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Symbiotic germination in orchids: An overview of *ex situ* and *in situ* symbiotic seed germination

Tadar Jamja[1], Sunil Bora[2], Ruthy Tabing[3*], Nangki Tagi[4], Arvind Kumar Chaurasiya [5]Sushma Ningombam Devi[6] and Mero Yangfo[7]

[1,2,6]Department of Horticulture, Assam Agricultural University, Jorhat, India
[3]Department of Plant Pathology, Assam Agricultural University, Jorhat, India
[4]Department of Nematology, Assam Agricultural University, Jorhat, India
[5]Department of Horticulture, NEHU, Tura Campus, West Garohills, Meghalaya, India
[7]Department of Forestry, North Eastern Regional Institute of Science and Technology, Nirjuli, India

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ABSTRACT

Plant family Orchidaceae is well-known in the global floriculture market for its beauty, long-lasting flowers, and exquisite variation in flower colour, shape, size, foliage form, and texture. They are thought to be one of the most varied and evolved vascular flowering plants. Orchids produce millions of dust-like seeds in a pod, but they have a unique and persistent problem with seed propagation due to a lack of food reserve 'endosperm' in their minuscule seeds, which often fail to germinate in a natural environment without being in symbiotic association with compatible mycorrhizal fungi. Symbiotic seed germination takes advantage of the fungal-orchid symbiosis interaction, in which orchid seeds are germinated in different growth media after being inoculated with orchid mycorrhizal fungi. It has been one of the most important and commonly used propagation techniques for orchid conservation and reintroduction programmes around the world. Prior to the introduction of the *in situ* technique in the early 1990s, symbiotic seed germination was performed using an *ex situ* procedure. The *in situ* technique was designed to improve the efficiency of orchid seed propagation and reduce acclimatisation challenges during the reintroduction programme. It emphasises germination in natural environmental settings, with the idea that the presence of suitable fungi in the surrounding environment can influence germination. *In situ* symbiotic germination is a relatively recent technique that has evolved over time and continues to be with increased interest and research in this area. This review article is an attempt to provide an overview of symbiotic seed germination in orchids- *ex-situ* and *in situ* techniques.

Key words: *Symbiotic germination, Orchids, Ex situ orchid conservation, In situ orchid conservation, Orchid mycorrhizal fungi*

Introduction

The family Orchidaceae, with more than 17,000 to 25,000 species recorded thus far, is the largest angiosperm plant family (Christenhusz and Byng, 2016) and is considered one of the most evolved and diverse flowering plant species (Nomura *et al*., 2013; Rafter *et al*., 2016; Suetsugu *et al*., 2020). Orchid species are known for their bewitching flowers, and rich diversity, viz. colors, form, textures, shape, size, fragrance, etc. (Peakall, 2007). Owing to their multiple uses, demands for orchids have increased manifold in recent decades (Shao *et al*., 2017a) rendering many species on the verge of extinction

across the planet (Dixon *et al*., 2003; Jones, 2006; Dearnaley, 2007a) due to unscrupulous extraction, drastic change in a forestland landscape, global climate change, etc. The endangerment of the orchid species and decline in their population are compounded by their high level of geographic endemism and complex relationship with other organisms (Swarts and Dixon, 2009; Orejuela-Gartner, 2012). Orchids are now considered one of the most endangered plant species (Cribb *et al*., 2003; Phelps and Webb, 2015) and are predicted to be among the first to decline due to habitat degradation (Backhouse, 2007). This makes it difficult for orchid conservationists worldwide to strike a balance between orchid conservation and market demands (Shao *et al*., 2017b).

The use of millions of dust-like, minute, light seeds produced in an orchid pod and their successful germination (Arditti and Ghani, 2000a) for mass propagation and rapid regeneration of orchids could be the best way to fill the gap between production and market demands as well as for conservational works. However, orchid seed have Unique and persistent problem for seed propagation. The minuscule orchid's seeds lack'endosperm' food reserves, and an embryo is surrounded by air space (Arditti and Ghani, 2000b; Barthlott *et al*., 2014). As a result, orchid seeds often fail to germinate in nature in the absence of compatible fungi called "orchid mycorrhizal fungi", which are crucial for the germination, initial seedling development, and subsequent growth and development of the plant (Rasmussen, 1995a). A mutualistic relationship is advantageous for the propagation of seeds devoid of nutritional reserves (Rasmussen, 1995c). To reap such beneficial association and to overcome the germination problem in orchid seeds,"symbiotic seed germination" was developed, wherein orchid seeds are germinated in media inoculated with compatible fungi. It has merit for both horticultural and conservational works (Aggarwal and Zettler, 2010) and has become an essential part of orchid propagation worldwide (Stewart *et al*., 2003; Batty *et al*., 2006a; Otero *et al*., 2013). Initially, symbiotic seed germinations were carried out in *ex-situ* conditions (conventional technique) which remained primary components for restoring many orchid species for decades (Shao *et al*., 2017c) despite various reports on slow growth, high mortality rates, delayed flower phenology (Shimura and Koda, 2005; Batty *et al*., 2006b; Stewart and Kane, 2007a; Wu *et al*., 2010), lacking genetic variation necessary for local adaptation and evolutionary potential (Zhou and Gao, 2016a), labor intensiveness, rendering it limited conservation value on a large scale (Shao *et al*., 2017d). Later, Rasmussen and Whigham (1993a) first developed the nonconventional *in situ* symbiotic germination technique which prompted many other researchers such as Zettler *et al*. (2011a) in the USA, Liu *et al*. (2014) in China, Higareda *et al*. (2015) in Brazil and Shao *et al*. (2017e) in China,to improve this technique with their ingenious research problems in addition to the difficulties and drawbacks stated by previous researchers. The continuous research and progress made by different researchers from different parts of the world significantly transformed *in situ* techniques over time. Growing research and literature suggest that the *in situ* symbiotic germination technique is comparatively advantageous over *ex-situ* symbiotic germination, especially for orchid reintroduction Programmes. However, compared to ex situ technique, the literature on in situ technique and its use is still limited. This review article attempts to provide an overview of ex situ and in situ symbiotic seed germination in orchids, its applications and benefits, and diverse empirical findings from various sources. This review article was prepared using a variety of sources, including e-journals, published research papers, articles, books, and so on.

