*, 2015). For all of these reasons, an increasing number of studies in recent years have focused on isolating specific fungi from protocorms rather than roots, and the use of protocorms is found to be more efficient in isolating germination-enhancing fungi (Wang *et al.*, 2011; Sheng *et al.*, 2012; Zi *et al.*, 2014).

Xishuangbanna is located on the northern margins of tropical Asia in southwestern China and is one of the most orchid-rich areas in China. A total of 426 orchid species in 115 genera have been identified in the area including 48 *Dendrobium* species, most of which were widely distributed in the area, but have been massively collected in the past 30 years (Liu *et al.*, 2015). *Dendrobium devonianum* is one of the most important medicinal species used in TCM, and the scale of its commercial cultivation is second only to *D. officinale* in China (Gao *et al.*, 2014). To enhance restoration-friendly cultivation of *D. devonianum*, we sought to isolate appropriate fungi for symbiotic seed germination from naturally occurred protocorms. Here we present our results, addressing three principal questions: (1) does *D. devonianum* have a highly specific relationship with mycorrhizae at the seed germination stage? (2) does the species-specific mycorrhizae extracted from the *D. devonianum* protocorms present more effectiveness on the success or otherwise of germination and subsequent seedling development than fungi isolated from related and/or more distant species? (3) in addition to the fungi, what is the role of incident light in further enhancing protocorm development and seedling establishment?

Materials and Methods

Species, seeds and protocorm samples of *Dendrobium devonianum* Paxton

Dendrobium devonianum Paxton is an epiphytic orchid widely distributed in the subtropical and tropical areas of SE Asia, including southwest China at altitudes from 1100 to 1600 m. In Xishuangbanna, it flowers from April to May and seeds mature in the following March (Gao *et al.*, 2014). The protocorms of *D. devonianum* we used in this study were harvested in a traditional tea garden at Jinuo village, Jinghong (21°59′05″ N; 101°05′07″ E; alt. 1150 m; Fig. 1A), in which *D. devonianum* grew naturally on tea trees (Fig. 1B).

Sampling took place in July 2012 when we found a small patch mixed with seedlings and protocorms on a branch of tea tree close to maternal plants of *D. devonianum* (Fig. 1C). Based on the observations on morphological character of

