In situ symbiotic seed germination in *Dendrobium* spp. (Orchidaceae): implications for orchid restoration

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Abstract – Orchidaceous taxa are known for symbiotic relationships that facilitate pollination as well as mycorrhizal associations that are necessary for seed germination. To enhance seed germination and the restoration of threatened orchid species, purified fungal strains typically found in host orchid protocorms can be isolated, verified to promote seed germination and subsequently applied to seed propagation *in situ*. Here, we describe and test the application of three fungal isolates on seed germination rates for three epiphytic species of orchids: *Dendrobium aphyllum*, *D. devonianum* and *D. nobile*. Additionally, we evaluate variance in seed germination rates on the host preferences and sowing time (all three species). Seed germination was highest for *D. nobile* (13.6%) and lowest for *D. devonianum* (9.4%); *D. aphyllum* had intermediate levels of germination (10.0%). The proportion of packets that produced seedlings varied slightly among species (77.3% for *D. aphyllum*, 73.8 % for *D. devonianum* and 76.9% for *D. nobile*, respectively). Germination rates differed significantly among hosts and sowing times and thus indicates there was significant association found between seed germination of orchids and host plants on different sowing time. The percentage germination on the trunk of *B. purpurea* was significant higher than those on the trunk of *Citrus maxima* and *Camellia assamica*. Seed germination rate also differed significantly on different sowing time for each species. Overall, our results indicate that *in situ* symbiotic seed germination is possible for epiphytic orchids and recruitment may be enhanced by species-specific symbiotic fungi and ecological and demographic parameters. Our study has broad implications for restoration efforts of rare and endangered epiphytic orchid as well as their commercial production for medicinal or horticultural applications.

Keywords – *Dendrobium aphyllum*, *Dendrobium devonianum*, *Dendrobium nobile*, germination time, host specificity, restoration

INTRODUCTION

Orchids produce tiny dust-like seeds (microspermy) that require essential carbon, water and mineral nutrient supply from their obligate mycorrhizal symbionts for germination (Rasmussen and Rasmussen, 2009; Tesitelova *et al.*, 2013). Although a number of studies have investigated the mechanisms of 1) rarity and distribution (McKendrick *et al.*, 2002; Tesitelova *et al.*, 2012) from the aspect of fungal assosication; 2) symbiosis between orchid seeds and fungi (Zettler and Hofer, 1998; Stewart and Kane, 2006; Nontachaiyapoom *et al.*, 2011; Sebastian *et al.*, 2014); 3) fungal specificity (Bidartondo and Read, 2008; Sheng *et al.*, 2012; Zi *et al.*, 2014); 4) nutrition flow from fungus to the host (McKendrick *et al.*, 2000; Kuga *et al.*, 2014) and 5) gene regulation (Zhao *et al.*, 2014; Liu *et al.*, 2015; Tsai *et al.*, 2016) that contribute to seed germination, symbiotic seed germination (SSG) remains a challenge for the reintroduction and conservation of orchid species (Batty *et al.*, 2006; Bustam *et al.*, 2014).

To date, SSG for the propagation of seedlings to be used in restoration has primarily been conducted in *in vitro* conditions because both the seeds of orchid species and their associated fungi are sensitive to microhabitat variance (Zettler and Hofer, 1997;