Orchid mycorrhizal fungi and their significance in orchid life

Mycorrhizae are symbiotic relationships between the roots of higher plants and fungi. Such symbioses are ubiquitous in nature (McCormick *et al*., 2018); however, the orchid mycorrhizal fungi are an exclusive symbiotic relationship between the plant family Orchidaceae and the fungi, rendering almost all the orchid species mycoheterotrophic at one point of their life (Rasmussen, 1995b). Orchid mycorrhizal fungi provide essential nutrition for germination, and protocorm formation until the green leaf stage (Dearnaley, 2007b; Smith and Read, 2008; Rasmussen and Rasmussen, 2009a; Rasmussen, 1995d). This fungal association is often maintained into adulthood, although the dependence of the adult plant on fungi may vary with life forms viz., epiphytic or terrestrial (Rasmussen and Rasmussen, 2009b). The actual symbiotic relationship begins with the infection of dust-like seeds by the fungus, after which the seed swells and protocorms are formed as the initial myco-heterotrophic stage

which leads to the development of seedlings. The fungus colonizes the tissue of the orchid and forms highly coiled hyphal structures called "pelotons" within the root cortex with the size of root cells (Sathiyadash *et al*., 2012). These pelotons are digested or lysed by orchid cells that access carbon, phosphorus, and nitrogen to the orchids and in return, orchids provide amino acids and sugar to the fungus (Dearnaley and Cameron, 2017). This digestion occurs in a controlled manner allowing successive waves of peloton formation, digestion, and reinfection within the same root cells (Smith and Read, 1997a) that maintain the duration of their active association (Brundrett, 2002). Thus, orchid roots contain pelotons either in lysed or intact conditions in different proportions at any given time (Smith and Read, 1997b).

Various studies have shown that both photosynthetic and mycoheterotrophic orchids associate with a range of fungal species (Shefferson *et al*., 2007; Jacquemyn *et al*., 2010; De Long *et al*., 2013; Waud *et al*., 2017) mostly with fungal taxa belonging to Rhizoctonia like species,a group of Basidiomycetes, specifically Ceratobasidiaceae, Serendipitaceae, and Tulasnellaceae (Dearnaley *et al*., 2012; Rasmussen and Rasmussen, 2014; Weiß *et al*., 2016). These mycorrhizal fungi play a significant role in rehabilitating threatened orchid species in their natural habitats (Dearnaley, 2007c) as they are believed to ensure orchid survival in habitats vulnerable to stressful conditions or habitats with limited resources. Thus, for successful *in situ* and *ex-situ* orchid conservation, the availability of suitable OMF and niche conditions is imperative (Rasmussen *et al*., 2015; Bidartondo and Read, 2008a and Reiter *et al*., 2016) and conservation efforts should involve a thorough understanding of the biology of orchids (Dearnaley, 2007d), their complex relationship and their interaction with fungi. However, the presence, distribution, andniche requirement of OMF are still poorly studied and understood (Muhammad *et al*., 2019).

Baiting techniques for symbiotic seed germination

A conventional technique, *ex-situ* symbiotic and asymbiotic seed germination, has remained the most widely used technique for orchid conservation for decades. In the symbiotic germination technique, orchid seeds are germinated with compatible fungal inoculation under controlled environmental conditions. *In situ* symbiotic seed germination is a nonconventional technique, wherein viable orchid seeds are baited in natural environmental conditions and retrieved later when protocorms/seedlings are developed. A growing body of literature suggests that *ex-situ* and *in situ* symbiotic germination techniques can be used in an integrated way for more efficient and effective results whereby the mycorrhizal fungi isolated from germinated protocorms or young seedlings from *in situ* are being used as inoculum for *ex-situ* germination for conservation and reintroduction (Huang *et al*., 2018a). In doing so, the *ex-situ* technique works as an extended technique of *in situ* symbiotic germination. The procedures followed in these two techniques are discussed below.