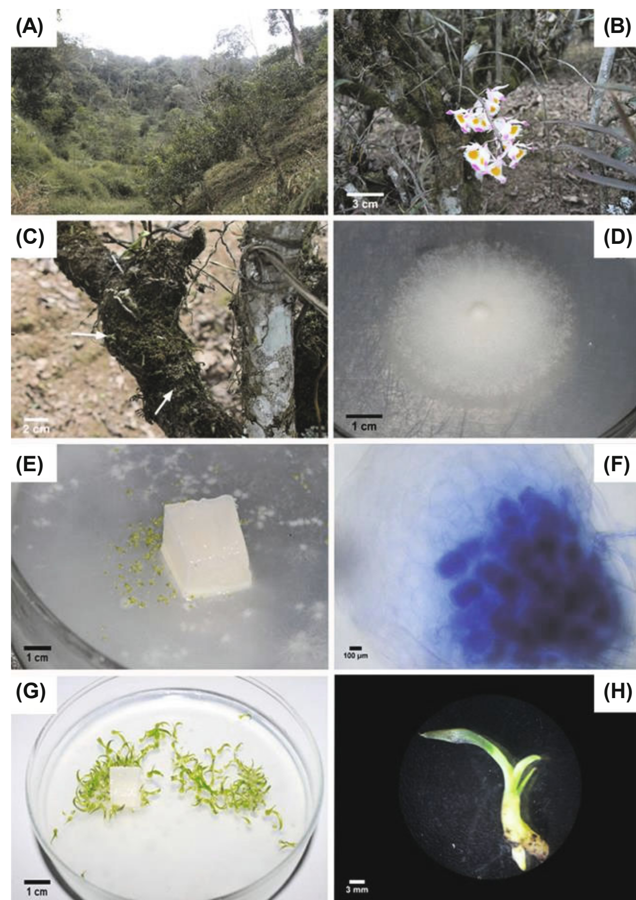

Fig. 1. **Sample site, inflorescence, fungal strain, protocorms, and seedlings of *Dendrobium devonianum*.** (A) Traditional tea garden in Longpa; (B) Inflorescence of *D. devonianum* which naturally grow in tea-plant tree; (C) Seedlings of *D. devonianum* in tea-plant tree, and arrows show the two-leaved seedlings and one-leaved seedling which just go through the protocorm stage; (D) Morphology characteristic of strain FDd1 (*Epulorhiza*) in PDA medium; (E) Protocorms of 20 days' inoculation; (F) The status of fungal colonization in protocorm of *Dendrobium devonianum* inoculated with FDd1; (G) Seedlings after 50 days' inoculation with FDd1 under light condition; (H) A single seedling with root after 50 days' inoculation with FDd1 under light condition.

seedlings and our monitoring on population dynamics of all orchids in this tea garden in the following 3 years, we assumed that both seedlings and protocorms acquired were those of *D. devonianum*. A total of 5 naturally occurred protocorms were obtained, and stored in polyethylene bags mixed with humid moss and transported to the laboratory for the study.

The resulting mature, close-to-dehiscent capsules of *D. devonianum* were harvested in the Jinuo traditional tea garden in March 2012, and seeds were dried and stored in the orchid seed bank using the same methods we have used in previous studies (Gao *et al.*, 2014).

Fungal isolation and identification

Protocorms were surface-sterilized using 1% (w/v) sodium hypochlorite solution (NaClO) for 3–5 min, and then rinsed 3–4 times with sterile water. One protocorm was cut into two halves and placed on potato dextrose agar (PDA) in Petri dishes under 25.0 ± 2.0°C. After 3–5 days, once fungal hyphae had emerged from the edge of the broken protocorms, the hyphae were excised from the medium and transferred to new plates containing fresh PDA and incubated at 25.0 ± 2.0°C in the dark. Fungal colonies from actively growing isolates were sub-cultured by excising the hyphae tips onto fresh PDA Petri dishes 3–4 more times until purified strains were obtained.

Purified fungal strains were sequenced for identification using the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA). The purified fungal strains were cultivated for 5–7 days in liquid PDA medium in Erlenmeyer flasks placed on a shaker (SPH-310A, Shanghai Baidian Instrument & Equipment Co., Ltd.), and total DNA was extracted using Cetyl trimethyl ammonium bromide (CTAB) methods. The ITS region of rDNA was amplified using the polymerase chain reaction (PCR) with ITS1 and ITS4 primers (White *et al.*, 1990) on a PCR Thermocycle Instrument (GeneAmp9700, Applied Biosystems). PCR reactions were followed the methods of our previous study (Zi *et al.*, 2014), and PCR products were purified and sequenced at Sangon Biotech Co., Ltd. All ITS-rDNA sequences obtained were compared with those deposited in the GenBank database (National Center for Biotechnology Information (NCBI) 2012) using the Basic Local Alignment Search Tool (BLAST) which allows the identification of isolates to the genus or species level when ITS sequence similarity exceeds 95% or 99%, respectively (Sanchez *et al.*, 2008).

Testing the fungal capacity in promoting seed germination

To test the capacity of isolated fungal in promoting seed germination in *D. devonianum*, the effectiveness of the fungal strain FDd1 obtained from protocorms of *D. devonianum* was compared with two other fungal strains: FDaI7 and FCb4. FDaI7 is a *Tulasnella* species and was isolated from protocorms of *D. aphyllum* - a congener of our focal species - and is known to enhance the seed germination of *D. aphyllum* (Zi *et al.*, 2014). FCb4 is an *Epulorhiza* species isolated from protocorms of *Cymbidium mannii*, and is known to enhance the seed germination of *C. mannii* (Sheng *et al.*, 2012).