Bruns and Read, 2000; Stewart and Kane, 2007; Park and Lee, 2013; Sathiyadash *et al.*, 2014; Tan *et al.*, 2014; Da Silva *et al.*, 2015). The fungi in SSG are usually from roots of orchid species (Zettler and Hofer, 1998; Zettler *et al.*, 2000; Stewart and Zettler, 2002; Liu *et al.*, 2010; Chutima *et al.*, 2011; Salifah *et al.*, 2011; Fracchia *et al.*, 2014; Sathiyadash *et al.*, 2014) since root-associated fungi is more accessible compared to fungal strains obtained from host protocorms which is inaccessible to pick up in nature at the initial stage of seed germination due to its tiny size and distribution haphazardness and can be time-consuming by *in situ* seed baiting technique to generate. For example, the induction of protocorms for fungal isolation can take upwards of nine months for *D. devonianum* (Zi *et al.*, 2014), seven months for *D. officinale*, *D. nobile* and *D. chrysanthum* (Wang *et al*., 2011), four months for *Cymbidium mannii* (Sheng *et al.*, 2012) and five months for *Papilionanthe teres* (Zhou and Gao, 2016). Although fungal isolation via roots is a faster technique and may facilitate an immediate need to generate orchid seedlings, a yet unresolved question in orchid biology is whether symbiotic mycorrhizal fungi (mycobionts) utilized as a carbon source by young seedlings (protocorms) are different from those utilized by the roots of adult plants (Zettler *et al.*, 2005); in other words, even fungi originated from roots can promote seed germination *in vitro*, but it isn't necessarily to stimulate seed initiation and further development in field because of narrower ecological specificity (Masuhara and Katsuya, 1994; Steinfort *et al.*, 2010). Hence, fungi supporting the symbiotic relationship in roots may not be the same fungi that promote seed germination and seedling formation: fungi isolated from protocorms and applied to seeds may result higher rates of germination (Sheng *et al.*, 2012; Zi *et al.*, 2014; Zhou and Gao, 2016; Shao *et al.*, 2017; Rasmussen and Whigham, 1993; Brundrett *et al.*, 2003; Ke *et al.*, 2007; Keel *et al.*, 2011), although previous studies are largely based on *in vitro* symbiotic germination and have rarely been evaluated *in situ* (Shao *et al.*, 2017).

Most orchid conservation efforts typically use *in vitro* asymbiotic seed germination techniques to generate seedlings for re-introduction or population augmentation of terrestrial species. Stewart and Zettler (2002) reported to establish seedlings of *Habenaria* spp. onto soil *ex vitro* by SSG. The theory of restoration-friendly cultivation using seedlings by tissue culture has been gradually applied in *D. catenatum* plantation and *D. nobile* plantation in Guangdong (Liu *et al.*, 2013, 2014), Chongqing, Guizhou, Hainan and Yunnan provinces, China (Personal observation), which benefits industrial cultivation and conservation. But there is rare report on *in situ* SSG (Shao *et al.*, 2017; Yang *et al.*, 2017). Although seedlings generated by SSG has great potential to enhance current restoration efforts in epiphytic orchid species (Wang *et al.*, 2011; Sheng *et al.*, 2012; Sathiyadash *et al.*, 2014; Zi *et al.*, 2014; Da Silva *et al.*, 2015; Khamchatra *et al.*, 2016; Zhou and Gao, 2016; Shao *et al.*, 2017; Yang *et al.*, 2017), there is a paucity of reports on restoration by *in situ* symbiotic seedlings even though its advantages for adaption to micro-environments, higher seed germination and seedling survival rates (Liu *et al.*, 2010; Shao *et al.*, 2017; Yang *et al.*, 2017).

Xishuangbanna Dai Autonomous Prefecture, Yunan Province, located in southwestern China, is a hotspot of biodiversity with > 430 orchid species (Gao *et al.*, 2014). *Dendrobium aphyllum* (Roxb.) C.E.C. Fischer, *Dendrobium devonianum* Paxt. and *Dendrobium nobile* Lindl. are commonly used in Chinese traditional herbal medicines (Bao *et al.*, 2001; Wang *et al.*, 2001). They are distributed widely in China and neighboring countries, but all the three species are on the Chinese Red List (Gao *et al.*, 2014) as endangered, rare, and extremely endangered, at least partly due to over-collection and habitat loss. Here, we describe and examine a new *in situ* SSG technique for three epiphytic *Dendrobium* species that utilizes fungi isolated from respective host protocorms. Using this technique we address the following four questions: (1) How does *in situ* seed germination rate responses for the species *Dendrobium aphyllum*, *D. devonianum* and *D. nobile*? (2) Do the seed germination rates vary among host trunks? (3) Does the sowing time influence germination rate? (4) What are the implications for *in situ* conservation and restoration of epiphytic orchid?

MATERIALS AND METHODS

Study sites

To evaluate the cultivation and restoration of endangered epiphytic orchid species, we examined on *D. aphyllum*, *D. devonianum* and *D. nobile* as our study species. All three species are epiphytic orchids on tree trunks in open forests or lithophytes on rocks in mountain valleys (c.a 500–1500 m for *D. aphyllum* and *D. nobile*, and 1100–1900 m for *D. devonianum*). All three species are widely distributed in southeastern Asian countries, such as Bhutan, India, Laos, Myanmar, Vietnam and China, including Guangxi, Guizhou, Yunnan (Chen *et al.*, 2011).

