Symbiosis
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# Improved use of mycorrhizal fungi for upscaling of orchid cultivation and population restoration of endangered orchids in China

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## Abstract
The orchid family (Orchidaceae) is one of the most diverse plant families in the world, but at the same time also contains one of the largest number of rare and endangered species. While conservation actions such as *in situ* and *ex situ* conservation and prohibition of international trade of wild orchids have achieved positive results to slow down the current decline of orchids, species with high medicinal or ornamental value may require more advanced measures. Recent pilot studies using novel cultivation techniques have successfully reintroduced endangered epiphytic orchids and facilitated the commercial cultivation of medicinal orchids. Because fungal partners play a key role in *in situ* symbiotic seed germination and industrial cultivation, we advocate for the development of fungus banks in laboratories engaging in orchid research, where fungi obtained from germinating seeds or seedlings can be studied and used to improve orchid germination under both *in vitro* and *in situ* conditions. Furthermore, these fungi could be shared nationally and internationally, enhancing orchid conservation efforts across the globe. Similar to seed banks, the development of fungus banks will reduce the possibility of fungi going extinct and ensure their availability for reintroduction programs and commercial cultivation. With the availability of both a fungus and seed bank, the conservation of threatened orchid species can be significantly enhanced by improving restoration programs and commercial cultivation of medicinal orchids and releasing the pressure on wild populations from harvesting.

**Keywords** In situ symbiotic seed germination · Medicinal orchid · Orchid mycorrhizal technique · Mycoheterotrophy · Reintroduction

## 1 Introduction

With their enormous diversity (no less than 28,000 species; Christenhusz and Byng 2016), fascinating flowers and complex interactions with pollinators and mycorrhizal fungi, orchids have always been an attractive study object that has appealed botanists and ecologists worldwide (Alcock 2006). Apart from their scientific, ornamental and aesthetic aspects, orchids have also been used for medicinal purposes for thousands of years (Bulpitt et al. 2007; Bazzicalupo et al. 2023) and for the production of beverages and desserts (Arditti 1992). For example, people in Turkey have used salep, a flour produced by grinding the tubers of bee and butterfly orchids, to produce drinks, sweets and slow-melting, stretchy ice cream. At the height of the Ottoman Empire in the nineteenth century, the warm, creamy drink made from salep gained popularity as far away as England and Germany. Similarly, traditional medicine in China has used orchids for thousands of years as primary constituents in medicinal formulations. For example, tubers of *Gastrodia* have been used to treat headaches and other illnesses (Shang 2008; Liu et al. 2017). Similarly, tubers of *Gymnadenia* have been widely used in traditional Chinese medicine, especially in Tibet and Mongolia (Han and Zeng 2010). However, this historical reliance on orchids has come at a severe cost, with

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millions of orchids being collected and their populations being severely reduced in size, if not outright threatened.

Currently, the harvesting of medicinal orchids has reached a point where major populations have disappeared and others are on the point of collapse (Zhou et al. 2021). To solve this problem, China took action by releasing the updated List of National Key Protected Wild Plants in Sept., 2021 (http://www.forestry.gov.cn/c/www/gkml/11057.jhtml). The most frequently used medicinal orchids in China have now been categorized as class I (Critially Endangered: *Dendrobium flexicaule* and *D. huoshanens*) and class II (Endangered/ Vulnerable: *Dendrobium* spp., *Gastrodia elata*, *Anoectochilus* spp., *Bletilla striata*, *Gymnadenia conopsea*, *Pleione* spp., *Cremastra appendiculata*) species in the Chinese National Key Protected Wild Plants List and are offered legal protection (Liu 2021). In addition, more anthropic stressors have recently affected orchid populations: land development and utilization, habitat fragmentation and degradation, global warming and nutrient enrichment pose additional challenges to orchid conservation in the Anthropocene (Wraith et al. 2020; Zhou et al. 2021).