Seeds of *D. devonianum* were sterilized with 1% (w/v) NaClO for 15 min and washed with sterile distilled water 3–4 times. Using a pipette, ca. 150 seeds, suspended in 150 μl agar solution, were transferred onto a Petri dish containing 20 ml of sterile 3 g/L oatmeal agar (OMA) medium adjusted to a pH of 5.6 before autoclaving. Once the seed transfer was completed, each dish was inoculated with one cubic centimetre of fungal inocula placed in the center of the Petri dish for each of the FDd1, FDaI7, and FCb4 treatments, or was maintained as a sterile control treatment without a fungal strain. To test the role of incident light, all treatment Petri dishes were randomly assigned to either a continuous dark treatment (0/24 h L/D) or a 12 h dark and 12 h light cycle (12/12 h L/D) at 25.0 ± 2.0°C. We used a cool white fluorescent light (cold cathode fluorescent lamp) with 2,000 $\mu mol/m^2/sec$ intensity for the period of light. All Petri dishes were assessed under the dissection microscope and the exact number of seeds was recorded for each Petri dish. Each fungal and light treatment was replicated in 18 Petri dishes which were placed in germination chambers (RXZ300B, Ningbo Southeast Instrument Co., Ltd.).

Six Petri dishes of each treatment were randomly selected to assess the seed germination and protocorm development status using a dissecting stereomicroscope (XTL-3400, Cany Precision Instruments Co., Ltd.) at 20, 50, and 90 days after incubation. Any Petri dishes (no more than 2 per treatment) that were contaminated with other fungi were discarded after visual examination.

To determine if mycorrhizal symbiosis were established *in vitro*, protocorms from FDd1 treatment were randomly selected, and examined for the presence of pelotons. Protocorms were cleared using 10% KOH solution, and washed with 1% HCl solution, and then stained in 0.05% (w/v) Trypan Blue in lactic acid overnight, following an adaptation of the procedure of Phillips and Hayman (1970). Stained pelotons were observed under microscope.

Data collection and statistical analysis

Seed germination and seedling development are generally divided into five stages (Table 1, adapted from Arditti, 1967). We used stages 0, 1, 2, and 4 to determine no germination, seed germination, protocorm formation, and seedling de-

Table 1. Description of different seed germination stages of *Dendrobium devonianum*

Seed germination stage	Description
0	No germination
1	Imbibed seed, swelled obviously and still covered by testa (germination)
2	Continued embryo enlargement, rupture of testa (protocorm formation)
3	Appearance of protomeristem (protocorm development)
4	Emergence of first leaf and continue development (early stage of seedling development)

Adapted from Arditti (1967)

Table 2. **The effect of fungi inoculation (CK, FDd1, FDaI7, FCb4,) and light condition (0/24 h L/D, 12/12 h L/D) on each seed germination stage in *D. devonianum* after twenty/fifty/ninety days**