We assessed SSG of *D. aphyllum* in a mixed forest of *Citrus maxima* and *Bauhinia purpurea* in Xishuangbanna Tropical Botanical Garden (21° 54′ 53″ N, 101° 16′ 09″ E; altitude 570 m; here after referred to as XTBG). For the species *D. devonianum* and *D. nobile*, we assessed SSG at Longpa traditional tea garden of Jinghong city (Longpa) (21° 59′ 05″ N; 101° 05′ 07″ E; altitude 1150 m) which is mainly dominated by mature *Camellia assamica.* At both sites, annual precipitation due to the tropical monsoon climate ranges between 1400–1800 mm/ yr, rainy season occurs from May to October and followed by misty cool season from November to February, and March to April is the dry hot season. Annual average temperature and mean relative humidity ranges between 18–20 °C and 86–89%, respectively. All laboratory experiments were carried out at the *Ex-Situ* Conservation & Re-introduction Group, XTBG.

Seed collection, activity testing and storage

Mature, un-dehisced, open-pollinated seed capsules were collected for each of the three species. Seed capsules of *D. aphyllum* were collected from XTBG in April, 2013. Seed capsules of *D. devonianum* were collected in April, 2014 from a *Dendrobium* plantation in Tengchong County, Yunnan province and seed sets of *D. nobile* were collected from *Dendrobium* plantation in January, 2015 in Lushui County, Yunnan province. All seed capsules were sterilized in 75% (v/v) ethanol for 2 min and dried at room temperature before being opened with a sterile scalpel under aseptic conditions. The extracted seeds were transferred to airtight glass containers containing calcium chloride anhydrous. After 4 days, the seeds were stored in glass vials at - 20 °C for long-term preservation. Seeds viability was assessed using a tetrazolium test (TTC). Based on an assessment of approximately 300 seeds per species, seed viability was tested with following values: 83.4%, 79.6%, 80.4%, for *D. aphyllum*, *D. devonianum* and *D. nobile*, respectively.

Isolation of mycorrhizal fungi

Fungal strains isolated from each of our study species have been previously shown to promote SSG in all three cases. The origin, identity, storage and capacity of the fungal strain FDaI7 to promote seed germination of *D. aphyllum* has been demonstrated and tested by Zi *et al.* (2014). The fungus FDd1 isolated from protocorms near maternal plants of *D. devonianum* in July 2012 has been shown to enhance *in vitro* seed germination for the same species (Huang *et al*., 2018). And the isolate JC-01 obtained from protocorms of *D. nobile* through *in situ* baiting techniques promotes *in vitro* seed germination of *D. nobile* (unpublished data). These strains were used to test *in situ* SSG at sites mentioned above.

Preparation of inoculated seed packets

For each treatment, we modified the methods of plastic wrap + packet in Shao *et al*. study (2017) that was shown to significantly promote seed germination of *D. devonianum* in field. Specifically, we used plastic wrap to secure a paper package containing a mix of fungal powder and orchid seeds to host trees. Prior to packet preparation, each fungal strain was cultured separately in 10–50 conical flasks (500 mL) with 100 mL sterilized potato dextrose broth (PDB) using a shaker (ZQZY-A, Shanghai Zhichu Instruments Co., Ltd., China) at 150 rpm and 25° C for 7 days. Fermented mycelia were filtered by medical gauze and washed 3 times with sterile distilled water to avoid dextrose residues (Shao *et al.*, 2017). One gram of fresh mycelium mixed with approximately 5 g agar was homogenized by blender and then dried at 30° C for two hours to maintain moderate humidity (Figure 1A). Because low seed density has been shown to facilitate relatively high percentage of germination for the species *D. devonianum* (Shao *et al.*, 2017), a certain amount of the seeds using a ear pick were added and stirred

to obtain a homogeneous mixture. Control test suggested that protocorms or seedlings could be formed only with the presence of symbiotic fungi in Shao *et al.* study (2017), so no further control tests had been replicated here. The mixture ca. 0.02 g was placed into each paper packet using spatula (Figure 1A) and then approximately one gram of sphagnum moss was added. Seed quantity in a packet was recorded by dissolving the mixture without sphagnum in 10 mL 0.1% agar solution and counted seed number under stereo microscope by placing 200 µL on glass slides using a pipette. This procedure was replicated 3 times per suspension and the seed quantity was calculated (Table 1).