## 2 Current limitations in orchid conservation/ restoration

To conserve orchid populations, integrated conservation measures and *in situ* conservation have been successfully applied. For example, 65% of all orchid species in China now occur in natural reserves, where the protected populations self-regenerate and persist (Zhou et al. 2021). Orchids living outside nature reserves are subject to *ex situ* conservation in botanic gardens, national parks, and conservation agencies. However, such initiatives often face challenges related to genetic drift due to smaller population size (Oosterhout 2020), emphasizing the ongoing importance of *in situ* preservation of sufficiently large populations. Unfortunately, the trend towards the extinction of medicinal orchid species persists, especially under the following three circumstances: (1) the discovery of a new medicinal value, (2) inability to meet market demand by commercial plantations, and (3) the public's recognition of substantial treatment effects for a specific disease. Similarly, the market demand for orchids involved in the production of salep has increased, even out of Turkey (Charitonidou et al. 2019), meaning that these orchids are still under high pressure and that effective conservation cannot be achieved. As long as people continue to collect orchids from natural populations, wild resources face significant threats. Ecological cultivation, i.e. *in situ* plantation of orchids, can possibly be gradually accepted as a valid alternative for wild specimens and thus change traditional perceptions about the use of wild orchids. If widely accepted, a balance between proper conservation of wild orchid populations and resource utilization can be achieved.

## 3 Industrial cultivation could alleviate pressure on natural populations

To release pressure on wild populations, domestic cultivation is considered beneficial for species conservation if it can meet the market demand and hence conserve the natural resources of the target species (Liu et al. 2019). Currently, the number of cultivated plants has never been so high (3,227 taxa) and continues to increase (Brinckmann et al. 2022), as domestication (the control of reproduction for production purposes) is a permanent and ongoing process (Murphy 2007). The first orchid cultivated on an industrial scale is the edible *Gastrodia elata* (Chen et al. 2019), with a total production of 100 million kg in China in 2020 (Qiong Wang, pers. comm.). However, the number of species that are cultivated in this way is still limited and some studies have suggested that cultivation of overexploited plants may not be beneficial and could, in the long term, be counterproductive because farmers may continue to harvest more wild resources rather than the opposite (Phelps et al. 2014; Williams et al. 2014). Nonetheless, improvements in cultivation techniques and genetic transformation to alter pathways for the biosynthesis of target metabolites may pave the way for more efficient use of wild resources (Canter et al. 2005).

The attitudes of consumers also play a critical role in the success of large-scale cultivation of medicinal orchids. Because consumers generally prefer 'authentic herbs' above cultivated medicinal plants, this may hinder widespread adoption, despite the misconception that health products from commercial cultivation have a different medicinal value as natural products. People often believe that wild plants are more potent than cultivated substitutes, emphasizing the importance of authenticity (地性 in Chinese) (Hinsley et al. 2018; Liu et al. 2019). In addition, the majority of herbs are cultivated in greenhouses and pushed to grow fast using fertilizers and pesticides and therefore cannot be considered as organic. The common belief that faster-growing plants contain fewer medicinal ingredients further complicates the shift towards cultivation. Although speed of growth may not directly correlate with medicinal value, the level of stress encountered during growth may influence secondary metabolite accumulation. As a result, most people are still collecting wild specimens, and despite efforts such as *in situ* and *ex situ* conservation, legal prohibitions and commercial plantations maintaining a balance between the conservation and utilization of natural species resources remains challenging and the illegal collection of wild specimens persists (e.g. http://www.ahhuoshan.jcy.gov.cn/zyaj/202307/t20230704_5287580.shtml). Besides, it is not always clear which extracts remain marketable (Canter et al. 2005) and farmers may invest in plants that finally fail to be sold. This was evident in the commercial cultivation of *Dendrobium* spp. (mainly *D. officinale*) in China. Between 1990 and 2012, commercial cultivation of *Dendrobium* spp. witnessed an enormous increase, but market demand suddenly declined because consumers considered cultivated plants as inorganic products and therefore they continued to seek authentic herbs (Yang et al. 2022; pers. obs. of S-C Shao). This prompted companies to develop new, more environmental-friendly cultivation methods, such as fixing seedlings grown from tissue cultures to the trunks of trees or rock surfaces (Liu et al. 2013). However, these methods had some serious drawbacks, including high mortality of seedlings, slower growth of established plants and higher costs. Moreover, what was worse, consumers did not recognize this cultivation method as a natural process and harvest pressure on wild resources increased again.