	Twenty days			Fifty days			Ninety days		
	Seed Germination	Protocorm Formation	Seedling Formation	Seed Germination	Protocorm Formation	Seedling Formation	Seed Germination	Protocorm Formation	Seedling Formation
Factor (*Chi*^{2})									
Light (*df*=1)	43.36***	17.32***	ng	5.48	80.48***	200.90***	4.90*	156.22***	311.59***
Fungi (*df*=3)	200.32***	843.10***	ng	2.41*	483.77***	429.20***	8.99*	335.69***	161.47***
Light × Fungi (*df*=7)	439.14***	1063.30***	ng	17.50*	923.90***	775.63***	32.95***	743.13***	569.44***
Multiple comparisons (estimation ± SE)									
Light (12/12)_Dark (0/24)	0.67 ± 0.10***	0.34 ± 0.08***	ng	-0.32 ± 0.14*	0.80 ± 0.09***	2.60 ± 0.25***	0.30 ± 0.14*	1.16 ± 0.10***	3.10 ± 0.25***
FDd1_CK	1.93 ± 0.16***	4.59 ± 0.33***	ng	-0.20 ± 0.21	2.85 ± 0.16***	3.37 ± 0.39***	0.08 ± 0.23	1.15 ± 0.17***	1.96 ± 0.24***
FDaI7_CK	1.68 ± 0.15***	4.68 ± 0.33***	ng	-0.18 ± 0.21	2.89 ± 0.17***	0.12 ± 0.48	-0.19 ± 0.21	0.04 ± 0.14	0.83 ± 0.25**
FCb4_CK	0.77 ± 0.13***	2.78 ± 0.33***	ng	-0.32 ± 0.21	1.79 ± 0.16***	-0.13 ± 0.50	-0.43 ± 0.20	-1.30 ± 0.14***	-0.09 ± 0.28
FDaI7_FDd1	-0.26 ± 0.17	0.09 ± 0.12	ng	0.02 ± 0.18	0.05±0.13	-3.25 ± 0.30***	-0.27 ± 0.20	-1.11 ± 0.16***	-1.13 ± 0.15***
FCb4_FDd1	-1.16 ± 0.15***	-1.80 ± 0.12***	ng	-0.12 ± 0.17	-1.05 ± 0.12***	-3.50 ± 0.34***	-0.51 ± 0.19*	-2.45 ± 0.15***	-2.04 ± 0.19***
FCb4_FDaI7	-0.90 ± 0.14***	-1.90 ± 0.12***	ng	-0.14 ± 0.17	-1.09 ± 0.12***	-0.25 ± 0.44	-0.24 ± 0.16	-1.34 ± 0.12***	-0.91 ± 0.20***

The values presented with factor effect are produced by Generalised Linear Mixed Models (chi-squared statistic). Multiple comparisons are given for each inoculum in comparison to the uninoculated sterile control with the estimates and their standard errors.
df degrees of freedom, Chi2 chi-squared, SE standard error, ng no seeds germinated to that stage with or without the fungi inoculating
*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$

velopment, respectively. The number of total seeds (t), germinated seeds (g), protocorms (p), and seedlings (s) were counted at 20, 50, and 90 days after incubation. The percentages of germinated seeds (G), protocorms (P), and seedlings (S) were calculated as: $G = (g + p + s) / t$, $P = (p + s) / t$ and $S = s / t$, respectively.

Generalised linear mixed models with a binomial distribution function were used to test the effectiveness of fungal inoculation, light condition and the interaction between these two factors. We coded each seed developmental stage as successful (= 1) or not successful (= 0) in reaching a particular developmental stage. Post-hoc pairwise comparisons were also made using Tukey tests. All analyses were performed in R (version 3.1.1).

Results

Isolating and identifying fungi

From five naturally occurred protocorms, only one fungal strain (FDd1) was isolated successfully. Combing morphological and molecular identification based on maximum similarity of ITS sequences of rDNA, stored in the NCBI gene database, the fungal strain was identified as a species of *Epulorhiza* (Fig. 1D). FDd1 fungal strain had 95% similarity with the fungus *Epulorhiza* Accession Number AJ313443.1 in the NCBI gene database.

The effects of different treatments on seed germination, protocorm formation, and seedling development

Twenty days after incubation : In the light treatments, there were no significant differences in seed germination across all treatments. In the dark treatments, however, the percentages of seed germination were all significantly higher in all three fungal inoculation treatments (FDd1, FDaI7, and FCb4) than in the control (Table 2 and Fig. 2). Protocorm formation was significantly enhanced with FDd1 or FDaI7 treatments (Fig. 1E), whereas FCb4 treatment was only effective under the light treatment (Table 2 and Fig. 2). No seedlings were developed in any treatment at this stage.