Sowing treatment

Once the seed packets prepared for each species, they were watered and fixed to the trunks of host species using a plastic wrap (Figure 1B). The seed packets of different sowing time could not be placed on the same trunk because of seedling colonization at the same site from previous plantation. To assess the effect of host preference on percentage of germination in *D. aphyllum*, two hosts *B. purpurea* (approximately 8 individuals/ tree; N=300) and *C. maxima* (approximately 16 individuals/ tree; N = 718) were chosen. Seed germination was conducted at two sites for *D. devonianum* (such as Longpa and Yiwu) to test the variation on seed germination between two sites (Table 1). Four sowing trials for *D. aphyllum*, *D. devonianum* and three sowing trials for *D. nobile* were conducted at different dates (Total N = 1376) and used to estimate the influence of sowing time on percentage seed germinatio presented in the Table 1. Plantation of *D. devonianum* and *D. nobile* were conducted on the trunks of *C. assamica* at Longpa site for four and three times, which included: Arpil 22, 2015 (N = 42), May 21, 2015 (N = 109), August 6, 2015 (N = 35) and March 26, 2016 (N = 38) for *D. devonianum*; October 12, 2015 (N = 56), March 25, 2016 (N = 43) and April 28, 2016 (N = 35) for *D. nobile* (Table 1).

Statistical analysis of seed germination rate

At the date of each sowing array after 3 months, the number of seedlings (few protocorms) for each treatment was counted and the percentage of germination was calculated as (the number of seedlings/ the number of viable seeds sown) x 100. The effect of species, host, site and sowing time on the percentage of germination rate (%) were analysed by Kruskal-Wallis chi-square test. All the statitisical analyses were performed by SPSS software version 22 (SPSS Inc., Chicago, USA).

RESULTS

Germination rates among species and hosts

Overall, seed germination rate of *D. aphyllum* for the 1018 replicates across both hosts varied from 0–59.9% (10.0 ± 9.63%). Percentage of germination of *D. devonianum*

Figure 1. Fungal powder mixed with seeds for sowing: **A**, fungal powder of FDdI7 for packaging by a spatula; **B**, tea packets fixed by plastic wrap on the trunk of *Camellia assamica.*

Table 1. Effect of sowing time (dates) on seed germination in of *D. aphyllum*, *D. devonianum* and *D. nobile* (the values provided with factor effect are Kruskal-Wallis chi-squared statistic and the associated significance values); significant level: *** $p<0.0001$; * $p < 0.01$.

Species	Sowing time	N	Seed number	df	Kruskal-Wallis chi square test
D. aphyllum	23 Oct 2015	2408	44	3	44.986 ***
	29 Oct 2015	210	53		
	6 Nov 2015	424	38		
	20 Nov 2015	144	36		
Total N		1018			
D. devonianum	22 Apr 2015	42	232	3	41.783 ***
	21 May 2015	109	87		
	6 Aug 2015	35	71		
	26 Mar 2016	38	40		
Total N		224			
D. nobile	10 Dec 2015	56	43	2	8.143 *
	25 Mar 2016	43	59		
	28 Apr 2016	35	11		
Total N		134			

for the 224 replicates and of *D. nobile* for the 134 replicates ranged between 0–36.1% (9.4 ± 9.8%) and 0–63.6% (13.6 ± 14.9%), respectively. The percentage of packets that produced seedlings of *D. aphyllum*, *D. devonianum* and *D. nobile* was 77.3%, 73.8%, 76.9%, respectively.

There was no significant differences found on seed germination among three *Dendrobium* species (Kruskal-Wallis chi-squared = 4.78; df = 2; $p = 0.092$; Figure 2).

