Mycorrhiza
DOI 10.1007/s00572-014-0565-8

ORIGINAL PAPER

In situ seed baiting to isolate germination-enhancing fungi for an epiphytic orchid, *Dendrobium aphyllum* (Orchidaceae)

Xiao-Meng Zi · Chun-Ling Sheng · Uromi Manage Goodale · Shi-Cheng Shao · Jiang-Yun Gao

Received: 29 November 2013 / Accepted: 30 January 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract Orchid conservation efforts, using seeds and species-specific fungi that support seed germination, require the isolation, identification, and germination enhancement testing of symbiotic fungi. However, few studies have focused on developing such techniques for the epiphytes that constitute the majority of orchids. In this study, conducted in Xishuangbanna Tropical Botanical Garden, Yunnan, China, we used seeds of *Dendrobium aphyllum*, a locally endangered and medicinally valuable epiphytic orchid, to attract germination promoting fungi. Of the two fungi isolated from seed baiting, *Tulasnella* spp. and *Trichoderma* spp., *Tulasnella*, enhanced seed germination by 13.6 %, protocorm formation by 85.7 %, and seedling development by 45.2 % (all $P<0.0001$). *Epulorhiza*, another seed germination promoting fungi isolated from *Cymbidium mannii*, also enhanced seed germination (6.5 %; $P<0.05$) and protocorm formation (20.3 %; $P<0.0001$), but *Trichoderma* suppressed seed germination by 26.4 % ($P<0.0001$). *Tulasnella* was the only treatment that produced seedlings. Light increased seed imbibition, protocorm formation, and two-leaved seed development of *Tulasnella* inoculated seeds ($P<0.0001$). Because the germination stage success was not dependent on fungi, we recommend that *Tulasnella* be introduced for facilitating *D. aphyllum* seed germination at the protocorm formation stage and that light be provided for increasing germination as well as further seedling development. Our findings suggest that in situ seed baiting can be used to isolate seed germination-enhancing fungi for the development of seedling production for conservation and reintroduction efforts of epiphytic orchids such as *D. aphyllum*.

Keywords *Dendrobium aphyllum* · Orchid conservation · Seed baiting · Symbiotic germination · *Tulasnella*

Xiao-Meng Zi and Chun-Ling Sheng contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00572-014-0565-8) contains supplementary material, which is available to authorized users.

X.-M. Zi · C.-L. Sheng · U. M. Goodale · J.-Y. Gao
Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan 666303, China

X.-M. Zi · C.-L. Sheng
University of Chinese Academy of Sciences, Beijing 100049, China

S.-C. Shao · J.-Y. Gao (✉)
Center for Integrative Conservation, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan 666303, China
e-mail: gjy@xtbg.org.cn

C.-L. Sheng
China Forestry Publishing House, Beijing 100009, China

Introduction

Due to overexploitation, habitat loss from land conversion, and climate change (Hágsater and Dumont 1996; Swarts and Dixon 2009a; Koopowitz and Hawkins 2012), members of the family Orchidaceae, the largest and most diverse of all flowering plant families (Dressler 1993), are threatened in the wild (Hágsater and Dumont 1996; Seaton et al. 2013). In orchid conservation efforts aimed at reintroduction, seeds, as opposed to tissue-cultured and mass-produced orchid seedlings, are preferred because they conserve the genetic diversity that can ensure the success and sustainability of the reintroduced population (Seaton et al. 2013). Although orchids produce large numbers of minute seeds and the collection of mature fruits from wild populations is possible, many species of rare and endangered orchids have limited germination success under controlled laboratory conditions (Knudson 1922 and see current review by Dearnaley et al. 2012). In addition, orchid seed germination and seedling development needs suitable fungi to provide the minute, energy-limited

Published online: 23 February 2014

seeds with carbon, water and other nutrients (Harley 1950; Arditti 1967; Arditti and Ghani 2000; Yoder et al. 2000; Dearnaley 2007; Rasmussen and Rasmussen 2009). This mycoheterotrophic association is known to increases the probability of successful seedling establishment (Dearnaley 2007; Eriksson and Kainulainen 2011, Bernard 1909 cf Selosse et al. 2011).

It may be cost effective to directly sow seeds into suitable habitats that naturally contain the seed germination-enhancing fungi. However, this may have limited success because of the low encounter probability, phylogenetic specificity and environmental conditions that mediate the association between the appropriate fungi and orchid seeds (Swarts et al. 2010, but see contrary evidence in Phillips et al. 2011 and McCormick et al 2012). Germinating seeds together with the species-specific mycorrhizal fungi could improve the success of seed-based conservation programs, both in in situ germplasm conservation and in reintroduction efforts (e.g. Keel et al. 2011). However, this requires the isolation, identification and germination enhancement testing of the fungi and a good understanding of the factors that determine the symbiotic association between the orchid seeds and the fungi (Hágsater and Dumont 1996; Dearnaley et al. 2012).

There are biological and methodological barriers that limit successful isolation of germination-enhancing fungi. As orchid seeds go through several germination stages, from imbibition to development of two-leaved seedlings (Arditti 1967), their relationship with the symbiotic fungi can also change (Swarts et al. 2010) and depend on environmental conditions, especially light (Wang et al. 2011; Sheng et al. 2012; Kartzinel et al. 2013; Park and Lee 2013). Symbiotic fungal diversity in germinating seeds, developing protocorms (Chen et al. 2012), and seedlings (Bidartondo and Read 2008) is generally lower than in the roots of adult plants, indicating that germination stages may also require a more specific mycorrhizal association than the seedling growth or mature stages. Most studies focused on extracting "germination-enhancing fungi" screen fungi from roots of mature plants (Zettler and Hofer 1998; Stewart and Zettler 2002; Massey and Zettler 2007; Chen et al. 2012; Chutima et al. 2011) which can be difficult and time consuming and such isolates may not be species-specific and/or germination stage-specific, resulting in limited success in facilitating seed germination (Bidartondo and Read 2008; Swarts and Dixon 2009a). There are only a few studies that empirically test the effect of fungal isolates on seed germination stage specificity.

Seed baiting, using seeds wrapped in muslin cloth and placed in the habitat of the adult plants (Rasmussen and Whigham 1993), has been successfully used for investigating in situ and ex situ orchid seed germination and seedling development (Masuhara and Katsuya 1994; Perkins and McGee 1995; Rasmussen and Whigham 1998a, b; Brundrett et al. 2003; Bidartondo and Read 2008; Wang et al. 2011). Although epiphytic orchids constitute 73 % of the estimated 17,000–35,000 of tropical orchid species worldwide (Atwood 1986; Cribb et al. 2003), a majority of these studies have focused on terrestrial orchids (but see Wang et al. 2011).

Here, we report on the isolation, identification, and testing of the effect of symbiotic fungi and light on the success of each seed germination stage in an epiphytic orchid, *Dendrobium aphyllum* (Roxb.) C.E.C. Fischer. This investigation was conducted as part of a conservation research program focusing on preventing the extinction of rare and endangered orchid species. *D. aphyllum* is found from Nepal to Southern China and Peninsular Malaysia (Romand-Monnier 2013) and is heavily collected throughout the region for floricultural and medicinal use (Subedi et al. 2013). In addition, in Yunnan, the region where this study was carried out, the recent rapid and permanent conversion of *D. aphyllum* prime habitat to monoculture rubber plantations (Li et al. 2009; Ziegler et al. 2009) has resulted in this species being listed as endangered in China in the Chinese Red List (Fu 1992; Bao et al. 2001; Gao et al. 2014). Although it is not listed in the International Union for Conservation of Nature (IUCN) red list, as with all orchids, it is listed in Appendix II of the Convention on International Trade in Endangered Species (CITES; Romand-Monnier 2013).

Using fungal isolates from in situ seed baiting near adult *D. aphyllum* plants, we tested the following hypotheses: (1) The species-specific mycorrhizae extracted from the protocorms developed near the adult *D. aphyllum* should have a greater impact on the success of germination and subsequent stages of seed development than fungi isolated from other orchids plants, or germination in the absence of symbiotic fungi. (2) In addition to the fungi, the presence of light should further enhance seed germination and developmental success of *D. aphyllum* orchid seedlings. (3) The facilitation from fungi and light for seed germination may be seed developmental stage-specific. This study is the first to report on culturable mycorrhizal fungi isolated from germinated protocorms of *D. aphyllum* and the first to empirically test whether the fungus is stage-specific in enhancing germination.