There was no significant difference in either seed germination or protocorm formation between light and dark treatments for the FDd1 treatment. For the FCb4 and FDaI7 treatments, light treatment either significantly decreased (FDaI7)

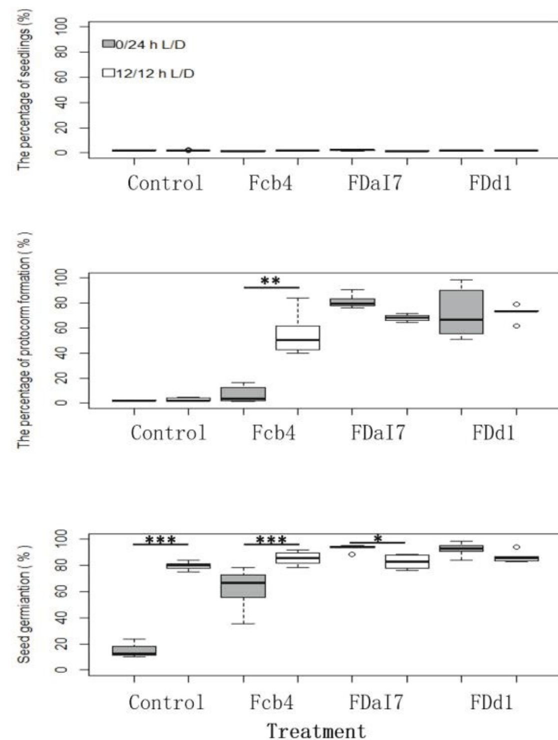

Fig. 2. **The effects of different treatments on each seed germination stage after 20 days' inoculation.** Asterisk denote statistical differences between light/dark treatment according to multiple comparisons (*** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$).

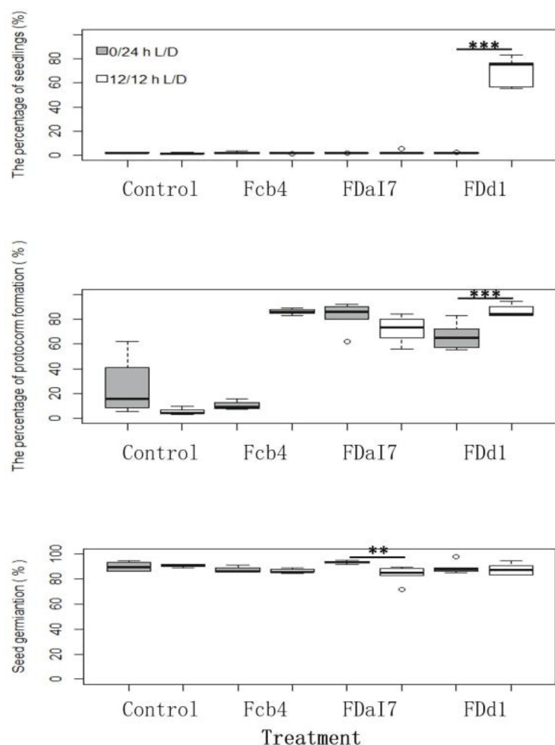

Fig. 3. The effects of different treatments on each seed germination stage after 50 days' inoculation. Asterisk denote statistical differences between light/dark treatment according to multiple comparisons (*** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$).

or increased (FCb4) in both seed germination and protocorm formation (Fig. 2). For protocorms of FDd1 treatment, the stained pelotons can be clearly observed under microscope at this stage (Fig. 1F).

Fifty days after incubation : At this stage, most seeds germinated under any treatments and there were no significant differences between the control and any of the inoculated treatments. The percentage of protocorm formation, however, differed significantly among treatments (Table 2 and Fig. 3). The percentages of protocorm formation were significantly higher in the FDd1 and FDaI7 treatments than in the control. For the FCb4 treatment, however, the protocorm formation was significantly higher only under the light treatment. In the control, protocorm formation was significantly lower under light than dark treatments (Table 2 and Fig. 3). Seedlings were only developed in the FDd1 and FDaI7 treatments under light (Fig. 1G), with the percentage of seedlings significantly higher in the FDd1 treatment (72.36 ± 11.7%) than the FDaI7 treatment (0.74 ± 1.7%; Table 2 and Fig. 3). Some seedlings developed with roots in the FDd1 treatment at this stage (Fig. 1H).