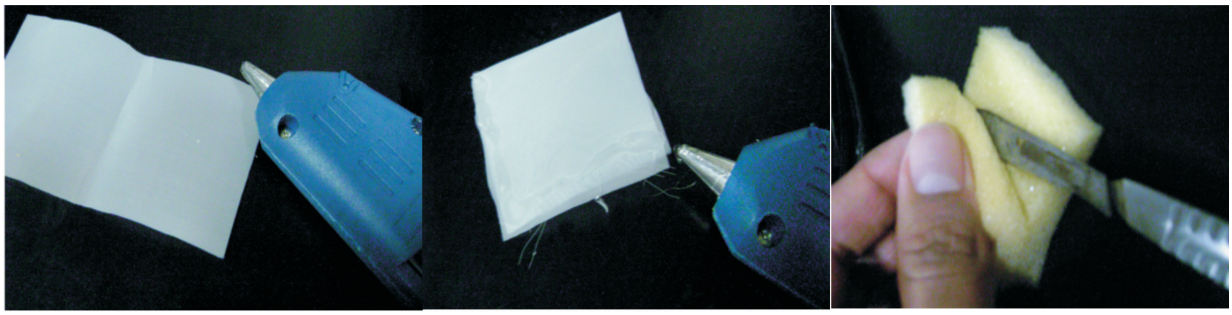
***In situ* symbiotic seed germination**

The initial *in situ* seed burial technique described as the "seed packet technique" was developed by Rasmussen and Whigham (1993b) and was based on the understanding that the patchy distribution of orchids may be influenced by the presence or absence of specific mycorrhizal fungi essential for the survival of orchids. In this technique, orchid seeds are sown and retrieved in the field under natural conditions. Later, Brundrett *et al*. (2003) modified the technique by placing orchid seeds and silica sand between two pieces of nylon mesh and held in place by a slide frame. However, this technique was confined mostly to terrestrial orchids, and poor survival during summer drought conditions, difficulty in retrieving the sample, and reported low retrieving percentage were the major drawbacks of this technique. These factors prompted many studies on *in situ* symbiotic germination in the following decades. Owing to their ascension into the tree canopy, researchers faced an additional burden to make *in situ* techniques practical for epiphytic orchids as well (Shao *et al*., 2017f). This led to the placement of orchid seeds in organic substrates such as sphagnum moss, leaf mold, or bark, which still proved unsuccessful (Kauth *et al*., 2008). However, it was found that sphagnum moss permits good light penetration and water retention, and prevents desiccation in the field which is necessary for acclimatization or seedling reintroduction (Zettler *et al*., 2007; Valadares *et al*., 2012; Zeng *et al*., 2012; Khamachatra *et al*., 2016a). This technique was further modified by Zettler *et al*. (2011b) and Zi *et al*. (2014a) by emphasizing affixing seed packets to arboreal substrates. Again, they indicated several challenges to keep substrates moistened and effective fungus growth and proliferation to enable endophytic seed germination. This moti-

vated Higareda *et al*. (2015) to develop a novel seed baiting technique wherein they used rectangular 10 x 5 cm plankton netting mesh packets with 65 µm pore size and rectangular 3.5 x 2.5 x 0.5 cm synthetic sponge to improve the water and moisture retention as well as to raise the possibility of priming of mycorrhizal fungi. A sponge with homogeneously dispersed seeds (without inoculation) was then placed into a nylon mesh packet and sealed, which was placed nearby young orchids in host trees (phorophytes). To mimic the natural environment, lichens or moss colonies from the same trees were used to cover each packet. This technique reported protocorm formation after 124 days of incubation (Fig. 1). Furthermore, Shao *et al*. (2017g) proposed a modified technique that they called a "novel *in situ* advanced restoration-friendly program" in which 1 g of cultured mycelium of *Tulasnella sp.* was homogeneously mixed with 50 ml of sterile deionized water to form a suspension, which was then sown on *Camellia assamica* trees using a medical syringe in two different locations and different treatments (Table 1). In all these studies, orchid seeds were placed in their natural habitat without inoculation, assuming that existing fungi in soil or substrate will colonize the baited seeds (Brundrett and Ramsay, 2001).

Ex situ symbiotic seed germination

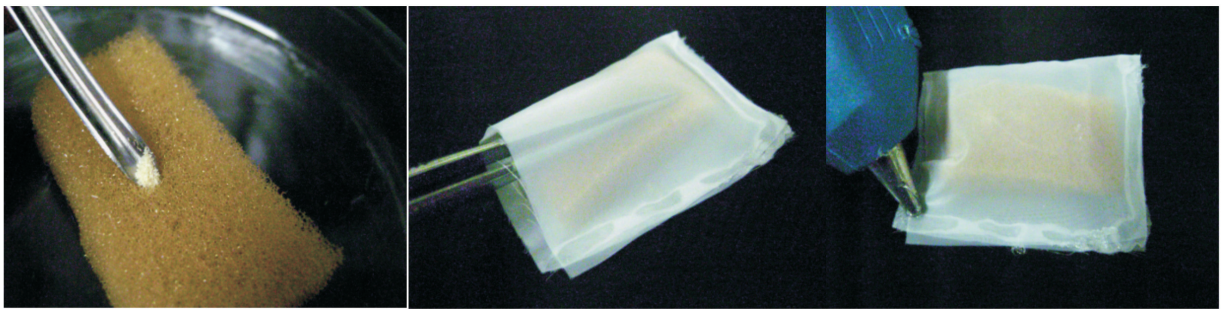
Traditionally, *ex situ* symbiotic or asymbiotic seed germination has been the main restoration technique and is considered cost-effective despite the limited genetic potential and high mortality rate in the field (Shao *et al*., 2017h). The *ex situ* method is used for both symbiotic and asymbiotic seed germination, however, our discussions here will focus on *ex situ* symbiotic germination, not asymbiotic germination. Reintroduction through *ex situ* symbiotic germination has been a primary component for the restoration of many orchid species to date. This technique does not always mean representing any realistic environmental conditions; rather, it acts as a replica of the microenvironment and may function as a tool for extensive studies (López-Chávez *et al*., 2016; Yamamoto *et al*., 2017; Valadares *et al*., 2020). Two important elements required for *ex situ* symbiotic culture are orchid seeds and a suitably isolated fungus on solid agar medium containing nutrients for the fungus and not the seeds assuming that seeds can absorb only water, not nutrients (Pujasatria *et al*.,