Material and methods

Species—*D. aphyllum*

D. aphyllum (Fig. 1a) has a wide microhabitat tolerance and can grow on tree trunks in open forests or on valley rocks in karst mountain forests at 400–1,500 m altitude. Flowering takes place from March to June after the leaves are shed and fruiting occurs from June to February (Yonzone et al. 2011). In China, *D. aphyllum* is distributed in northwest Guangxi (Longlin, Xilin, Leye), southwest Guizhou (Xingyi), and southeast to west Yunnan (Funing, Jianshui, Jinping, Mengla, Menghai, Lushui; Chen 1999).

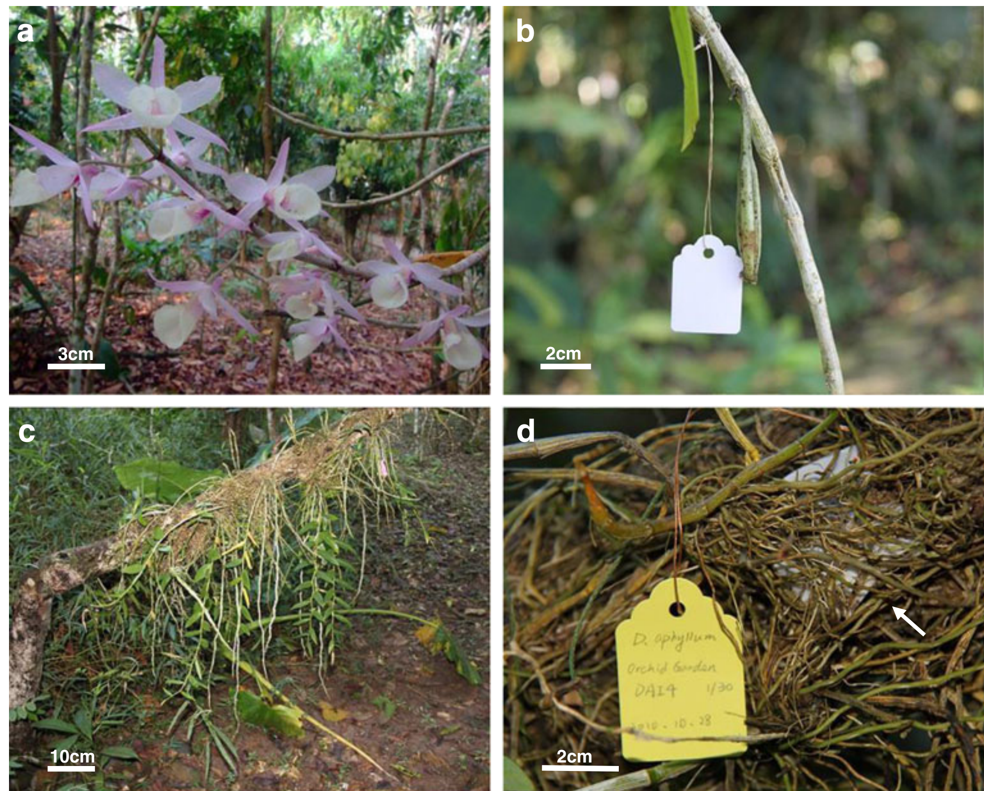


Fig. 1 Flower, fruit, and seed packets of *D. aphyllum* in XTBG Orchid Garden. **a** Inflorescence of *D. aphyllum*. **b** Outcross-pollinated fruit of *D. aphyllum*. **c** One of the locations we placed the seed packets in Orchid Garden. **d** Position and placement of seed packets

Study site

The study site, Xishuangbanna Tropical Botanical Garden (XTBG; 21° 45′ N, 101° 02′ E; altitude, 580 m), in Yunnan, is well placed for this investigation, not only because of its tropical location but also because of laboratory facilities that can conduct advanced research on orchid conservation (Swarts and Dixon 2009b). In this study, seed collection was done from the Orchid Garden of XTBG. The Orchid Garden, which has a mean canopy height of 30 m, is situated in natural tropical rainforest valley dominated by *Pometia tomentosa* and *Terminalia myriocarpa* (Zhang and Cao 1995). This area has an annual precipitation of 1,557 mm due to the tropical monsoon climate resulting from the southwest monsoon occurring from May to October. The rainy season is followed by a misty cool season from November to February and March and April are dry hot months. The annual average temperature is 21.5 °C with average minimum temperatures falling to 7.5 °C. Average annual relative humidity is 86 % (Zhang and Cao 1995). In addition to the orchid garden, seed baiting experiments were conducted at the Tropical Rain Forest of XTBG, which belongs to the Xishuangbanna National Nature Reserve in Mengla district (21° 41′ N, 101° 25′ E; altitude, 580 m) and the XTBG Green Stone Forest, which is an original Karst monsoon forest found at 580 m elevation in a 225 ha area. It has a unique physiognomy, which consists of clumped limestone and dark brown soil rich in humus that collects in the cracks of clumping lime stone rocks (Wang et al. 1997). All laboratory experiments were conducted at the XTBG orchid seed bank of the Ex Situ Conservation & Re-introduction Group, Center for Integrative Conservation research facility.

Seed collection and storage

Seeds of *D. aphyllum* were obtained from a population of wild-collected plants grown on tree trunks from the in situ conservation collection at the Orchid Garden of XTBG. To ensure seed availability, we conducted assisted outcross-pollination trials between different individuals in March (2010) on the *D. aphyllum* population maintained in the Orchid Garden. In October 2010, the resulting mature, nearly dehisced capsules (Fig. 1b) were collected in sterilized Petri dishes and transported to the XTBG orchid seed bank.

To harvest seeds, we surface-sterilized the capsules with 75 % ethanol, rinsed three times with sterile double distilled water and opened them with a scalpel under sterile conditions. Seeds were then air-dried over color-changing silica gel with water absorption capacity between 20 and 50 % (Qingdao Yubao Fine Chemical Industry Co., Ltd., Shandong, China) for 24 h at 22 °C. A subsample of seeds was germinated in sterile MS Medium to test for seed viability. Another two subsamples were stored in the orchid seed bank in airtight glass containers; at 4 °C for short-term preservation and at −20 °C for long-term preservation.

Fungal baiting using in situ symbiotic germination of *D. aphyllum* seeds

In October 2010, we selected seven adult *D. aphyllum* populations from three sites that consisted of both naturally growing and ex situ collections for the seed baiting experiments. This included two populations from the Orchid Garden ex situ collection, two naturally occurring populations from the same tropical rain forest in XTBG, and three additional populations from the Green Stone Forest. Of the three Green Stone Forest populations, two were naturally growing and one was propagated and maintained as an ex situ collection.

To attract fungi appropriate for germination, we placed *D. aphyllum* seeds in 4 cm×6 cm nylon packets with 45 μm diameter holes, which are large enough to allow fungal hyphae to enter but small enough to retain seeds inside the packet (Rasmussen and Whigham 1993; Batty et al. 2001). All packets were secured on to the tree bark, near the adult roots and were then covered with a thin layer of moss to prevent desiccation but allowing for light to penetrate (Fig. 1c, d). Thirty packets, each containing 80–100 seeds, were placed in each of the seven populations. In August 2011, seed packets were gently taken from the buried location from each population, placed between moist sheets of sterilized wet moss and transported back to the laboratory.

Isolation of endophytic fungi from seed baiting packets

Using methods described in Zettler and Piskin (2011), mycorrhizal fungi were isolated from the developing protocorms found in the seed baiting packets. The developing protocorms were extracted and surface-sterilized using sodium hypochlorite solution containing 1 % available chlorine for 3–5 min and rinsed three or four times with sterile water. The protocorms were then broken open using a sterile blade to release endogenetic symbiotic fungi and were placed in Petri dishes containing potato dextrose agar (PDA) medium. After 3–5 days, once the 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 subcultured by excising the hyphal tips onto fresh PDA Petri dishes three to four more times until purified strains were obtained (Wang et al. 2007).