Ninety days after incubation : At this stage most seeds had germinated and significantly larger proportions of protocorm development were observed in the FDd1, FDaI7 treatments. Significantly larger proportions of protocorm development was also observed in the FCb1 and control treatments provided that the seeds were in the light treatment. Seedlings had developed in all treatments under light, but no seedlings were found in the dark treatments (Fig. 4). The percentage of seedlings was highest in the FDd1 treatment (71.17 ± 15.64%), and this was significantly higher than in FDaI7 treatment (23.59 ± 24.52%), FCb4 treatment (9.08 ± 12.51%) and control treatment (8.75 ± 10.71%), suggesting that FDd1 is effective in promoting seedling development in *D. devonianum* under light conditions (Table 2 and Fig. 4).

Discussion

Symbiotic seed germination has been practically used in orchid recovery project worldwide and is considered as an effective way for orchid conservation (Stewart *et al.*, 2003; Batty *et al.*, 2008; Otero *et al.*, 2013). This is particularly useful in cultivating over-collected epiphytic medicinal orchids under natural conditions as a meaning of restoration-friendly cultivation (Gao *et al.*, 2014). To obtain compatible fungi is a key step for orchid symbiotic seed germination. Traditionally, the fungi were isolated and screened from roots of wild mature plants (*e.g.*, Zettler and Hofer, 1998; Stewart and Zettler, 2002; Massey and Zettler, 2007; Nontachaiyapoom *et al.*, 2010). Because there are a large numbers of fungi in the roots of wild mature plants that have little or unknown function, the process is extremely complex and the fungi may be obtained randomly in this way. Moreover, it is still not sure if the fungi in the roots of mature plants are compatible for seeds germination (Zettler *et al.*, 2005).

In this study, we sampled the 5 protocorms from a small patch

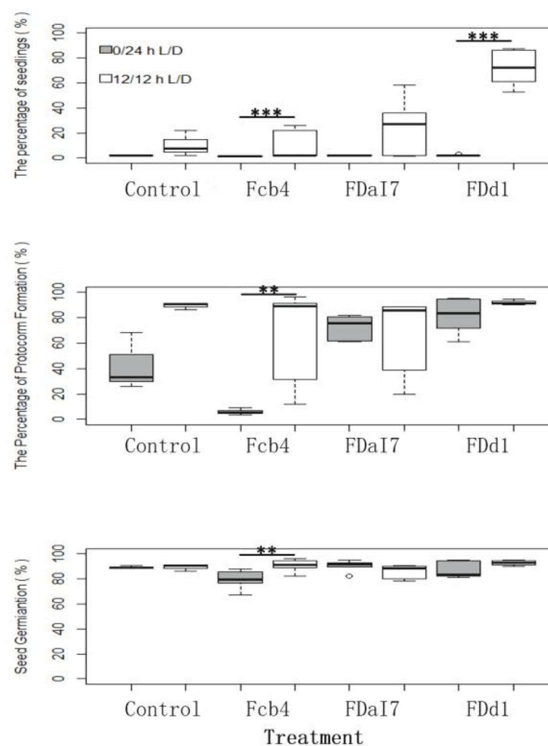

Fig. 4. The effects of different treatments on each seed germination stage after 90 days' inoculation. Asterisk denote statistical differences between light/dark treatment according to multiple comparisons (*** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$).

mixed with seedlings and protocorms on a branch of tea tree close to maternal plants of *D. devonianum*. Because in this study site, a traditional tea garden, we have been conducted different studies on orchids (e.g., Liu *et al*., 2015), including monitoring on population dynamics of all orchids, we observed that the seedlings and remaining protocorms all grew up to the plants of *D. devonianum* in the following years. However, to make sure the protocorms we sampled are from *D. devonianum*, molecular identification is still needed in future studies. Using the five naturally occurred protocorms, we successfully isolated only one orchid-seedling mycorrhizae (OSM) fungal strain, which suggested that *D. devonianum* holds a highly specific relationship with mycorrhizae at the seed germination stage. The fungal strain (FDd1) has been molecularly identified as a species of *Epulorhiza*, which is the teleomorphs stage of *Tulasnella*. Species of *Tulasnella* are among the most common symbiotic fungi in orchids. In our previous study, the fungal strain FDaI7, also isolated from protocorms and identified as a species of *Tulasnella*, can effectively increase seed germination and support subsequent seedling development in *D. aphyllum* (Zi *et al*., 2014).