## 4 Novel techniques to (re-)introduce orchid populations

The "dust-like" seeds of orchids typically lack carbon reserves, implying that seed germination cannot occur spontaneously under natural conditions. Consequently, a substantial number of seeds fail to establish as a seedling (Rasmussen et al. 2015). Noël Bernard's pioneering work showed that compatible fungi are needed to induce germination (Selosse et al. 2017) by providing carbon to seedlings, a nutritional process called mycoheterotrophy. The fungi subsequently establish a mycorrhizal relationship and form a symbiosis with the roots where plant carbon is exchanged for mineral nutrients from the soil (Smith and Read 2008; Dearnaley et al. 2012), since most orchids become photosynthetic at later stages. The symbiotic relationship between orchids and their mycobionts has been investigated in a large number of studies during the last hundred years (Rasmussen 1995). The symbiosis significantly increases germination and decreases the germination period (i.e. the time needed for a seed to develop into a seedling) compared to asymbiotic germination, where seeds in sugar-containing media lack compatible fungi (some species may not germinate under such conditions; Dearnaley et al. 2016). These results support that symbiotic germination or the active providing of suitable fungi increases the chance of seedlings to establish, especially in natural habitat restoration projects (McKendrick et al. 2000; Shao et al. 2017; Jiang et al. 2022).