Molecular identification of fungal isolates

Fungal strains isolated from the developing protocorms were identified using the internal transcribed spacer (ITS) region sequences of nuclear ribosomal DNA (rDNA). After cultivating the purified fungal strains for 5–7 days in liquid PDA medium in Erlenmeyer flasks placed on a shaker (SPH-310A, Shanghai Baidian Instrument & Equipment Co., Ltd., China), total DNA extraction of the 9 strains was performed using the cetyl trimethyl ammonium bromide method (Doyle and Doyle 1987). 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, Foster, USA). For the amplification, we used 25 μl reaction volumes each containing the following: 2.5 μl of 10× PCR buffer (10 mM KCl, 10 mM $MgCl_2$, 20 mM Tris–HCl at pH 8.8, and 0.1 % Triton X-100), 1.5 μl of 1.5 mM Mg^{2+}, 0.4 μl of 10 mM dNTP, 1.5 μl of each primer (ITS1 and ITS4), 2.0 μl of template DNA, 0.2 μl of 2 U Taq DNA polymerase, and 15.4 μl of dd water. The fungal rDNA was denatured using the following cycling scheme: 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 51 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 10 min (Duan et al. 2010). The PCR products obtained from this procedure were electrophoresed in 1 % agarose gel, stained with ethidium bromide, and visualized under UV light to check for the quality and quantity of rDNA. They were then purified and sequenced at Sangon Biotech (Shanghai) Co., Ltd. (No. 698, Xiangmin Road, Songjiang District, Shanghai, China.). All rDNA sequences obtained from each isolate were compared to those in the GenBank database in the National Center for Biotechnology Information (NCBI 2012, USA), 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 isolates' capacity to promote germination of *D. aphyllum* seeds

The ability of the fungal partner to enhance seed germination was tested using two representative fungal isolates out of the nine isolates obtained from the above procedure (Electronic Supplementary Material [ESM] 1). The two isolates, FDaI2 representing *Trichoderma* species and FDaI7 representing *Tulasnella* species hereafter referred to as *Trichoderma* and *Tulasnella*, respectively, were selected because they had the fastest and most sustained growth in the PDA culture media under laboratory conditions. In addition to FDaI2 and FDaI7, we used another previously isolated fungal strain, FCb4, representing *Epulorhiza* species (from here on referred to as *Epulorhiza*) known to enhance the seed germination of *C. mannii*, a terrestrial orchid native to Xishuangbanna (Sheng et al. 2012).

Seeds of *D. aphyllum* were removed from storage at −20 °C and moved to ambient temperature for 10 h. Seeds were sterilized by first washing with distilled water for 5–10 min, then with sodium hypochlorite solution containing 1 % available chlorine and one drop of detergent for 10 min, and finally with sterile distilled water three to four times. The sterile seed packets were opened using sterile scissors and the

seeds were transferred to 0.1 % sterile agar suspension solution. Using a pipette, ca. 140 seeds each, suspended in 150 μl agar solution, were transferred onto a semicircular nylon cloth with a radius of 6 cm placed in a 9 cm diam. Petri dish containing 20 ml of sterile 3 g/L oat meal agar (OMA) medium adjusted to pH of 5.6 prior to autoclaving. Once the seed suspension transfer was completed, each dish was inoculated with one 0.5-cm^3 piece of fungal inoculum placed in the center of the Petri dish for each of the FDaI2, FDaI7, and FCb4 treatments, or was maintained as a sterile control treatment without a fungal strain. Fungal-inoculated 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 with 2,000 lx intensity in the photoperiod cycle condition. All Petri dishes were assessed under the dissection microscope and the exact number of seeds placed was recorded for each Petri dish. Each fungal and light treatment was replicated in seven Petri dishes which were placed in germination chambers (RXZ-300B, Ningbo Southeast Instrument Co., Ltd., China).

After 39 days, seed germination and protocorm development were assessed for each Petri dish using a dissecting stereomicroscope (XTL-3400, Cany Precision Instruments Co., Ltd. China). At this time, any Petri dishes that were contaminated with other fungi were discarded.

Statistical analysis of germination and seedling development

Germination and seedling development were divided into six stages including stage 0 (Table 1) and scored as described by Arditti (1967). We calculated the number of seeds that successfully reached or passed each germination stage out of the total seeds placed in each treatment as successful events for the specific stage being assessed. We determined those seeds that did not reach or pass the developmental stage being assessed as failures for that stage. For example, any seed that reached or passed stage 1 was considered as germinated and any seed that reached or passed stage 2 in development were recorded as having reached the protocorm formation stage and so on. Seeds that remained in stage 0 were considered as failures for the complete germination process.

We tested whether the presence or absence of the fungal inoculate, the presence or absence of light, and the interaction between these two factors, influenced the success of reaching or passing each seed germination stage (1–5; see Table 1). We modeled the success and failure to reach each developmental stage using generalized linear mixed models with a binomial distribution function in R project (version 3.0.1; R Development Core Team 2011) and *epicalc* package (Sileshi 2012). We coded each seed developmental stage as having a success (=1) or not reaching that development stage as a failure (=0) for each seed in replicate in each treatment combination. Using the Tukey test in the *multicomp* package of R, we conducted multiple comparisons to test within-treatment differences after adjusting for alpha at 0.05 (Bretz et al. 2010).

Results

Seed baiting for fungal isolation

The ex situ outcrossed seed source for the seed baiting experiments had 68 % seed viability. We had high recovery rates from the buried seed packets (Fig. 2); after 10 months 161 packets out of 210 were recovered from the seven locations. However, the encounter probability of fungi for the 10-month duration was 19 %; only 11 packets from four locations contained germinated seeds. In these 11 packets we found 55 developed protocorms and seedlings (Table 2). Although seed packets from both ex situ (five packets) and naturally growing populations (six packets) contained germinated seeds, naturally growing populations had a higher number of germinated seeds compared to packets recovered from the ex situ locations (43 and 12 protocorms or seedlings, respectively).

Molecular identification of endophytic fungi

We were able to successfully isolate nine fungal strains (named as FDaI1 to FDaI9; see rDNA sequence of ITS region for each strain in EMS_2) from 7 protocorms out of the 55 or seedlings that were cut open for cultivating any fungi that may have infected the developing seeds. The morphological and molecular identification based on maximum similarity of ITS sequence of rDNA stored in the NCBI gene database confirmed that these fungal strains belonged to two different species; *Tulasnella* (EMS 1 and Fig. 3a, b) and *Trichoderma*

Table 1 Description of different seed germination stages of *D. aphyllum*

Seed germination stage	Description
0	No germination
1	Imbibed seed, swollen 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 (early stage of seedling development)
5	Emergence of second leaf and continue development

Adapted from Arditti (1967)