A. Rectangles of plankton netting mesh.

B. Folding plankton netting mesh and sealing

C. Construction of synthetic sponge rectangles



D. Seeds dispersed on the surface of the sponge

E. Placing sponge with seeds into nylon mesh packet

F. The packet was finally sealed with glue

Fig. 1. A novel seed baiting by Higareda et al. (2015) in Lankesteriana, vol. 15, issue 1 (pp. 67-76) (with permission)

2020). This technique can be modified based on personal convenience (Hoang *et al*., 2016). The stages involved in this technique are as follows;

Seed collection

Mature orchid seed capsules are collected in a sterile glass vial containing desiccant $CaSO_4$ (Higareda *et al*., 2015). During collection, care must be given to maintaining reproductive potential in the wild. It is advisable to collect only 10% of the total seeds available on the day of collection (Brundrett and Ramsay, 2001). Proper labelling should be done.

Seed sterilization, viability test, andpretreatment

A collected seed's capsules aresurface sterilized with 75% ethanol for 2 minutes (Meng *et al*., 2019a; Shao *et al*., 2017i). The capsules are opened to release the seeds with the help of a scalpel under sterile conditions. Seeds are air dried over $CaCl_2$ for 4 days at 25±2 °C and then stored at 4 °C fora short period (Higareda *et al*., 2015; Meng *et al*., 2019b), after which they are stored at -20 °C (Meng *et al*., 2019c; Shao *et al*., 2020). Seeds canthen bepre-treated with NaClO 1% (w/v) for 3 minutes to improve the hydrophilicity and permeability (Chen *et al*., 2022) followed by rinsing with deionized water. Pretreatment of seeds is not a mandatory prerequisite, as Aggarwal and Zettler (2010b) successfully germinated *Dactylorhiza hatagirea* without pretreatment. The viability of the seed is tested with 1% TTC in deionized water (Vujanovic *et al*., 2000; Higareda *et al*., 2015; Shao *et al*., 2017j) for 72 hours at 30 °C in darkness (Higareda *et al*., 2015) followed by washing the seeds in sterile distilled water for 3-4 minutes (Meng *et al*., 2019d). The seed with a red, pinkish brown, ovoid shape embryo under light microscopy (Aggarwal and Zettler, 2010c) observation is viable whereas the unstained embryo is unviable as described by Van Waes and Debergh (1986).

Seed sowing and fungal inoculation

A range of asymbiotic nutrient media, such as OMA, MS-Media, Vacin and Went media, and Thomale GD (Khamachatra *et al*., 2016b) can be used for symbiotic seed germination. A 0.5 cm^3 (Higareda *et al*., 2015) or 1 cm^3 (Aggarwal and Zettler, 2010d; Chen *et al*., 2022) sample isolated, the identified mycorrhizal fungus is inoculated on one side of the paper strip and sealed immediately to retain moisture and prevent contamination. Seed sowing can be done by following the general protocols reported by Stewart and Zettler (2002). Petri dishes with seeds are then incubated at 22°C (Higareda *et al*., 2015).

Seed germination

Depending upon the orchid species, media used, and fungal isolates, germination will occur with swelling of seeds, followed by protocorm formation and seedling development. The different germination stages can be observed by Arditti (1967) as shown in Table 2.

Growing

Well-developed seedlings are transferred into a growing container with suitable media in ambient environmental conditions for acclimatization before reintroduction into the wild.

Table 2. Stages of symbiotic germination and seedling development by Arditti (1967) and modified by Shao *et al.* (2020) and Chen *et al.* (2020)

Seed germination stage	Description
Stage 0:	Seed, no germination
Stage 1:	Embryo swollen, turned light green, no germination (covered by testa)
Stage 2:	Embryo continue enlargement and discharged from testa (germination)
Stage 3:	Protocorms formation and development, appearance of protomeristem
Stage 4:	Emergence of first leaf (seedling)
Stage 5:	Elongation of first leaf
Stage 6:	Seedlings with two leaves