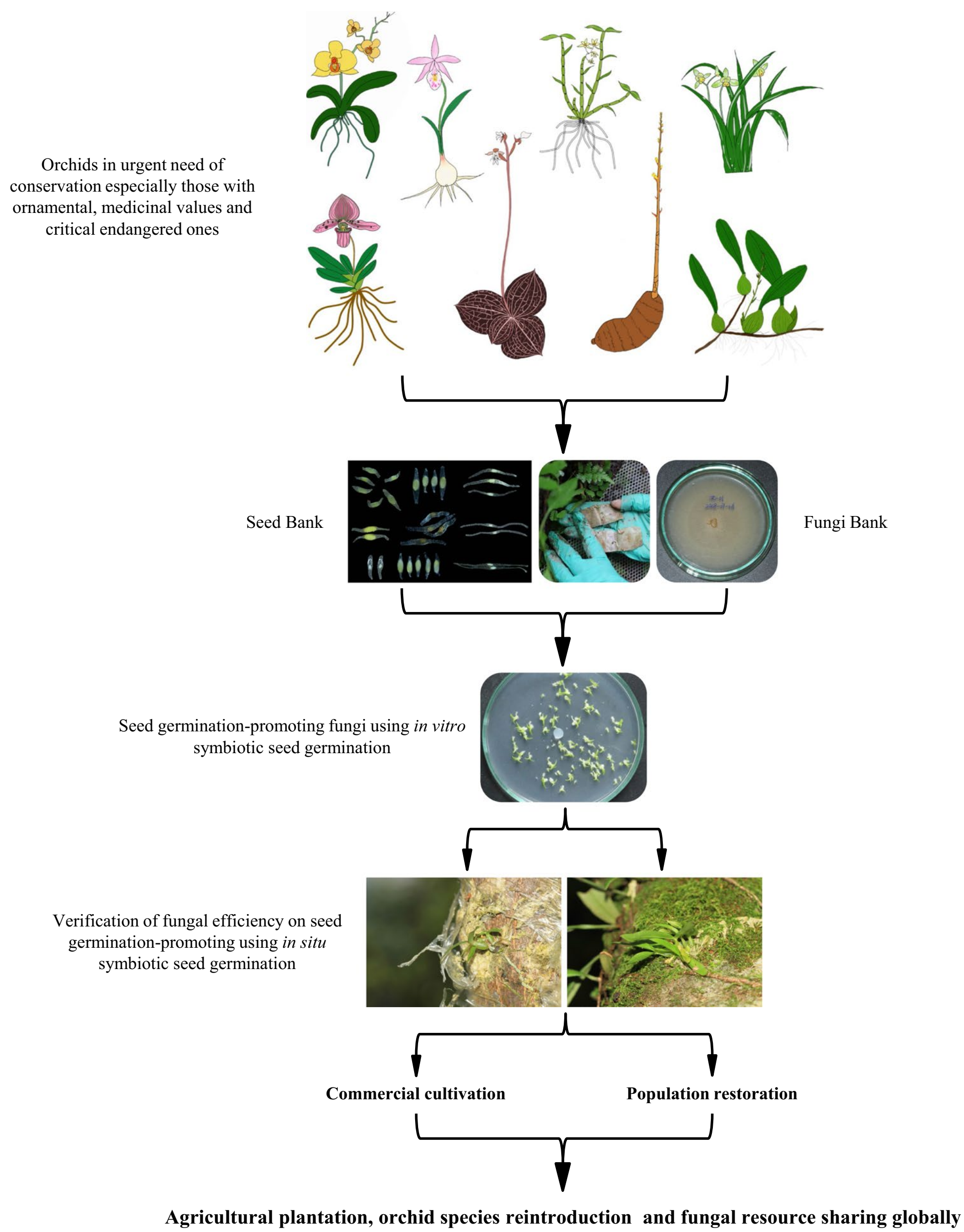
Recent progress in orchid mycorrhizal research and seed germination has expanded the possibilities for cultivating medicinal orchids (Fig. 1) and at the same time alleviating pressure on wild orchid populations. Successfully reintroducing orchids in their natural environment using natural seeds and a fungal powder (Shao et al. 2018) and afterwards obtaining new plants through natural regeneration (Shao et al. 2022) comes closest to the natural process of


**Fig. 1** Examples of medicinal orchid species that have declined substantially as a result of over-collection and have been successfully used in restoration and commercial cultivation programs using mycorrhizae. **a** *Dendrobium devonianum*, **b** *Dendrobium nobile*, **c** *Gastrodia elata* and **d** *Cremastra appendiculata*

population regeneration. Symbiotic seed germination has already been instrumental in the reintroduction and artificial establishment of populations of *Dendrobium devonianum* (Fig. 1a; Shao et al. 2022) and *Dendrobium nobile* (Fig. 1b). Furthermore, this approach has proven successful in the commercial cultivation of *Gastrodia elata* (Fig. 1c) and *Cremastra appendiculata* (Fig. 1d). In the case of the latter, the symbiotic fungus (*Coprinellus disseminatus*; identified by professor Bo Xiao from the Research Institute of Medicine Plantation of Chongqing, China) was used to promote seed germination and plants reached maturity within a single year. Consequently, this method safeguarded wild specimens of *C. appendiculata* from harvest. Similarly, Jiang et al. (2022) showed that the addition of fungal inoculum to seed packages increased seed germination and seedling formation in the endangered terrestrial orchid *Gymnadenia conopsea* in China, while seeds failed to germinate when no inoculum was added.