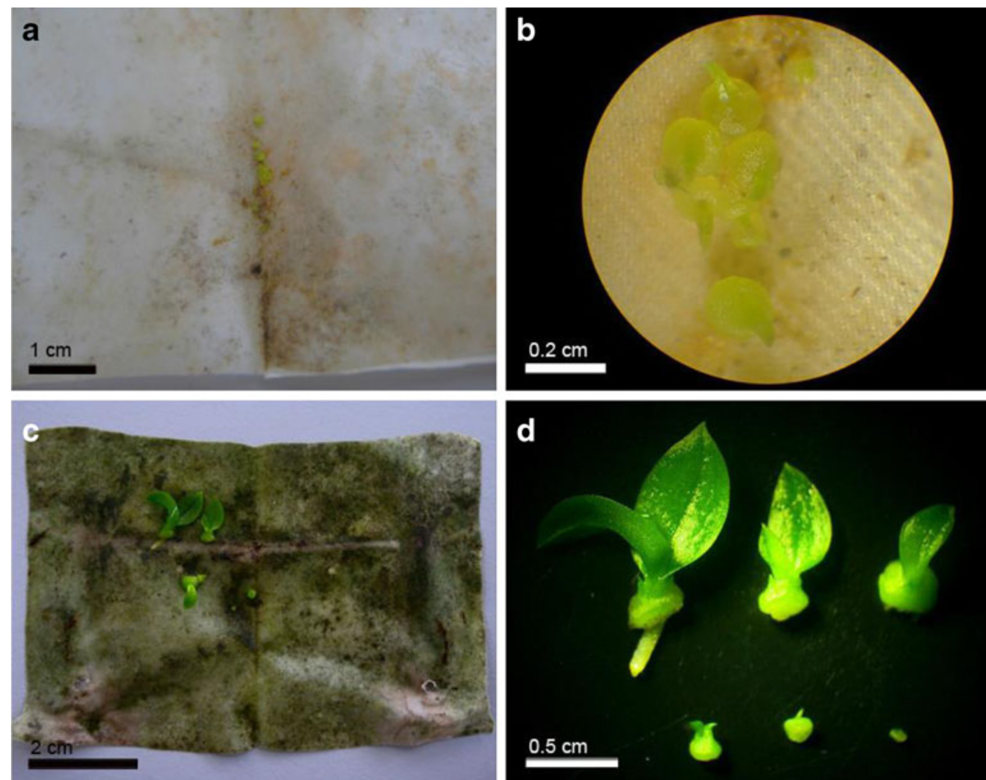


Fig. 2 Protocorms and seedlings in seed packets recovered from in situ seed baiting experiments of *D. aphyllum*. **a** Seed packet recovered form Green Stone Forest, naturally growing populations with protocorms and seedlings. **b** Enlarged drawing of a. **c** Seed packet recovered form XTBG Rain Forest, naturally growing populations with protocorms and seedlings. **d** Enlarged drawing of protocorms and seedlings of (**c**)

(Fig. 3c, d). Fungal strains FDaI1 and FDaI4 to FDaI9 had a 99 % similarity with the fungus *Tulasnella calospora* (accession number: GU166410.1, EMS 1 and EMS 2) and strains FDaI2 and FDaI3 matched 97 % with the rDNA sequence of *Trichoderma viride* (accession number: HM037928.1, EMS 1 and EMS 2).

The effect of fungal isolates and light on each germination stage of *D. aphyllum* seeds

Stage 1—germination seed imbibition Both the fungal isolates and light availability significantly affected the germination of *D. aphyllum* seeds ($P<0.0001$; Table 3). However, the effect of fungi was dependent on the inoculum. The seeds inoculated with *Tulasnella* showed a 13.6 % increase in germination compared to the control treatment lacking fungi ($P<0.0001$; Fig. 4a, b; Table 3) whereas *Trichoderma* isolated from seed baiting had a significant negative effect on germination (26.4 % decrease compared to the control treatment; $P<0.0001$; Fig. 4a; Table 3). The fungal isolate, *Epulorhiza*, despite being obtained from a different orchid, also enhanced germination (6.5 % increase of seeds in stage 1 compared to the control treatment; $P<0.01$; Fig. 4a; Table 3). Seeds that received light showed a significant increase in germination only in the presence of *Tulasnella* (8.5 % increase in germination compared to dark treatment, $P<0.001$; Fig. 4a; Table 3).

Table 2 Result of in situ seed baiting experiments of *D. aphyllum* after 10 months

Study site and population information	Number of seed packet placed in each location	Number of seed packets within protocorms or seedlings/total number of recovered seed packets	Number of protocorms and seedlings in each location
GSF-natural growth	30	0/25	0
GSF-natural growth	30	4/24	30
GSF-ex situ collection	30	0/24	0
GSF- ex situ collection	30	2/23	6
OG- ex situ collection	30	3/25	6
RF-natural growth	30	2/28	13
RF-natural growth	30	0/12	0

GSF green stone forest, *OG* orchid garden, *RF* Rain Forest in Xishuangbanna Tropical Botany Garden

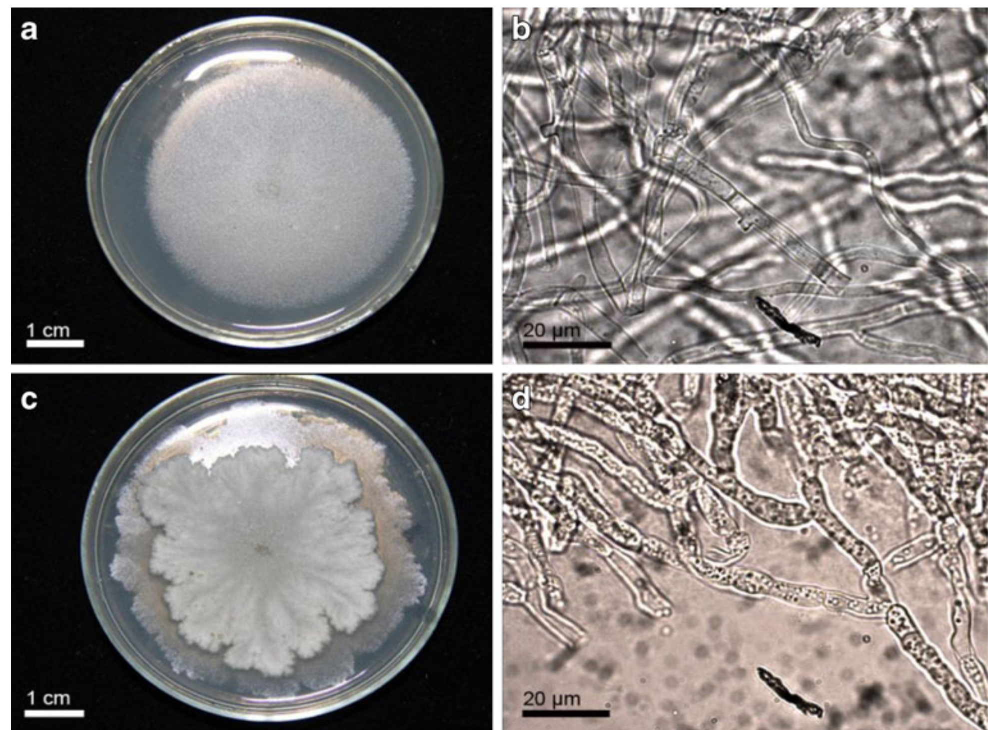


Fig. 3 Morphology and microscopic characteristics of FDaI1 strain (*Tulasnella*) and FDaI2 strain (*Trichoderma*). **a** Morphology of FDaI1 strain in PDA medium. **b** Microscopic characteristics of FDaI1 strain. **c** Morphology of FDaI2 strain in PDA medium. **d** Microscopic characteristics of FDaI2 strain

Stage 2—protocorm formation The effect of fungi on achieving or surpassing protocorm development was positive when seeds were inoculated with *Tulasnella* as well as *Epulorhiza* (85.7 and 20.3 % increase compared to the control; $P<0.0001$ for both treatments; Fig. 4c, d; Table 3), and as in stage 1, light further increased the development (11.8 and 43.6 % increase compared to the dark germinated seeds, $P<0.0001$ for both *Tulasnella* and *Epulorhiza*; Fig. 4c). Seeds lacking fungi inoculum or seeds that were inoculated with *Trichoderma* failed to develop beyond the initial protocorm stage (Fig. 4c).

Stage 3—developed protocorms We found a significant effect of seeds reaching or passing the developed protocorm stage due only to *Tulasnella* (65.7 % compared to the control treatment; $P<0.0001$; Fig. 4e, f; Table 3). Seeds in other treatments did not reach this developmental stage. At this stage, light did not have an effect on the number of developed protocorms.