Environmental conditions for symbiotic seed germination

The growth of orchids depends upon environmental factors, viz. temperature, soil type, competition, pollination, and the presence of mycorrhizal fungi in the ecosystem (Gregga and Kéryb, 2006; Swarts *et al*., 2010). Various *ex-situ* and *in situ* symbiotic germination studies have established that different environmental conditions during the incubation period and the sites of seed placement have significant effects on germination, protocorm formation, and subsequent seedling development. The major advantage of *ex situ* over *in situ* conditions is that the environmental conditions that in *ex situ* conditions can be regulated or maintained as per need throughout the study period which is not possible in *in situ* conditions. In *in situ*, seeds are brought back to the

Table 1. *In situ* symbiotic seed germination reported from different places

Orchid	Epiphytic/ Terrestrial	Location	Experiment conditions	Experiment outcomes	References
Dendrobium devonianum	Epiphytic	China	A liquid suspension of the seed of *D. devonianum* 4 mlwas sown on a tree trunk using a medical syringe. Treatments were; sphagnum wrap, directly applied on the tree trunk, seed suspension mixed with cow dung directly applied on the tree trunk, plastic wrap, plastic wrap + suspended seeds in nylon packets, and control where suspension of seeds was mixed with 0.1% sterile agar without fungal inoculum. These treatments were placed on *Camellia assamica* tree in two different locations.	After 3 months, the germination % was found to vary among treatments and locations. Control treatment did not germinate in both locations. The highest germination % was found in treatment plastic wrap + suspended nylon seed packet in both locations. Regardless of the season, microclimate conditions plastic wrap + seed packet having the highest *in situ* germination rate (0.94-1.44%) with no significant variation among sites, supported by a warm, moist, and fixed site that allowed for light penetration.	Shao *et al.* (2017)
Dendrobium chrysotoxum, D. nobile, D. catenatum, D. devonianum	Epiphytic	China	Seeds of *D. chrysotoxum* were homogenized with sterilized agar suspension (0.1% agar). 1 ml viable seed mixture was dispensed into a nylon mesh packet with 45 µm pores, using a standard baiting protocol described by Rasmussen and Whigham (1993) and Zi *et al*. (2014). The seeds were placed on trees with plastic wrap to retain a sufficient amount of humidity within the packet and avoid desiccation in different locations.	After 4 months of seed baiting, 32% (34 out of 107) of the packets were retrieved and were effectively germinated and developed into protocorms. Protocorms were colonized by seven different fungal isolates. Four different strains isolated from *D. chrysotoxum* were *Tulasnellaceae sp.*, *Coprinellus subdisseminatus*, Uncultured *Tulasnellaceae* clone, and Uncultured *Tulasnellaceae* (clone). One strains each from *D. nobile* (*Sebacinaceae sp*.), *D. catenatum* (Uncultured *Tulasnellaceae* clone) and *D. devonianum* (*Epulorhizasp*.)	Shao *et al.* (2020)
Rhynchostele cervantesii	Epiphytic	Mexico	The sponge was used to retain moisture, where the matured dried seed of 100 mg was homogeneously dispersed. The sponge was then placed under a nylon mesh packet which was later on installed on the host tree given that that tree harbored at least one species of *Rhynchostele cervantesii.*	Sponge acted as a moisture retainer and mimics the natural conditions. Of the total 22 samples installed, 3 samples were found developing protocorms after 3-4 months (124 days).	Higareda *et al.* (2015)
D. aphyllum	Epiphytic	China	Seeds of *D. aphyllum* were placed in a 4x 6 cm nylon packet with 45µm diameter pore to facilitate fungal	After 10 months, 161 packets out of 210 total packets were recovered and it was found that the probability of	Zi *et al.* (2014)

Table 1. *Continued ...*

Orchid	Epiphytic/ Terrestrial	Location	Experiment conditions	Experiment outcomes	References
			hyphae growth inside the packet. All 210 nylon packets containing 80-100 seeds were placed on the tree bark and covered with moss to prevent desiccation and facilitate proper light penetration.	encountering fungi during 10 months was just 19% wherein only 11 packets of the total were found germinated. Developed protocorms and seedlings were found in these 11 packets. The fungus was identified as *Tulasnella sp., Epulorhiza sp., and Trichoderma sp.*	

natural environment, and exposed to different biotic and abiotic factors.

Maintaining ambient environmental conditions for *in situ* germination is a challenging task. A warm microhabitat with high and constant humidity and proper light penetration to the site of seed placement affects germination and protocorms formations in *in situ* seed baiting has been reported by Shao *et al*. (2017) and Zi *et al*. (2014b). Maintaining favorable microclimatic conditions is necessary for the maintenance of seedlings and fungal vitality (Shao *et al*., 2017), which otherwise may prove to be fatal to successfully symbiotically germinated seedlings as reported in *Caladenia arenicola* by Batty *et al*. (2006c). To maintain sufficient humidity within the seed packet and to prevent desiccation, a synthetic sponge (Higareda *et al*., 2015) and plastic (degradable and eco-friendly) are used to wrap the seed packet placed on the tree (Shao *et al*., 2020). The rapid development of orchid seedlings (epiphytic in particular) *in situ* may be advantageous in moisture deficit places, especially during the dry season (Shao *et al*., 2020).