However, scaling up commercial orchid cultivation requires a much better understanding of the germination process and access to the specific orchid symbiotic fungus in culture (Zhao et al. 2021) since the association between an orchid and a fungus is often highly specific (Swarts et al. 2010). Consequently, the fungus becomes integral to set up large-scale orchid cultivation programs. Two points need to be emphasized here. First, the efficiency of germination-promoting fungi needs to be investigated under both *in vitro* and *in situ* conditions (Fig. 2) since not all fungal strains that

**Fig. 2** Schematic overview of all necessary steps to setup successful orchid restoration and cultivation programs aimed at alleviating harvest pressures on wild orchid populations



enhance seed germination *in vitro* can replicate the same effect in the wild (Batty et al. 2006; Rasmussen et al. 2015; Těšitelová et al. 2022). One reason for this discrepancy is that the physiology of the fungi may differ substantially between natural and laboratory conditions (Rasmussen et al. 2015; Zhao et al. 2021). Secondly, germination-enhancing fungal taxa most likely differ between terrestrial and epiphytic orchids and between orchids with different trophic modes (McKendrick et al. 2002; Leake et al. 2004; Bidartondo and Read 2008; Martos et al. 2012; Rasmussen et al. 2015; Gao et al. 2022). Generalizing the use of a single fungal strain for several orchid species is impossible, necessitating a case-by-case study for each orchid species (Swarts et al. 2010; Martos et al. 2012; Xing et al. 2019). Moreover, different fungi may require different culture media and conditions (Freestone et al. 2022). The most common medium, potato dextrose agar (PDA), is suitable for typical orchid mycorrhizal fungal taxa (the rhizoctonias, a polyphyletic grouping of three Basidiomycota lineages, namely Tulasnellaceae, Serendipitaceae, and Ceratobasidiaceae; Dearnaley et al. 2012), but may not be the most effective medium for culturing symbiotic fungi associated with partially or fully mycoheterotrophic orchids (Hynson et al. 2013; Selosse et al. 2022). Given the challenge of inducing seed germination using *in situ*/ *ex situ* baiting, we strongly recommend adjusting culturing conditions (medium nutrition, incubation temperatures) and exploring different pH gradients to cultivate a broader range of symbiotic fungal strains. For example, plain oatmeal agar (OMA) is suitable to promote germination of *Cremastra appendiculata* with the symbiotic *C. disseminatus* under *in vitro* conditions (Gao et al. 2022). However, when sowing seeds in the field, rotting wood is needed to grow this saprophytic fungus and to obtain seedling development (pers. obs. of Bo Xiao).

## 5 The importance of fungal origin for *in situ* symbiotic seed germination and industrial application

The relationship between orchids and their fungi is complex and requires detailed investigations (Swarts et al. 2010). A few successful applications of symbiotic fungi in commercial plantations have used fungi that were isolated from germinated seeds or young seedlings (Huang et al. 2018; Shao et al. 2020b), suggesting that these have the largest potential for *in situ* symbiotic seed germination and industrial applications. The mycorrhizal symbioses between orchids and fungi may be influenced by inherent differences among closely related fungi. While the exact mechanisms are still unclear, they may involve fungal effector and plant receptor genes similar to other mycorrhizas or plant-pathogen interactions (Fuji et al. 2020). Therefore, obtaining efficient ecological/habitat-specific fungi for seed germination is crucial in conservation practices for orchids (Masuhara and Katsuya 1994).

At present, there is no conclusive evidence that mycorrhizal fungi isolated from adult roots consistently contribute to *in situ* symbiotic seed germination (Meng et al. 2019) and commercial applications. However, there is evidence that germination-enhancing fungi are present and retained in the roots of mature individuals (McCormick et al. 2021). We therefore recommend a more systematic investigation into the efficiency of fungi isolated from roots in promoting seed germination, both under *in vitro* and *in situ* conditions, with comparison to fungi isolated from germinating seeds. The latter can be obtained by *in* and *ex situ* seed baiting (Meng et al. 2019), where seeds are put in their natural habitats using seed bags and subsequently fungi are isolated from developing seeds (Rasmussen and Whigham 1993; Brundrett et al. 2003). Subsequently, the effectiveness of these fungi to initiate germination can be tested under *in vitro* and *in situ* conditions. Seed baiting typically takes one month (for epiphytic orchids) to two months (for terrestrial orchid) (Swarts and Dixon 2017) or even longer. Furthermore, seed baiting does not guarantee consistent success and may require repeated experimentation.