Stage 4—seedling formation The effect of fungal inoculation was significant for seedlings in stage 4 as was observed for seeds at the developed protocorm stage ($P<0.05$; Fig. 4g, h;

Table 3 The effect of fungi inoculation (Control, *Tulasnella*, *Epulorhiza*, and *Trichoderma*) and light condition (0/24 h L/D, 12/12 h L/D) on each seed germination stage in *D. aphyllum* (the values presented with factor effect are Chi-squared statistic and the associated significance)

	Stage 1 Seeds imbibition	Stage 2 Protocorm formation	Stage 3 Protocorm development	Stage 4 Seedling formation	Stage 5 Seedling development
Factor (Chi^2)					
Light (df=1)	19.42 ***	165.12 ***	8.33	19.59 ***	19.42 ***
Fungi (df=3)	774.74 ***	4318.90 ***	3308.70 ***	2068.70 *	774.74 ***
Light × Fungi (df=7)	832.16 ***	4783.00 ***	3308.80	2075.60	832.16 ***
Multiple comparisons (estimation±SE)					
*Tulasnella*_Control	1.28±0.12 ***	6.80±0.29 ***	5.65±0.28 ***	4.83±0.28 ***	2.47±0.30 ***
*Epulorhiza*_Control	0.35±0.10 **	3.50±0.29 ***	ng	ng	ng
*Trichoderma*_Control	−1.22±0.08 ***	ng	ng	ng	ng

Multiple comparisons are given for each inoculum in comparison to the uninoculated sterile control with the estimates and their standard errors, and the associated significance

df degrees of freedom, *Chi*^{2} *Chi*-squared, *SE* standard error, *ng* no seeds germinated to that stage with or without the fungi inoculating

***$P<0.0001$; **$P<0.01$; *$P<0.05$

Table 3). However, light became more important for seedling formation ($P<0.0001$; Fig. 4g, h; Table 3).

Stage 5—seedling development Only 11.2±9.9 % of seeds developed to the final germination stage during the 39 day experimental period. All of them grew in the *Tulasnella* inoculum with 12 h light treatment (all comparisons $P<0.0001$; Fig. 4i, j; Table 3).

Storage and management of live fungi for future studies and conservation

The two strains that were effective in enhancing germination were stored in China General Microbiological Culture Collection Center (CGMCC, http://www.cgmcc.net/) under the accession number 7552 for FDaI7 and 7553 for FCb4. We are also maintaining live cultures in storage at 4 °C temperature at the XTBG orchid seed bank laboratory.

Discussion

Our results partially support the hypothesis that mycorrhizal fungi extracted from protocorms developed near the adult *D. aphyllum* would enhance germination and seed development compared to having fungi isolated from other orchid species or not having a fungal symbiont (Fig. 4, Table 3). *Tulasnella* supported germination and seedling development, but *Trichoderma*, also obtained from *D. aphyllum* using seed baiting techniques, had a negative effect on germination. The effectiveness of *Epulorhiza* in supporting the formation of a two-leaved seedling of *C. mannii* was not seen with *D. aphyllum* seeds. This observation supports the species-specificity hypothesis. Availability of light further enhanced seed germination, but was dependent on the germination stage. Fungal facilitation was stage-specific because the seed germination stage could happen independently of a symbiont while protocorm formation was supported by both *Tulasnella* and *Epulorhiza*. Further development towards a two-leaved seedling was only supported by *Tulasnella*.

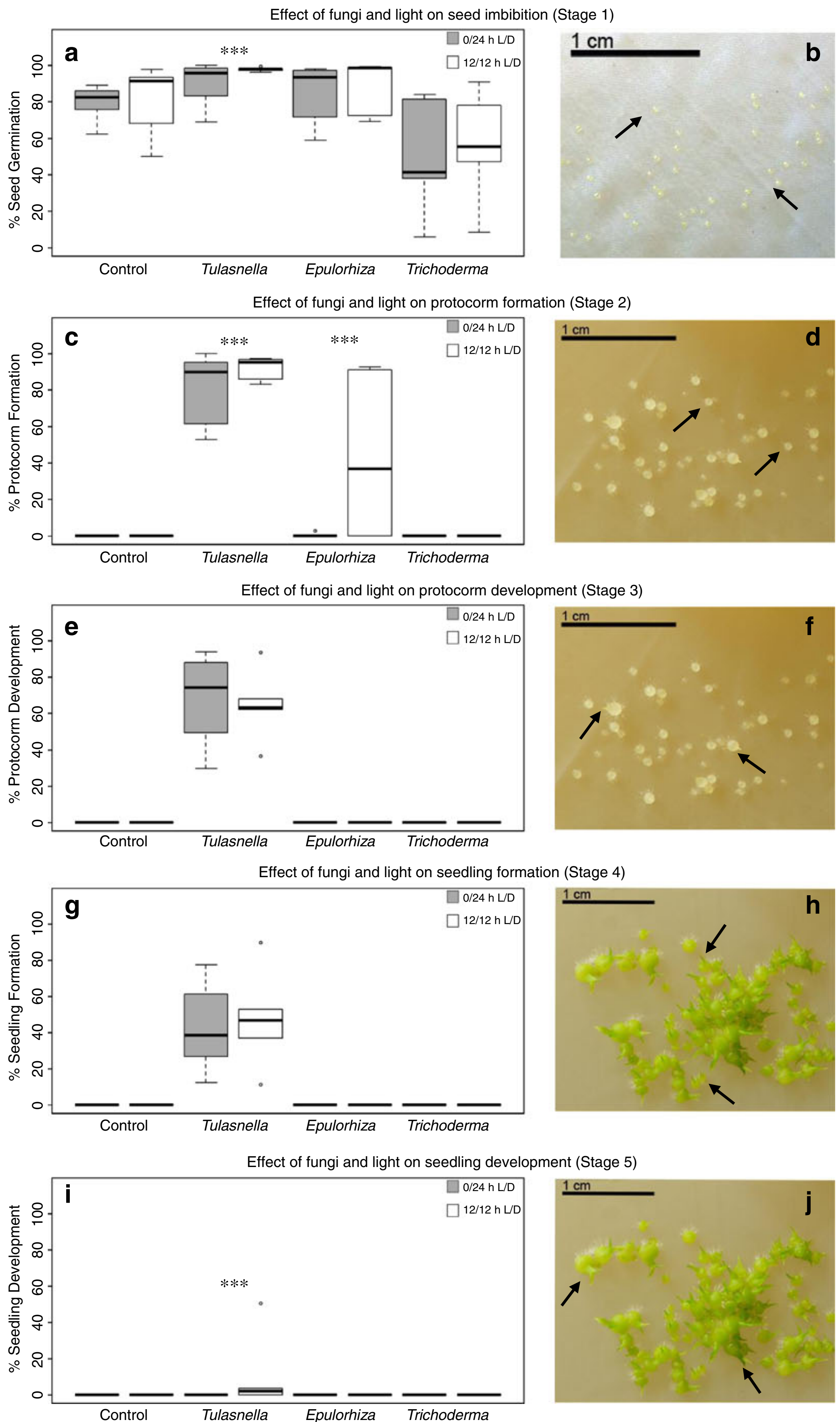
The majority of symbiotic fungi found in orchids belong to *Rhizoctonia* or *Rhizoctonia*-like fungi (Arditti 1992; Otero et al. 2002; Rasmussen 2002; Dearnaley 2007; Shimura et al. 2009), which includes the anamorphic (asexual) genera *Ceratorhiza* and *Epulorhiza* (Moore 1987) and the telemorphic (sexual) genera *Ceratobasidium*, *Sebacina*, and *Tulasnella* (Warcup and Talbot 1967, 1971, 1980). Tullasnellaceae, including the anamorphic *Epulorhiza* and the telemorphic *Tulasnella* are the most common symbiotic fungi to support the germination of *Dendrobium* species (Liu et al. 2010; Nontachaiyapoom et al. 2010; Chen et al. 2012). Our study confirms this general trend. The seedling stage of *D. aphyllum* was supported only by *Tulasnella* not by

Fig. 4 Symbiotic seed germination of *D. aphyllum* (Success at each stage is presented as a percentage. The *thick black line* in each plot indicate the 50th percentile values of each species, the *lower and upper side of the box* represent the 25th and 75th percentile, and the *error bars* the 10th and 90th percentile. The *circles above or below the 10th and 90th percentile line* represent data points that are more than three times the standard error for the observation (outliers). The *line and stars above the boxplots* connecting the light and dark treatments are for those comparisons that are significantly different. The significance *P* values are given as ***$P<0.0001$.) **a** Seed imbibition/ germination (Stage 1) when inoculated with different fungal strains under light or dark conditions. **b** *Arrows* show the imbibed seeds (Stage 1). **c** Protocorms formation (Stage 2) when inoculated with different fungal strain under light or dark condition. **d** *Arrows* show the formatted protocorms (Stage 2). **e** Protocorms development (Stage 3) when inoculated with different fungal strain under light or dark condition. **f** *Arrows* show the developed protocorms (Stage 3). **g** Seedlings formation (Stage 4) when inoculated with different fungal strain under light or dark condition. **h** *Arrows* show the formatted seedlings (Stage 4). **i** Seedlings development (Stage 5) when inoculated with different fungal strain under light or dark condition. **j** *Arrows* show the developed seedlings (Stage 5)

Epulorhiza, which does support the germination and protocorm stages, confirming that there is a fungal specificity bottleneck at the seedling stage compared to the early germination stages (Bidartondo and Read 2008).