Effect of host and sowing time

In spite of the lack of species effect on seed germination, there were significant differences found with host plants (Kruskal-Wallis chi-squared = 50.66; df = 2, $p < 0.0001$; Figure 3). Besides, the sowing time showed significant differences on the percentage of seed germination of *Dendrobium* species (Kruskal-Wallis chi-squared = 104.79; df = 10, $p < 0.0001$; Table 1).

In *D. aphyllum*, the percentage of seed germination showed significant association and differences according to the host plant, *Bauhinia purpurea* and *Citrus maxima* (Kruskal-Wallis chi-squared = 52.05; df = 3; $p < 0.0001$).

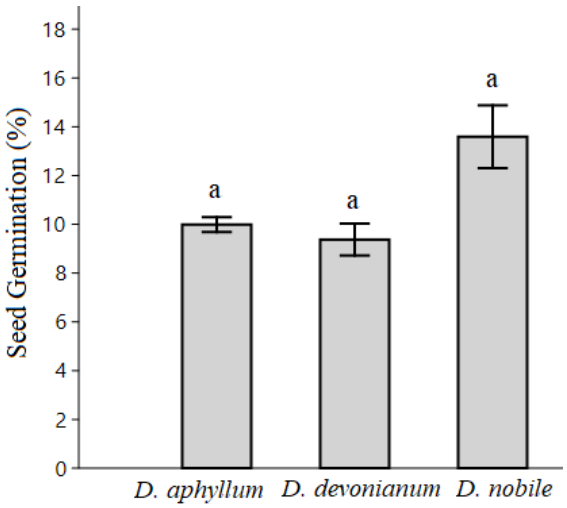

Figure 2. *In situ* symbiotic seed germination rate of *D. aphyllum*, *D. devonianum* and *D. nobile*; the same letters above bars indicate no significant differences among species according to the Kruskal-Wallis rank sum test**.**

In addition, the sowing time (dates) showed significant differences on seed germination of *D. aphyllum* (Kruskal-Wallis chi-squared = 44.99; df = 3; P < 0.0001; Table 1). Similarly, this effect is significant on both

host plants, *Bauhinia purpurea* (Kruskal-Wallis chi-squared = 14.5; df = 2; p = 0.001) and *Citrus maxima* (Kruskal-Wallis chi-squared = 28.5; df = 3; p = 0.0001). The percentage of seed germination showed significant differences of *D. devonianum* and *D. nobile* on different sowing times (Kruskal-Wallis chi-squared = 41.78; df =3; $p < 0.0001$; Kruskal-Wallis chi-squared = 8.14; df = 2; p = 0.017; Table 1) respectively.

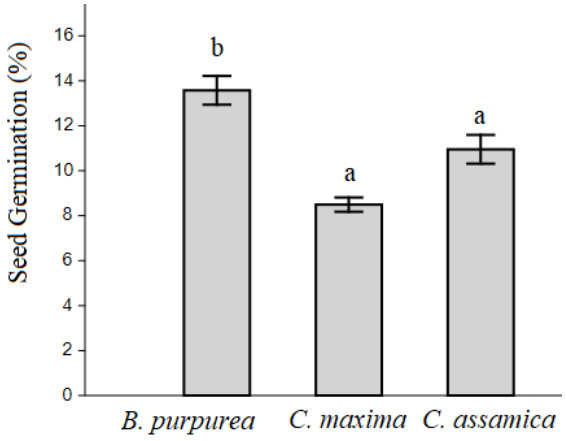

Figure 3. The effect of host plants on *in situ* symbiotic seed germination (%) for three *Dendrobium* species; small letter 'b' indicates significant different on seed germination according to the Kruskal-Wallis rank sum test.

DISCUSSION

In situ seed germination rates

The treatment plastic wrap + packet (paper) mixed with seeds, fungal powder and sphagnum proved an effective method for seed germination *in situ*. In the current study, we adjusted our methods from those of previous studies (Shao *et al*., 2017) to account for the potential loss and drying out seeds sown *in situ*. As a result, the germination rate of *D. devonianum* by this new treatment increased from ~1.4 ± 0.4% in Longpa and 0.9 ± 0.4% in Yiwu (Shao *et al*., 2017) to 9.4 ± 9.8% in this experiment.