## 6 Importance of a seed and fungus bank

To safeguard rare orchid mycorrhizal resources for future cultivation programs, the concept of a fungus bank has been proposed and, to some extent, implemented (Krupnick et al. 2013; Liu et al. 2015; Swarts and Dixon 2017; Mujica et al. 2018; Zettler and Dvorak 2021). Given the large advancements in fungal applications for orchid population restoration and commercial cultivation, we advocate for the further development of fungal banks in laboratories engaged in orchid research. This would contribute to improving orchid germination under *in vitro* and *in situ* conditions (Fig. 2) and facilitate sharing of resources at a national and international level. Despite the perceived challenges, evidence suggests that harvesting of orchid germinating seeds is easier than it looks (Kuga et al. 2014; Zhou and Gao 2016; Swarts and Dixon 2017; Shao et al. 2020a, 2020b) (Fig. 2). Therefore, we strongly recommend prioritizing fungal isolation once seedlings are obtained. While most orchids have a mycorrhizal relationship with rhizoctonias, some associate with ectomycorrhizal fungi (e.g. Russulaceae, Tuberaceae) and purely saprotrophic fungi (Dearnaley et al. 2012; Selosse et al. 2022). *In vitro* preservation of rhizoctonia strains is easy and feasible (Freestone et al. 2022). Currently, Index Fungorum, an international program that aims at indexing all recognized names in the fungal kingdom (http://www.indexfungorum.org/names/Names.asp), lists at least 363 rhizoctonia-like fungal strains (119 strains in the genus *Tulasnella*, 47 in *Ceratobasidium*, 175 in *Sebacina* and 22 in *Serendipita*, respectively). However, it is highly unlikely that these 363 species would induce seed germination in each of the > 28,000 orchid species described so far (Christenhusz and Byng 2016). Therefore, the development of a fungus bank focusing on germination-promoting fungal strains represents a crucial step towards achieving the goals of the Millennium Seed Bank partnership for Orchidaceae (Swarts and Dixon 2009). With two complementary banks – one for orchid seeds and one for their symbiotic fungal partners – we possess all the tools to enhance the conservation and propagation of many threatened orchid species and populations.

## 7 Fungal turnover deserves more attention

All orchid species are mycoheterotrophic during seed germination. Once seeds have germinated and developed into seedlings, about 98.6% species gradually form chlorophyll and start to perform photosynthesis (Merckx et al. 2009). Therefore, the dependency on fungal partners decreases over time. However, some adult orchids, including achlorophyllous orchids and chlorophyllous orchids that do not use it for photosynthesis (e.g. *Neottia nidus-avis*), still rely on symbiotic fungi (Fig. 3). There is increasing evidence that in some species the fungi that promote germination differ from those required by adult mycoheterotrophic plants. For example, seed germination of *Gastrodia elata* is initiated by fungi of the genus *Mycena*, while the fungus *Armillaria mellea* is needed to initiate growth into adult plants (Chen et al. 2019) (Fig. 3). Similarly, seed germination and subsequent growth to an adult stage in *Gastrodia confusoides* require a distinct switch in mycorrhizal partners: seedlings associate with a fungal strain from the genus *Mycena*, while adults mainly associate with a *Gymnopus* strain (Li et al. 2022). In these cases, fungal turnover is a crucial step during seedling propagation and the cultivation of adult achlorophyllous orchids, and a similar phenomenon has been observed in some chlorophyllous orchids (Rammitsu et al. 2023). However, further research is needed to identify which orchids undergo a partner switch during their development (Ventre Lespiaucq et al. 2021). In such cases, fungi need to be isolated from both germinating seeds and the roots of adult plants, and their ecological functions, including effects on growth, rooting and survival need to be thoroughly assessed. Furthermore, it is crucial to investigate the dynamics of microbial communities at different developmental stages of orchid species propagated *in situ* using fungal inoculum. This could help discovering interesting patterns of interactions, as some root-associated fungi may only be exerting indirect effects (Almario et al. 2017; Selosse et al. 2022; Pyles 2023).