Orchid seed baiting bioassays provide an effective technique to detect the inoculum potential of mycorrhizal fungi and to obtain endophytes that support early seed germination and protocorm development in native habitats (Wang et al. 2011). The success that we observed in using seed baiting techniques to attract these fungi has been reported by other research programs (Rasmussen and Whigham 1993; Batty et al. 2001; Wang et al. 2011). Although we were successful, the number of seed packets with developing protocorms was low, possibly because our sample size was small. However, a detailed study outlining recommendations for seed packet sampling to attract seed germination-enhancing fungi by Bidartondo and Read (2008), states that 20 packets per treatment is sufficient for terrestrial orchids. We placed 30 seed baiting packets in each of our locations. We assume that instead of low sampling, the low encounter probability of seed germination-enhancing fungi in the wild may be the reason for the low retrieval rates in this epiphytic orchid (Hollick 2004; Wang et al. 2011). The low retrieval rate of seeds naturally inoculated with fungi confirms the importance of symbiotically propagated seedlings in reintroduction efforts (Batty et al. 2006; Johnson et al. 2007).

Timing the seed packet retrieval from the field is important for the fungal isolation since we were only able to successfully obtain fungi from the protocorm stages and not from the seedling stages. The fact that none of the seedling stages contained the fungi suggests that *D. aphyllum* is able to control the rate of infection beyond the protocorm stage and may have digested all mycelia that infected the protocorm stage in preparation for other seedling growth fungi to inoculate, as in *Goodyera pubescens* (McCormick et al. 2006). Our



Effect of fungi and light on seed imbibition (Stage 1)
Effect of fungi and light on protocorm formation (Stage 2)
Effect of fungi and light on protocorm development (Stage 3)
Effect of fungi and light on seedling formation (Stage 4)
Effect of fungi and light on seedling development (Stage 5)
a
b
c
d
e
f
g
h
i
j
% Seed Germination
% Protocorm Formation
% Protocorm Development
% Seedling Formation
% Seedling Development
0/24 h L/D
12/12 h L/D
***
1 cm
0
20
40
60
80
100
Control
Tulasnella
Epulorhiza
Trichoderma

experiment in the laboratory on seed germination in the presence of *Tulasnella* shows that close to 43 % of seeds are at the protocorm stage by 39 days. Therefore, in using seed packets for baiting with *D. aphyllum*, we recommend a step-wise retrieval of seed packets for maximum success in extracting fungi from protocorm stages.

Seed baiting instead of root-based fungal isolation techniques are more efficient in extracting only the seed germination-enhancing members of the fungal community. In this study, we obtained only two kinds of endophytic fungi (one *Tulasnella*, one *Trichoderma*) from the in situ symbiotic germinated protocorms making it much easier to test for the ability of these species to enhance germination. However, it is important to note that root-based fungal isolation techniques attract an important component of the fungal community associated with orchids.

In *Dendrobium*, studies have shown that root colonizing endophytic fungi can stimulate host plant growth (Herre et al. 2007; Hou and Guo 2009; Yuan et al. 2009), increase concentrations of total alkaloids and polysaccharides (Chen and Guo 2005), and even exhibit strong antibacterial activity (Xing et al. 2012). Hence the ability to market these as medicinal plants grown with such isolates may counter the argument that in vitro asymbiotically produced *Dendrobium* species are inferior in their therapeutic qualities and reduce the collection on wild plants. Therefore, we recommend an integrated conservation approach in using seed germination-enhancing fungi for obtaining seedlings, but the growth of mature adults with root-extracted fungi for producing *D. aphyllum* for the medicinal use market. Although we expect that symbiotically grown seedlings will do well in the wild, site and species-specific trials should be established before executing a large-scale conservation program.

Conservation programs need to consider two important issues during the fungal extraction and isolation process. It is not enough to report on the identification of the fungal isolates in the form of the genetic code. Conservation efforts have to carefully consider maintaining live cultures of the isolated fungi for not only further testing of their effectiveness in germination enhancement of the species being studied but also as a future reference for using on other orchid species. For example, *Trichoderma* in our study suppressed germination, but it has the potential to facilitate the growth of developed seedlings of other orchids (e.g., Huang et al. 2004) or facilitate the mature plants due to antagonistic effects on other plant pathogens (Catalano et al. 2011), even for *Dendrobium* (Zhu et al. 2011).

When assessing the effect of symbiotic fungi on seed germination stages, studies generally separately report the exact number of developing seeds found in each of these six stages: no germination, germination, protocorm formation, protocorm development, seedling formation and two-leaved seedling stage (Stewart and Zettler 2002). These data were then directly used for statistical analysis. Our approach here was to calculate the number of seeds that were either found in a given stage or have surpassed that stage for testing the treatment effects on stage specificity. We recommend this approach because it is more accurate, given that any seed that already surpassed a previous stage should be included in the count for how many seeds were able to arrive and/or surpass that stage.

A key goal of our conservation program is to understand the germination and seedling developmental physiology of orchids because this can guide our seedling propagation protocols. Our finding that *D. aphyllum* seeds can germinate and form protocorms in the absence of light, but that seedling development was significantly facilitated by light, was also seen in the congeneric species, *Dendrobium officinale* and *Dendrobium mobile* (Wang et al. 2011), as well as in the terrestrial orchid, *Calopogon tuberosus* (Kauth et al. 2006). However, the seeds of *C. mannii*, an epiphyte, do significantly better in the dark during the protocorm formation and protocorm development stages (Sheng et al. 2012). This variety in response to dark and light conditions during germination of epiphytic orchids (Arditti 1967; Arditti and Ernst 1984) is also observed in terrestrial orchids. For example, some terrestrial orchids may have reduced (Rasmussen and Rasmussen 1991; Zettler and McInnis 1994) or inhibited (*Calanthe tricarinata*; Godo et al. 2010) germination in the dark while in others dark conditions may stimulate germination (*Cypripedium reginae*; Harvais 1973, *Habenaria macroceratitis*; Stewart and Kane 2006). In *Habenaria radiata*, there was no difference in germination between the dark and the light conditions (Takahashi et al. 2000). Therefore, we recommend that the light requirement for germination be individually tested for each species as well as for each germination stage.

Conclusions

The benefits of understanding the ecology of orchids and their fungal associates in natural habitats include improved management and translocation opportunities. Seed baiting is a valuable tool for detection of compatible mycorrhizal fungi for enhancing orchid seed germination. We found that *D. aphyllum* orchid seed germination enhancement due to light availability and supporting fungal inoculum is germination stage-specific and that fungal enhancement of germination can be species-specific. In addition to gaining a better understanding of the biology of orchid symbiotic seed germination, these findings have a broader impact of improving seedling generation for conservation and reintroduction programs aimed at reducing the wild collection pressure on this species.

Acknowledgments This research was supported by the National Natural Science Foundation of China (grant no. 31170358) to JYG and manuscript writing was supported by China National Post Doctoral Fund 2013 to UMG and Chinese Academy of Science CAS 135 program (XTBG-T01). We thank Richard T. Corlett and two anonymous reviewers for constructive comments on a previous version of the manuscript. We are also grateful to Wenliu Zhang, Xuli Fan, Hua Lin, Qiang Liu for field and laboratory assistance.