In the laboratory experiment, at 90 days after incubation, all three fungal treatments showed significantly greater seedling development than the control treatment (Fig. 4 and Table 2), suggesting that *D. devonianum* may have a low *in vitro* specificity. However, the specific OSM fungi of *D. devonianum*, strain FDd1, was the most effective symbiont at the seedling stage (Fig. 4 and Table 2), indicating that this strain has high level of compatibility and specificity in seedling development stage of *D. devonianum*. Although, both FDd1 and FDaI7 fungal strains showed greater effectiveness on seed germination and subsequent seedling development than did the *Cymbidium*-related FCb4 and control treatments, FDd1 did much better than FDaI7, especially on seedling formation and development. At 50 days after incubation, the percentage of seedlings was significantly higher in FDd1 treatment (72.36 ± 11.7%) than in FDaI7 treatment (0.74 ± 1.7%), and kept stable subsequently as indicated by the percentage of seedlings at 90 days after incubation (71.17 ± 15.64%; Table 2, Figs. 3 and 4) . These results suggested that FDd1was the most effective fungi among the three fungi used in this study and capable of quickly promoting seedling formation and development of *D. devonianum* under light conditions. The quick seed germination and seedling formation might be vital to seedling establishment for epiphytic orchids under natural conditions. Because both FDd1 and FDaI7 fungal strains were obtained from protocorms of *Dendrobium* species, and belong to the genus *Epulorhiza*, our results also suggested a strong specific relationship between fungi FDd1and *D. devonianum* and supported the hypothesis that mycorrhizae associated with orchid seedlings are highly host-specific, and the degree of specificity can be life stage-specific under *in vitro* conditions.

In many studies, the fungi obtained from roots of wild mature plants have also been considered capable of promoting seeds germination (*e.g.*, Zettler and Hofer, 1998; Stewart and Zettler, 2002; Massey and Zettler, 2007; Nontachaiyapoom *et al*., 2010), however, the success of seed germination mainly depend on the stage 1–3 of the germination criteria (Arditti, 1967), and the status of seedling formation and development were not clear. More recent studies focused on isolating specific fungi from protocorms rather than roots, and suggested that orchids in the seed germination stage require more specific mycorrhizal association, and non-compatible fungi may stimulate germination per se, but not support subsequent seedling development (Bidartondo and Read, 2008; Zi *et al*., 2014; Rasmussen *et al*., 2015).

Light is often regarded as an inhibitory factor to germination in orchids as a seed needs to avoid desiccation before germination (Rasmussen *et al*., 2015). Although we found that *D. devonianum* seeds can germinate and form protocorms in the absence of light, seedling development required exposure to light. This effect has also been observed in the congeneric species, *D. officinale*, *D. nobile*, and *D. aphylum* (Wang *et al*., 2011; Zi *et al*., 2014), as well as in terrestrial orchids (Kauth *et al*., 2006). Seeds under the FDaI7 treatment, however, displayed enhanced development in darkness in the early stage of seed germination and protocorm formation at 20 days of inoculation (Fig. 2).

Conclusion

The integration of *in situ* conservation and reintroductions have been shown to be effective in protecting endangered orchid species. Improvement of such practices demands understanding of the symbiotic relationship between orchids and their mycorrhizae. We show that isolating fungal strains from protocorms can be effective in identifying and culturing germination-promoting strains. For *D. devonianum*, seed germination was greater with fungal isolates and incident light. However, effectiveness of fungal inoculation other than its own FDd1 was stage-specific, and seedling development was generally lower than that of FDd1.

In the previous studies, we successfully isolated germination-enhancing fungi using *in situ* seed-baiting in *Dendrobium aphyllum* (Zi *et al*., 2014). We suggest that naturally occurred protocorms could be used to isolate fungi for enhancing germination for the development of seedling production and that this could be effective for conservation and reintroduction.

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