## 8 Challenge of orchid conservation using mycorrhizal fungi

The development of mycorrhizal technology based on symbiotic biology holds promising prospects for orchid conservation in natural habitats. Our previous successful experiences in reintroduction practices with three *Dendrobium* species (Shao et al. 2018, 2022) and the commercial cultivation of *Cremastra appendiculata* and *Gymnadenia conopsea* (unpublished data) are clear examples showing how mycorrhizal fungi can effectively contribute to the restoration of orchid populations in the wild or the large-scale cultivation of orchids in an industrial setting. However,

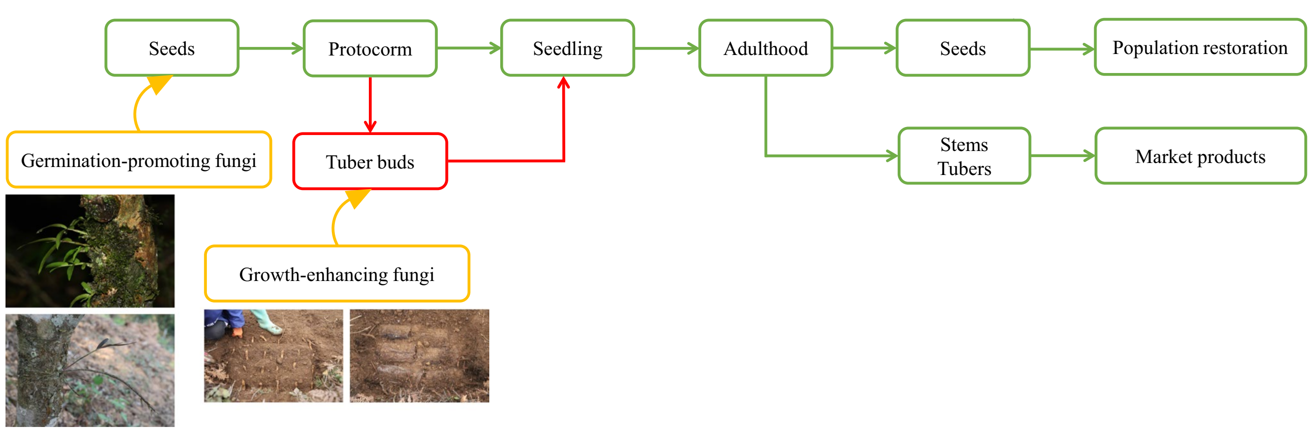



**Fig. 3** Various stages in the life cycle of orchids that are important for the development of successful restoration and cultivation programs. Light green boxes represent different life cycle stages of orchid species that develop to adults with a single fungal partner, while the red box represents a part of the life cycle of orchids species that require a shift in fungal partner when growing into adulthood. Orange boxes represent different symbiotic fungi that are required to complete the life cycle

caution is needed when applying mycorrhizal fungal strains in the long run.

Firstly, the availability of new cultivation techniques and the mass production of orchids could divert attention and resources away from other pressing biodiversity issues, such as the conservation of endangered species. There is a risk that people may become less concerned about orchid conservation, assuming that the cultivation of endangered species alone satisfies their needs. Furthermore, the significance of wild populations remains paramount as they contain the genetic diversity and microbiota resources (endophytic fungi, bacteria) that are needed for sustaining successful cultivation programs and potential future applications. Even with new breakthroughs in orchid cultivation, the conservation of wild populations is essential, because the individuals that are cultivated in commercial plantations typically originate from a narrow range of source populations, even for regionally widespread species, contributing to concerns about genetic bottlenecks (Ramsay and Dixon 2003). Therefore, it is imperative to strike a balance between cultivation and conservation, creating a win–win situation for both partners.