References

Arditti J (1967) Factors affecting the germination of orchid seeds. Bot Rev 33:1–97

Arditti J (1992) Fundamentals of orchid biology. Wiley, New York

Arditti J, Ernst R (1984) Physiology of germinating orchid seeds. In: Arditti J (ed) Orchid biology, reviews and perspectives, 3rd edn. Cornell University Press, New York, pp 170–222

Arditti J, Ghani AKA (2000) Numerical and physical properties of orchid seeds and their biological implications. New Phytol 145:367–421

Atwood JT (1986) The size of the Orchidaceae and the systematic distribution of epiphytic orchids. Selbyana 9:171–186

Bao XS, Shun QS, Chen LZ (2001) The medicinal plants of *Dendrobium* (Shi-Hu) in China, a coloured atlas. Fudan University Press, Shanghai (in Chinese)

Batty AL, Dixon KW, Brundrett M, Sivasithamparam K (2001) Constraints to symbiotic germination of terrestrial orchid seed in a Mediterranean bushland. New Phytol 152:511–520

Batty AL, Brundrett MC, Dixon KW, Sivasithamparam K (2006) In situ symbiotic seed germination and propagation of terrestrial orchid seedlings for establishment at field sites. Aust J Bot 54:375–381

Bidartondo MI, Read DJ (2008) Fungal specificity bottlenecks during orchid germination and development. Mol Ecol 17:3707–3716

Bretz F, Hothorn T, Westfall P (2010) Multiple comparisons using R. CRC, Boca Raton

Brundrett MC, Scade A, Batty AL, Dixon KW, Sivasithamparam K (2003) Development of in situ and ex situ seed baiting techniques to detect mycorrhizal fungi from terrestrial orchid habitats. Mycol Res 107:1210–1220

Catalano V, Vergara M, Hauzenberger JR, Seiboth B, Sarrocco S, Vannacci G, Kubicek CP, Seidl-Seiboth V (2011) Use of a non-homologous end-joining-deficient strain (delta-*ku70*) of the biocontrol fungus *Trichoderma virens* to investigate the function of the laccase gene *lcc1* in sclerotia degradation. Curr Genet 57:13–23

Chen XQ (1999) Orchidaceae. In: Flora of China 19. Science Press, Beijing, pp 106–108, in Chinese

Chen XM, Guo SX (2005) Effects of four species of endophytic fungi on the growth and polysaccharide and alkaloid contents of *Dendrobium nobile*. Zhongguo Zhong Yao Za Zhi 30:253 (in Chinese)

Chen J, Wang H, Guo SX (2012) Isolation and identification of endophytic and mycorrhizal fungi from seeds and roots of *Dendrobium* (Orchidaceae). Mycorrhiza 22:297–307

Chutima R, Dell B, Lumyong S (2011) Effects of mycorrhizal fungi on symbiotic seed germination of *Pecteilis susannae* (L.) Rafin (Orchidaceae), a terrestrial orchid in Thailand. Symbiosis 53:149–156

Cribb PJ, Kell SP, Dixon KW, Barrett RL (2003) Orchid conservation: a global perspective. In: Dixon KW, Kell SP, Barrett RL, Cribb PJ (eds) Orchid conservation. Natural History Publications, Kota Kinabalu, pp 1–24

Dearnaley JDW (2007) Further advances in orchid mycorrhizal research. Mycorrhiza 17:475–486

Dearnaley JDW, Martos F, Selosse MA (2012) Orchid Mycorrhizas: molecular ecology, physiology, evolution and conservation aspects. In: Hock B (ed) Fungal associations. Springer, Berlin, pp 207–230

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15 obtained from http://irc.igd.cornell.edu/Protocols/DoyleProtocol.pdf. Accessed 23 January 2011

Dressler RL (1993) Phylogeny and classification of the orchid family. Cambridge University Press, New York

Duan CF, Li ZL, Fang F, Yang GH, Chen SS, Hong QY (2010) Isolation and identification research of mycorrhizal isolated from several orchids in Yunnan Province. Xi Nan Nong Ye Xue Bao 23:756–759, in Chinese

Eriksson O, Kainulainen K (2011) The evolutionary ecology of dust seeds. Perspect Plant Ecol Evol Syst 13:73–87

Fu LK (1992) China plant red data book: rare and endangered plants. I. Science Press, Beijing (in Chinese)

Gao JY, Liu Q, Yu DL (2014) Orchids in Xishuangbanna: diversity and conservation. China Forestry Publishing House, Beijing (in Chinese)

Godo T, Komori M, Nakaoki E, Yukawa T, Miyoshi K (2010) Germination of mature seeds of *Calanthe tricarinata* Lindl., an endangered terrestrial orchid, by asymbiotic culture in vitro. In Vitro Cell Dev Biol Plant 46:323–328

Hágsater E, Dumont V (1996) Conservation threats. In: Prindgeon AM (ed) Orchids: status, survey, and conservation action plan. IUCN, Gland, pp 6–9

Harley JL (1950) Recent progress in the study of endotrophic mycorrhiza. New Phytol 49:213–247

Harvais G (1973) Growth requirements and development of *Cypripedium reginae* in axenic culture. Can J Bot 51(2):327–332

Herre EA, Mejía LC, Kyllo DA, Rojas E, Maynard Z, Butler A, Van Bael SA (2007) Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. Ecology 88:550–558

Hollick PS (2004) Mycorrhizal specificity in endemic Western Australian terrestrial orchids (tribe Diurideae): implications for conservation. Doctoral dissertation, Murdoch University

Hou XQ, Guo SX (2009) Interaction between a dark septate endophytic isolate from *Dendrobium* sp. and roots of *D. nobile* seedlings. J Integr Plant Biol 51:374–381

Huang L, He XR, Zheng LM, Cai J (2004) Preliminary studies on mycorrhizal fungi in promoting the growth of orchid seedlings from tissue culture. Re Dai Zuo Wu Xue Bao 25:36–38 (in Chinese)

Johnson TR, Stewart SL, Dutra D, Kane ME, Richardson L (2007) Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)—preliminary evidence for the symbiotic culture advantage. Plant Cell Tissue Organ Cult 90:313–323

Kartzinel TR, Trapnell DW, Shefferson RP (2013) Highly diverse and spatially heterogeneous mycorrhizal symbiosis in a rare epiphyte is unrelated to broad biogeographic or environmental features. Mol Ecol. doi:10.1111/mec.12536

Kauth PJ, Vendrame WA, Kane ME (2006) In vitro seed culture and seedling development of *Calopogon tuberosus*. Plant Cell Tissue Organ Cult 85:91–102

Keel BG, Zettler LW, Kaplin BA (2011) Seed germination of *Habenaria repens* (Orchidaceae) in situ beyond its range, and its potential for assisted migration imposed by climate change. Castanea 76:43–54

Knudson L (1922) Nonsymbiotic germination of orchid seeds. Bot Gaz 73:1–25

Koopowitz H, Hawkins BA (2012) Global climate change is confounding species conservation strategies. Integr Zool 7:158–164

Li H, Ma Y, Liu W, Liu W (2009) Clearance and fragmentation of tropical rain forest in Xishuangbanna, SW, China. Biodivers Conserv 18: 3421–3440

Liu H, Luo Y, Liu H (2010) Studies of mycorrhizal fungi of Chinese orchids and their role in orchid conservation in China-a review. Bot Rev 76:241–262

Massey EE, Zettler LW (2007) An expanded role for in vitro symbiotic seed germination as a conservation tool: two case studies in North