The efficiency of the new technique varied among species. In the current study, *D. devonianum* showed the lowest mean percentage germination (9.3%), which was still far much higher than rates achieved for *D. devonianum* in previous work (Shao *et al.*, 2017). Although the germination percentage in this study is obviously lower than that in Zi and Gao's trails in which seeds grown to protocorms with percentage 44.4% and further developed to seedlings with rate 42.9% when co-cultured with the same fungus FDd1 (Zi and Gao, 2014), their trial was conducted on sterile artificial substrate under controllable conditions but not in the field. Yang *et al.* (2017) test impacts of host trees and sowing conditions on seed germination of *Dendrobium sinense* and indicated germination rate was lower than 1.0% in most cases and rarely up to 9.6% under *ex situ* conditions on preferential host tree substrate. Percentage germination was not kept stable in Yang *et al.* study since no symbiotic fungi were inoculated. The unreliable germination process may be caused by opportunistic concurrence between seeds and specific fungi, which contributed the conclusion that *in situ* a positive correlation was found between the seed germination rate and the distance of the seeds from the adult *D. sinense*. Aewsakul *et al.* (2013) achieved higher germination rates, up to 67.6%, in *Spathoglottis plicata* under sterile commercial cultivation substrates, consisting of potting soil, coir dust, and peat moss, which push the method forwards for practical application compared to *in vitro* SSG on OMA (oat meal agar) for its economical facilities and uninoculated conditions for mass seedling propagation and horticultural purposes (Quay et al., 1995). *Ex vitro* SSG of *Papilionanthe teres* by Zhou and Gao (2016) with different bark substrates without sterilization and inoculation showed 0.42% seeds developed to protocorms (stage 3) on preferential host barks and no further development at 45 days after sowing; however, 47.5% seeds cocultured with compatible fungus *in vitro* formed seedlings (stage 4) on the same host substrate at the times. Even though the germination rate decreased from *in vitro* SSG via *ex vitro* SSG to *in situ* seed sowing; seedling process in this study showed practice and potential to population construction of other epiphytic *Dendrobium* spp. in the field.

Host Preference

Although significance exists between two hosts, both showed higher percentage reports in field up to 13.6% and 8.5%, respectively, which imply certain colonial preference on the host of *B. purpurea* possibly due to its roughness, capability of maintaining moisture

and other potential chemical and physician characteristics, such as pH, nitrogen and carbon components. Harshani *et al*. (2013) suggest the prohibition of seed germination of *D. aphyllum* may be interacted by the chemical composition of the non-host barks. Some other factors such as the water holding capacity of the bark, bark stability, presence of facilitators and microclimate also may contribute to the distribution of *D. aphyllum* (Rafter *et al*., 2016). Zhou and Gao (2016) disclosed preference of *Papilionanthe teres* seed germination on three host plants (*Averrhoa carambola*; *Lagerstroemia villosa*, *Callistemon rigidus*) and no germination on bark substrates of *Butea monosperma*. The inclination was also shown with significant differences in the average fresh weight of a single germinated seed, average developmental stages, and percent of seed number with developmental stage 4. Host preference was also exhibited in Yang *et al*. (2017) studies in which *in situ* seed germination successfully occurred with the highest germination rates on three host tree species with *Dendrobium sinense* but failed to germinate *in situ* on *Cyclobalanopsis blakei* and regardless of setting (*ex situ* or *in situ*) on *Exbucklandia tonkinensi*, the latter two hosts with no associations to *D. sinense*.

Sowing time

Time is an important factor influencing germination in three species by temperature and air humidity. Germination rates varied significantly among sowing times for each of the three species screened in this study. All the sowing times of *D. aphyllum* belonged to the misty cool season and in proximity, however, similar significant difference was found among trials on the trunk of *B. purpurea* and of *C. maxima*, which was possibly caused by microhabitat and other factors. Significant differences on seed germination trials of *D. devonianum* and *D. nobile* were found at different times which might be probably caused by climate factors in three seasons, including moisture and temperature in microhabitat.