Shao *et al*. (2017) also reported that seed germination in *in situ* is influenced by the season of sowing in *Dendrobium devonianum* where high germination was recorded during the cool misty season, followed by the dry hot season, whereas the lowest germination was recorded during the rainy season. To obtain immediate fungal colonization from the natural environment in *in situ* seed baiting, it is advisable to place the seed nearadult plants of the same species to obtain high germination (Batty *et al*., 2001). However, Kartzinel *et al*. (2013) in *Epidendrum firmum* and Shao *et al*. (2020) in *Dendrobium chrysotoxum* reported successful germination even when seed packets were not placed in proximity toany adult plant.

Likewise, in *ex-situ* conditions, the length of light duration during the incubation period accelerates protocormformation and subsequent seedling development (Aggarwal and Zettler, 2010e; Huang *et al*., 2018b; Shao *et al*., 2020). A significant influence of light on seedling development was also reported by Zi *et al*. (2014c) in *D. aphyllum* and Wang *et al*. (2011a) in *D. nobile* and *D. officinale* as well as in the terrestrial orchid *Calopogontuberosus* (Kauth *et al*., 2006). Huang *et al*. (2018c) reported that seeds developed into protocorms regardless of the presence of light, whereas protocorms failed to develop into seedlings unless illumination was provided in *D. devonianum*. Rasmussen (1995e) reported that the seeds of temperate orchids do not respond well to light conditions (Rasmussen, 1995f). Different responses to light and dark conditions during germination are observed in epiphytic and terrestrial orchids (Zi *et al*., 2014d). The temperature in incubation is usually maintained at 25±1 °C (Zhang *et al*., 2020a) in *D. officinale*, and Li *et al*. (2021a) in *G. elata*.

Source of fungal isolates and their specificity

Very little is known about the niche requirement for the germination of orchids in *in situ*, especially the role and specificity of mycorrhizal fungi during germination and in subsequent seedling development (Stewart and Kane, 2007b; Rasmussen *et al*., 2015). At any given time, orchid species may associate with one or more Rhizoctonia-like fungal species (Dearnaley *et al*., 2012), thus their relationship is very complex. The degree of specificity between orchids and their mycorrhizal fungi is influenced by environmental conditions and surrounding plant species and is highly variable (Waterman and Bidartondo, 2008; Selosse and Roy 2008; Otero *et al*.

Table 3. *Ex situ* symbiotic seed germination reported from different places

Orchid	Epiphytic/ Terrestrial	Fungus isolates inoculated	Location	Culture conditions	Experiment outcomes	References
Dendrobium chrysotoxum	Epiphytic	*Tulasnella sp. Sebacina sp. Coprinellus sp.*	China	Petri dishes containing seeds, inoculated with fungus or control treatments were randomly cultured in incubators at 25±1°C under complete darkness or (12/12h) light/dark conditions with a cool white fluorescent lamp with a light intensity of 8000 lux. The experiment was conducted for 90 days.	Fungal inoculation, light conditions, and their interaction were found to have a significant effect on protocorm formation and seedling development. Fungus isolated from the host *(D. chrysotoxum*) produced the highest number of seedlings after 50 days. It was found that light conditions and origin of fungal isolates the had a strong effect on germination and seedling formation.	Shao *et al.* (2020)
D. lindleyi, *D. fimbriatum*, *D. findlayanum*	Epiphytic	*Tulasnella deliquescens* (Juel)	Thailand	Three different concentrations of oat medium 1, 5, and 10 g/l were used to evaluate the effect of media concentrations on symbiotic germination inoculated with *Tulasnella deliquescens.* Uninoculated control treatment contains 10 g/l OMA, 1/5th MS medium containing 6 g/l glucose.	Germination and protocorms development in *D. lindleyi* were found enhanced by the higher concentration of OMA. Treatment containing OMA 10 g/l inoculated with fungal inoculum outperformed the control. The germination % was similar in all three treatments. However, the symbiotic method was found more effective than the control treatment with 1/5th MS media in protocorms development.	Mala *et al.* (2017)
Gastrodia elata	Terrestrial	*Mycena sp.*	China	Under a laminar flow hood, *Quercus* leaves were fully colonized with fungal hyphae (*Mycena sp.*) and then placed on a water agar medium in Petri plates. Approximately 100 sterilized seeds were inoculated on the surface of each leaf, and then sealed with paraffin. Sealed petri plates were then placed in a dark at 25±1pC.	After the week of inoculation, the seed embryo started to enlarge and embryo cells became highly vacuolated. Enlarged embryos, later on, turned into ovoid protocorms. Fungal hyphae form pelotons in epidermal cells.	Li *et al.* (2020)
Paphiopedilum villosum (Lindl.) Stein	Epiphytic	*Tulasnella sp., Ceratobasidium sp.,* and *Flavodon sp.,*	Thailand	The sterile seeds of *P. villosum* were sown in sterile Whatman no. 4 filter paper and placed onto media in a petri dish containing 20 ml of sterile	The germination rate index (GRI) and developmental rate index (DRI) were significantly higher in fungus-inoculated	Kamachatra *et al.* (2016)