Second, some fungi (e.g. species from the genus *Ceratobasidium*) may exhibit different ecological roles and can act as typical orchid mycorrhizal mycobionts or as pathogens (Veldre et al. 2013). In fact, most rhizoctonias are likely to function as endophytic, biotrophic colonizers of non-orchids roots (Selosse and Martos 2014; Selosse et al. 2022). Introducing these fungi into new environments may pose potential ecological risks. Recent studies in Australia have shown that both orchid-associating and pathogenic fungi within *Ceratobasidium* are phylogenetically closely related and do not form distinct clusters in a phylogenetic tree (Freestone et al. 2021). Veldre et al. (2013) further showed that orchids can associate with both weakly pathogenic and aggressive *Ceratobasidium* spp. although they tend to associate more with soil-dwelling, likely saprobic, species. Mosquera-Espinosa et al. (2013) even found *Ceratobasidium* as biocontrol agents of *Rhizoctonia solani* sheath blight of rice. Therefore, the ecological roles (endophytes, orchid symbionts, pathogens, saprotrophs and ectomycorrhiza) of rhizoctonia spp. should be verified in detail in future studies. Similarly, some strains of *Armillaria* can cause large damage and are considered as forest pathogens (Coetzee et al. 2003). However, observations suggest that after interacting with seedlings of *G. elata*, the fungus causes little harm and is mainly beneficial for its orchid host (pers. obs. of S-C Shao). So far, commercial cultivation of *G. elata* or the medicinal *Polyporus umbellatus* inoculated with *Armillaria* spp. (Xing et al. 2022) at least fifty years ago have not caused any ecological problem.

Given the large diversity of orchid species and their complex interactions with mycorrhizal fungi, priority should be given to the collection and preservation of seeds and fungi of rare and threatened species, including orchid species used for nutritional purposes (such as salep) and medicinal applications (e.g. *Dendrobium* spp., *Gastrodia* spp., *Bletilla* spp., *Cremastra* spp., *Pleione* spp.,), ornamental herbs (e.g. slipper orchids and *Cattleya* spp.), and early-diverged taxa (*Apostasia* spp.). Besides, emphasis should be placed on preserving rhizoctonias that have been shown to promote seed germination of a large diversity of orchids, especially those validated through *in situ* symbiotic seed germination and/or plantation practices (Gao et al. 2022; Harzli and Kompe 2023, Shao et al. 2022; Yu et al. 2022). While endophytic fungi have shown potential to promote seed germination *in vitro* (Jiang et al. 2019), their effectiveness needs to be validated under realistic *in situ* conditions.

## 9 Conclusion

We have highlighted the potential of orchid mycorrhizal fungi to improve the conservation and industrial cultivation of endangered orchids. Obtaining compatible fungi that promote germination both *in vitro* and *in situ* is a prerequisite to propagate seedlings under both field and laboratory conditions and in some cases involves more than one fungus. Establishment of a fungus bank that comprises germination-enhancing fungi and that is accessible to orchid conservationists around the world will undoubtedly contribute to species conservation. Unfortunately, up till now there are very few fungal strains whose seed germination enhancing capabilities have been tested under *in situ* conditions. Future studies should further investigate whether naturally restored orchid populations can regenerate and build up viable populations over the long term. Additionally, assessing whether propagated orchids produce extracts of medicinal ingredients similar to those of wild specimens is crucial to convince people that cultivated orchids are as valuable as wild specimens. Fortunately, commercial cultivation using mycorrhiza collected from natural populations comes closest to the natural process of population dynamics (Fig. 1d). If confirmed that there are no significant differences in medicinal ingredients between cultivated and wild orchids, the technique has the potential to substantially, if not entirely, alleviate the harvest pressures on wild medicinal orchid resources.

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## Declarations

**Competing interests** The authors declare that they have no competing interests that may otherwise influence the research presented in this study.

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