Orchid seed germination is in some cases strictly seasonal, employing a short time window between seed ripening and seed vitality loss, as the seedling stages before the development of leaves and storage tissues are highly dependent on sustained moisture and appropriate thermal regimes, synchrony of germination and time and conditions for sufficient growth before an unfavorable season can be critical for germination and establishment (Rasmussen *et al*. 2015). Some cases showed the key season to seed germination: Wang *et al*. (2011) harvested packets of *in situ* seed baiting in June 2008 in order to con-occur with the growing season; orchid seeds in Madagascar dispersed during the dry season would not have such access to water and would probably need to undergo a dormant period and wait for the subsequent wet season for germination (Rafter *et al*., 2016); Seeds of *Dendrobium sinense* sown *ex situ* and *in situ* had the highest rates of germination in July, coincided with the raining season in Hainan during which the fruits of *D. sinense* dehisce from May to July and high rainfall and humidity prevails (Yang *et al*. 2017); the season of seed sowing of *D. devonianum* was considered as an important factor influencing seed germination rate, which is proved by the significantly highest seed germination during the misty cool season (*Shao et al*., 2017).

Conservation implications

SSG can be used to study fungal activity and investigate the effect of fungal ecological distribution on host dynamics for terrestrial orchids by burying the seed packets. There are seldom references to apply the packets with seed and fungi to promote seed germination for terrestrials or epiphytes in the field. Before this study, at least two obstacles limit the development of SSG. One is the fact that, when the seeds germinate and form seedlings a period after introducing, it will be constrained in the nylon net packets and cannot grow out through it. The other problem is the difficulty in dealing with the fungi. It is an issue to keep seeds and fungi together during germination stages and the appropriate proportion for substantial balance between seeds and fungi. For epiphytic orchids, how to retain the moisture is also problematic but crucial to seed germination. By the new methods, all the obstacles above can be resolved. All the three species showed the potential reflected by relatively high germination rate although variable repeats.

The technique is novel and applicable for *Dendrobium* species' conservation through *in situ* cultivation and restoration. Nylon net packets are replaced by degradable tea packets

so that the seedlings can grow out the bags naturally. Sphagnum contained in the packets with seeds and fungi are rolled and covered by plastic wrap to keep moisture during the early germination stage even in the dry season. Fungal mycelium mixed with agar was made into powder by blender rather than suspension, therefore the number of seeds and powder is controllable in each packet which can be easy preserved in fridge and operated during the procedure of sowing.

However, a prerequisite must be emphasized that fungal origin and effectiveness should be originated from corresponding protocorms and proved. Although many fungal strains observed by trials promoted orchid seed germination, it is also important to question the compatibility between the fungi and orchid seedlings development (Nontachaiyapoom *et al*., 2011) in nature. Based on the previous work (Rasmussen and Whigham, 1993; Wang *et al*., 2011; Zi *et al*., 2014), we predict the best method to obtain the fungal strains from corresponding orchid protocorms formed in nature or by *in situ*/ *ex situ* baiting but not plants roots-associated and heterogeneous protocorms because specificity is higher in the field than that *in vitro* symbiotic germination (McKendrick *et al*., 2002). The right origin could initiate seed germination and support seedling further development. An example in case is fungi from protocorms of *Cymbidium manii* which can support seed germination of *Dendrobium aphyllum* but not plantlets augmentation (Zi *et al*., 2014). The role of fungal specificity remains elusive and needs to be tested. In addition, given the fact that tiny seeds and microorganism are susceptible to the microhabitat, several factors effect on the seed germination percentage, including sowing time and/ or host, should be paid much more attention.

Future directions

We propose that the populations can be constructed after two or three years' growth using SSG by introducing seeds with effective fungus/fungi directly on the trunks, the fresh stems used as Chinese medicinal herbs have the same quality to the wild plants, which can be applied for the industrial plantation. The seeds germinated and formed protocorms 30 days (Figure 4D) after sowing and further developed to plantlets at 60 days (Figure 4A, B). Puncturing a hole in the plastic wrap to help seedlings grow out of the plastic wrap is the last step and then the seedlings grow naturally without management (Figure 4C). The plantlets survived by forming pseudostems during the winter and sprouted at the beginning of the next spring. Profit-motivated conservation can benefit local people through such a friendly cultivation method and reducing dependency on the over/ illegal collection of wild plant resources. The method will contribute to *in situ* SSG for rare and endangered epiphytic orchid reintroduction and conservation.

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