Table 3. *Continued ...*

Orchid	Epiphytic/ Terrestrial	Fungus isolates inoculated	Location	Culture conditions	Experiment outcomes	References
				oatmeal agar with 6 pH. The plates were inoculated with 5 mm diameter actively growing five different fungal inoculums (isolates) viz., PVCP01, PVCP05, PVCP06, PVCP08, and PVCP09. Whereas, control treatment was uninoculated. Sealed petri plates were placed in dark condition at 25 ^{o}C for 16 weeks.	treatment than in the control treatment. The highest GRI was recorded in treatment with fungal isolate PVCP01 (28.36% per week). This fungal isolate supported the advance protocorm developmental stage up to the 5th stage resulting in the highest DRI (0.59% per week). All the sampled seedlings survived and grew vigorously after transplantation in a greenhouse.	
Chloraea grandiflora, *C. crispa*, *C. gavilu*, *C. chrysantha*, *C. bletioides*, *C. longipetala*, *B. fimbriata*,	Terrestrial	*Tulasnella sp.*, *Ceratobasidium sp.*, and *Thanatephorus sp.*	Chile	Viable sterilized seeds were made and suspended with sterile deionized water. This seed suspension was placed on OMA media (4 g/l oats + 10 g agar at pH 5.6) inoculated with different fungal strains. These inoculated petri plates were placed in the dark at 25±1^{o}C for 8 weeks.	The fungus isolated could induce germination at different efficacies and showed low specificity. Germination was recorded as low but protocorms development continued for 60 days.	Harrera *et al*. (2017)
Dactylorhiza hatagirea (D. Don) Soo	Terrestrial	*Ceratobasidium sp.*	India	The test tube containing OMA medium and seed was inoculated with 1 cm^{3} fungal inoculum with one control and was incubated at 25^{o}C for 20 days under a 12/12 light/dark photoperiod. A cool white fluorescent bulb with 40 µmol/m^{2} /s^{-1} was used for irradiance. Germination was inspected weekly basis.	Seeds inoculated with fungus showed 100% germination within 10 days of sowing without pretreatment of seeds. Seedlings with well-developed roots, leaves, and tubers were obtained after 3 months.	Aggarwal and Zettler (2010)
Arundina graminifolia	Terrestrial	*Tulasnella sp.* 1, *Tulasnella sp.* 2, *Fusarium solani*, *Cylindrocarpon sp.*, *Acremonium sp.*, and *Phlebiopsis flavidoalba*	China	Sterilized seeds of *Arundina graminifolia* were sown on petri plates (120 seeds/plate) either fungus inoculated or axenically (MS-nutrient rich medium or OMA-nutrient poor medium). Petri plates containing seeds,	Four no standard mycorrhizal fungi (*Fusarium solani*, *Cylindrocarpon sp.*, *Acremonium sp.*, and *Phlebiopsis flavidoalba*) did not support germination beyond the imbibition and greening of seed in the first 35 days.	Meng *et al*. (2019)

Table 3. *Continued ...*

Orchid	Epiphytic/ Terrestrial	Fungus isolates inoculated	Location	Culture conditions	Experiment outcomes	References
				inoculated with 6 different fungi isolated were placed in a germination chamber at 25±2°C, providing 12/12h L/D photoperiod.	*Tulasnella sp.*1 isolated from adult mycorrhiza induced protocorm formation but not further development. Whereas, *Tulasnella sp.* 2 isolated from advanced seedlings facilitated protocorm formation to seedling development.	
D. officinale	Epiphytic	*Tulasnella sp.* and *Sebacina sp.*	China	Petri plates containing sterilized OMA medium and *D. officinale* seeds were inoculated with fungal inoculum and then placed under a dark environment at 25±1pC for 7 days. The culture room was illuminated with a fluorescent tube of 20W at 12/12h L/D photoperiod.	All strains of *Tulasnella sp.* and *Sebacina sp.* were found to induce germination after two weeks with different efficacies. However, after 5th week, two strains of *Tulasnella sp.* S6 and S7 showed a higher germination rate than other fungal strains. Embryo swelled in control but no further seedling development was noticed in the control treatment.	Zhang *et al.* (2020)
D. draconis	Epiphytic	*Tulasnella sp.*		25°C darkness for 1 week in an incubator, followed by 16 h PP for the next 15 weeks at 25°C	Tulasnella isolates DT-TC-1, Pv-PC-1-1, and C3-DT-TC-2 were found most effective in germination in 2 weeks and promote protocorms at 13 weeks and seedling	

2007). The literature suggests that the maximum fungal specificity bottle neck occurs particularly at the advanced seedling stage compared to the early germination stage (Shao *et al*., 2020; Bidartondo and Read, 2008b). Again, such studies are mostly reported from temperate, European orchid species, mostly heterotrophic in particular, and do not associate with Rhizoctonia (Bidartondo and Read 2008c; Tìšitelová *et al*., 2013a). To substantiate such claims, more evidence is required by investigating the developmental stages on a finer scale.

It has also been reported that different strains of the same genus of orchid mycorrhizal fungi may also induce protocorm formation and seedling development with different efficacies (Zhang *et al*., 2020b). Empirical findings show that mycorrhizal fungi isolated from host protocorms has ten the germination and seedling development (Sebastian *et al*., 2014; Zhou and Gao, 2016b; Huang *et al*., 2018d; Meng *et al*., 2019f) compared to those isolated from other orchid species or not having a fungal symbiont (Zi *et al*., 2014e) or fungi isolated from closely related orchid species possibly due to cross-compatibility between orchid species (Shao *et al*., 2020). Shao *et al*. (2020) showed that *Tulasnella species* isolated from *D. nobile,* which is phylogenetically closely related to *D. chrysotoxum* (Xiang *et al*., 2016), failed to facilitate germination in the symbiotic culture of *D. chrysotoxum*. Likewise, a mycorrhizal fungus isolated from *D. devonianum*, which was previously found to enhance seed germination in *D. devonianum* by Huang *et al*.