America (*Platanthera leucophaea* and *Epidendrum nocturnum*). Lankesteriana 7:303–308
Masuhara G, Katsuya K (1994) In situ and in vitro specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon) Ames, var. *amoena* (M. Bieberstein) Hara (Orchidaceae). New Phytol 127: 711–718
McCormick MK, Whigham DF, Sloan D, O'Malley K, Hodkinson B (2006) Orchid-fungus fidelity: a marriage meant to last? Ecology 87: 903–911
McCormick MK, Lee Taylor D, Juhaszova K, Brunett RK, Whigham DF, O'Neill JP (2012) Limitations on orchid recruitment: not a simple picture. Mol Ecol 21:1511–1523
Moore RT (1987) The genera of *Rhizoctonia*-like fungi: *Ascorhizoctonia*, *Ceratorhiza* gen. nov., *Epulorhiza* gen. nov., *Moniliopsis*, and *Rhizoctonia*. Mycotaxon 29:91–99
Nontachaiyapoom S, Sasirat S, Manoch L (2010) Isolation and identification of *Rhizoctonia*-like fungi from roots of three orchid genera, *Paphiopedilum*, *Dendrobium*, and *Cymbidium*, collected in Chiang Rai and Chiang Mai provinces of Thailand. Mycorrhiza 20:459–471
Otero JT, Ackerman JD, Bayman P (2002) Diversity and host specificity of endophytic Rhizoctonia-like fungi from tropical orchids. Am J Bot 89:1852–1858
Park EJ, Lee WY (2013) In vitro symbiotic germination of myco-heterotrophic *Gastrodia elata* by *Mycena* species. Plant Biotechnol Rep 7:185–191
Perkins AJ, McGee PA (1995) Distribution of the orchid mycorrhizal fungus, *Rhizoctonia solani*, in relation to its host, *Pterostylis acuminata*, in the field. Aust J Bot 43:565–575
Phillips RD, Barrett MD, Dixon KW, Hopper SD (2011) Do mycorrhizal symbioses cause rarity in orchids? J Ecol 99:585–869
R Development Core Team (2011) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/. Accessed 30 June 2013
Rasmussen HN (2002) Recent developments in the study of orchid mycorrhiza. Plant Soil 244:149–163
Rasmussen HN, Rasmussen FPN (1991) Climatic and seasonal regulation of seed plant establishment in *Dactylorhiza majalis* inferred from symbiotic experiments in vitro. Lindleyana 6:221–227
Rasmussen HN, Rasmussen FN (2009) Orchid mycorrhiza: implications of a mycophagous life style. Oikos 118:334–345
Rasmussen HN, Whigham DF (1993) Seed ecology of dust seeds in situ: a new study technique and its application in terrestrial orchids. Am J Bot 80:1374–1378
Rasmussen HN, Whigham DF (1998a) Importance of woody debris in seed germination of Tipularia discolor (Orchidaceae). Am J Bot 85:829–834
Rasmussen HN, Whigham DF (1998b) The underground phase: a special challenge in studies of terrestrial orchid populations. Bot J Linn Soc 126:49–64
Romand-Monnier F (2013) *Dendrobium aphyllum*. IUCN 2013. IUCN red list of threatened species. Version 2013.1. http://www.iucnredlist.org/details/15053780/0. Accessed 19 November 2013.
Sanchez MS, Bills GF, Zabalgogeazcoa I (2008) Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses. Fungal Divers 33:87–100
Seaton P, Kendon JP, Pritchard HW, Puspitaningtyas DM, Marks TR (2013) Orchid conservation: the next ten years. Lankesteriana 13:93–101
Selosse MA, Boullard B, Richardson D (2011) Noël Bernard (1874–1911): orchids to symbiosis in a dozen years, one century ago. Symbiosis 54:61–68
Sheng CL, Lee YI, Gao JY (2012) Ex situ symbiotic seed germination, isolation and identification of effective symbiotic fungus in *Cymbidium mannii* (Orchidaceae). Zhi Wu Sheng Tai Xue Bao 36: 859–869 (in Chinese)
Shimura H, Sadamoto M, Matsuura M, Kawahara T, Naito S, Koda Y (2009) Characterization of mycorrhizal fungi isolated from the threatened *Cypripedium macranthos* in a northern island of Japan: two phylogenetically distinct fungi associated with the orchid. Mycorrhiza 19:525–534
Sileshi GW (2012) A critique of current trends in the statistical analysis of seed germination and viability data. Seed Sci Res 22:145–159
Stewart SL, Kane ME (2006) Symbiotic seed germination of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell Tissue Organ Cult 86:159–167
Stewart SL, Zettler LW (2002) Symbiotic germination of three semi-aquatic rein orchids *Habenaria repens*, *H. quinquiseta*, *H. macroceratitis* from Florida. Aquat Bot 72:25–35
Subedi A, Kunwar B, Gravendeel B et al (2013) Collection and trade of wild-harvested orchids in Nepal. J Ethnobiol Ethnomed 9:64
Swarts ND, Dixon KW (2009a) Terrestrial orchid conservation in the age of extinction. Ann Bot 104:543–556
Swarts ND, Dixon KW (2009b) Perspectives on orchid conservation in botanic gardens. Trends Plant Sci 14:590–598
Swarts ND, Sinclair EA, Francis A, Dixon KW (2010) Ecological specialization in mycorrhizal symbiosis leads to rarity in an endangered orchid. Mol Ecol 19:3226–3242
Takahashi K, Ogiwara I, Hakoda N (2000) Seed germination of *Habenaria* (*pecteilis*) *radiata* (Orchidaceae: Orchideae) in vitro. Lindleyana 15:59–63
Wang H, Zhu H, Li BG (1997) Vegetation on limestone in Xishuangbanna Southwest China. Guangxi Zhi Wu 17:101–117 (in Chinese)
Wang D, Jia SH, Zhang ZX, Cai YP, Lin Y (2007) Isolation and culture of an endophytic fungus associated with *Dendrobium huoshanense* and its effects on the growth of plantlets. J Fungal Res 5:84–88 (in Chinese)
Wang H, Fang H, Wang Y, Duan L, Guo S (2011) In situ seed baiting techniques in *Dendrobium officinale* Kimuraet Migo and *Dendrobium nobile* Lindl.: the endangered Chinese endemic *Dendrobium* (Orchidaceae). World J Microbiol Biotechnol 27: 2051–2059
Warcup JH, Talbot PHB (1967) Perfect states of *Rhizoctonias* associated with orchids. New Phytol 66:631–641
Warcup JH, Talbot PHB (1971) Perfect states of rhizoctonias associated with orchids. II. New Phytol 70:35–40
Warcup JH, Talbot PHB (1980) Perfect states of rhizoctonias associated with orchids. III. New Phytol 86:267–272
White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: a guide to methods and applications. Academic, New York, pp 315–322
Xing X, Ma X, Deng Z, Chen J, Wu F, Guo S (2012) Specificity and preference of mycorrhizal associations in two species of the genus *Dendrobium* (Orchidaceae). Mycorrhiza 23:317–324. doi:10.1007/s00572-012-0473-8
Yoder JA, Zettler LW, Stewart SL (2000) Water requirements of terrestrial and epiphytic orchid seeds and seedlings, and evidence for water uptake by means of mycotrophy. Plant Sci 156:145–150
Yonzone R, Lama D, Bhujel RB, Rai S, Kendra DKV, Viswavidyalaya UBK, Kalimpong PO (2011) Epiphytic orchid species diversity of Darjeeling Himalaya of West Bengal, India. Asian J Pharm Life Sci ISSN 2231:4423
Yuan ZL, Chen YC, Yang Y (2009) Diverse non-mycorrhizal fungal endophytes inhabiting an epiphytic, medicinal orchid (*Dendrobium nobile*): estimation and characterization. World J Microbiol Biotechnol 25:295–303
Zettler LW, Hofer CJ (1998) Propagation of the little club-spur orchid *Platanthera clavellata* by symbiotic seed germination and its ecological implications. Environ Exp Bot 39:189–195
Zettler LW, McInnis JTM (1994) Light enhancement of symbiotic seed germination and development of an endangered terrestrial orchid, *Platanthera integrilabia*. Plant Sci 102:133–138
Zettler LW, Piskin KA (2011) Mycorrhizal fungi from protocorms, seedlings, and mature plants of the eastern prairie fringed orchid,

Platanthera leucophaea: a comprehensive list to augment conservation. Am Midl Nat 166:29–39

Zhang J, Cao M (1995) Tropical forest vegetation of Xishuangbanna, SW China and its secondary changes, with special reference to some problems in local nature conservation. Biol Conserv 73: 229–238

Zhu JM, Zhao YM, Bai J, Li YT, Zhang KM, Chen JW, Wu JB, Wang HZ (2011) Preliminary studies on the antagonism of *Dendrobium* mycorrhizal fungi (*Trichoderma persoon*). J Hangzhou Normal Univ (Nat Sci Ed) 10:340–344 (in Chinese)

Ziegler AD, Fox JM, Xu J (2009) The rubber juggernaut. Science 324(5930):1024–1025