(2018e), failed to induce protocorms formation in *D. chrysotoxum*. In contrast, low specificity was reported by Liu *et al*. (2010) and Zhang *et al*. (2012), who found that different mycorrhizal fungi can promote germination in the same orchid species.

In *in situ* culture, it has been reported that orchids have different symbionts at different stages and that only a subset of the fungi-promoting germination phase may support later developmental stages (Bidartondo and Read, 2008d; Jacquemyn *et al*., 2011; Long *et al*., 2013) whereas in *ex situ* conditions, more diverse fungi are involved stimulating early seed germination (Wang *et al*., 2011b; Rasmussen *et al*., 2015). This ability of orchids to switch their fungal association (McCormick *et al*., 2004; Tĩšitelová *et al*., 2013b) causes orchids to switch their association from one fungal species to another at different developmental stages. Sufficient literature is available to support such claims in *ex situ* conditions; however, elucidating the roles of diverse fungi in determining seedling establishment still requires more experiments in *in situ* conditions.

Structural changes in seeds during symbiotic germination

Despite numerous studies on symbiotic germination, providing considerable information about the interaction between mycorrhizal fungi and orchid seeds, structural and ultrastructural changes in particular (Peterson *et al*., 1998; Chen *et al*., 2014), proper information on sequential changes in seed structure during symbiotic germination with a defined time scale is still deficient (Li *et al*., 2021b). Generally, after infection, mycorrhizal fungi penetrate the embryo and form hyphal coil"pelotons" enveloped in the plasma membrane in the host cell. These pelotons collapse and undergo lysis and digested products are absorbed by the host cell. That is a general idea of what mycorrhizal fungus does, and their fates after infection. However, such studies have mostly been performed on green orchids, and very few have been performed on achlorophyllous orchids (Li *et al*., 2021c).

According to Li *et al*. (2021d) studied *G. elata*, the first developmental stages during symbiotic germination are characterized by the thickening of the cell wall with papillae-like structures penetrating the suspensor end cell, epidermal cells, and cortical cells of the embryo. Embryo cells continue to enlarge and become highly vacuolated. After 2 weeks of inoculation, the seed coat ruptured to form protocorms. At this phase, cells at the apical of the protocorms frequently divided to generate a meristematic zone, but cells at the basal parts of the protocorms do not divide further. The protocorms continue to elongate, and the fungal colonization is restricted to basal protocorms. Some fungi are digested during this phase, but many fungal hyphae remain vigorous within the suspensor end cell. Soon ovoid protocorms are formed as a result of the continuous enlargement of the embryo. Epidermal cells and cortical cells are frequently penetrated by fungal hyphae, in the due process some are soon digested and become compressed. In ultrastructural observations numerous electron-dense tubular networks are visible, hyphae penetrate enlarged digestion cells, and the plasmalemma and fungal wall are surrounded by the radiating endocytic tubules to mark the final stage of fungal hyphal-breakdown (Wang *et al*., 1997). Fungal hyphae appear to be digested through endocytosis (Li *et al*., 2021e).

Conclusion

It is well established that orchid mycorrhizal fungi play a significant role in the life of an orchid from germination to its further growth and development, as they help in nutrition and to withstand with biotic and abiotic stresses. Additionally, it is understood that orchid mycorrhizal fungi influence the population distribution, survival of orchids, and their rarity due to the fungal species specific nature of orchids and vice versa. Thus, it is crucial to understand the diverse benevolent mycorrhizal fungi associated with different orchid species to utilize them for propagation through symbiotic seed germination. Although orchids are propagated vegetatively for commercial production, various empirical findings have reported that orchid seedlings derived from symbiotic germination or raised under conditions inoculated with orchid mycorrhizal fungi have better chances of survival even under adverse environmental conditions, especially when they are reintroduced for conservational works. Germination of millions of dust-like orchid seeds through symbiotic germination technique can help bridging the gap between market demand and supply as well as the need for orchid conservational works. Both *ex situ* and *in situ* techniques can be used in an integrated way for more effective for orchid propagation and mass production. Symbiotic germination thus holds great potential for orchid propagation from both

horticultural and conservational perspectives. Nonetheless, In comparison to the ex situ approach, the literature on the in situ technique and its application is still limited, and it requires further development to make it more convenient in terms of applicability. Ex situ symbiotic seed germination, on the other hand, has become one of the most extensively used germination techniques for orchid seed propagation and conservation efforts. In comparison to the ex situ approach, the literature on then in situ technique and its application is still limited, and it requires further development to make it more convenient in terms of applicability. Ex situ symbiotic seed germination, on the other hand, has become one of the most extensively used germination techniques for orchid seed propagation and conservation efforts.

Conflict of interest

The authors declare no conflict of interest

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