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Orchid mycorrhizal interactions on the Pacific side of the Andes. A review.

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Abstract

In order to confront the constant decline in global biological diversity, amelioration strategies are needed for threatened species to design reintroduction policies, particularly in plants with critical reproduction steps, such as orchids. Orchids are part of a highly diverse plant family, with several species under imminent extinction risk. This is the case of Chilean Orchidaceae, which have shown a constant decay in their populations due to an increase in the alteration processes of their natural distribution habitats. Successful orchid reintroductions require a full understanding of orchid mycorrhizal fungi and their dynamic according to different developmental stages and environmental conditions because orchid seeds need mycorrhizal fungi to obtain nutritional compounds at early developmental stages. This article performed a critical literature review of the ecological studies conducted on Chilean orchids and their relationships with mycorrhizal fungi in order to focus on the best scientific approach to achieve successful restoration programs involving orchid seeds and compatible mycorrhizal fungi.

Keywords

Mycorrhiza. *Rhizoctonia*-like fungi. Andean orchids. Mycoheterotrophy. Seed germination.

BACKGROUND

Mycorrhizal interactions are complex plant-fungi interactions present in most earth ecosystems and are usually associated with a mutualistic symbiosis in which both partners have ecological advantages to survive under different natural or stressed conditions (Smith and Read, 2010). These benefits are often related to improved water and mineral nutrient uptake, tolerance to stress and pathogen attacks, beneficial metabolic properties for the associated plant, stabilizing soil aggregates, or as a reward for photosynthetically fixed carbon and other compounds for the fungi (Six *et al.*, 2004; Giovannetti *et al.*, 2017; Jiang *et al.*, 2017; Kaur and Garg, 2017). These symbiotic mechanisms have been widely studied in arbuscular mycorrhizae due to their wide distribution and importance for agricultural plants (Barea and Jeffries, 1995; Jeffries *et al.*, 2003; Abbott and Manning, 2015). Arbuscular mycorrhizal fungi require an ecological association to grow and survive in symbiosis with almost 80% of vascular plant species (Smith *et al.*, 2003; Davison *et al.*, 2015). These mycorrhizal fungi include 25 genera (~300-1600 species) from the classes Glomerales, Diversisporales, Paraglomerales, and Archaesporales in the subphylum Glomeromycotina (Johnson and Jansa, 2017; Santander *et al.*, 2017). Such mechanisms are different in the symbiotic interaction established between orchids and their mycorrhizal fungi (Rasmussen

and Rasmussen, 2009). There is no more enigmatic symbiotic relationship than the symbiosis established between the tiny orchid seeds and the soil-borne fungi, because under natural conditions orchid seeds need to be colonized by compatible mycorrhizal fungi for their seeds to germinate (Otero *et al.*, 2004; Dearnaley, 2007). At this developmental stage all orchids are parasites on fungi, obtaining the water and mineral nutrients directly from the colonizing hyphae (Valadares *et al.*, 2014). These processes are needed to promote embryo development and plant establishment, because orchid seeds do not have sufficient nutritional reserves to promote successful seed germination (Herrera *et al.*, 2017; Yeung, 2017). Thus, mycorrhizal fungi are essential for orchids, especially to achieve seed germination under both, laboratory and field conditions (Shimura and Koda, 2005; Batty *et al.*, 2006a; Fracchia *et al.*, 2014b; Khamchatra *et al.*, 2016b).

Orchids seeds are produced in high amounts, but this process does not translate into higher orchid populations, which trigger most symbiosis-dependent orchids to become successfully established (McCormick *et al.*, 2016; Waud *et al.*, 2016b). Thus, to protect orchid populations, knowledge is needed of the mycorrhizal fungi associated with orchids and the dynamic of the mycorrhizal interactions under different ecological conditions (Ercole *et al.*, 2015; Jacquemyn *et al.*, 2015; Oja *et al.*, 2015). The orchid mycorrhizal fungi associated with most orchids involves ectomycorrhiza-forming fungi and different saprophytic, endophytic, and different free-living fungi from the *Rhizoctonia*-like complex, which is an artificial polyphyletic group of unrelated fungi in the anamorphic stage (Otero *et al.*, 2002). These fungal groupings include Basidiomycetes from the genera *Ceratobasidium*, *Tulasnella*, *Thanatephorus,* and *Sebacina* associated with most orchids according to the trophic nature of orchids (fully photoautotrophic, partially mycoheterotrophic, fully mycoheterotrophic) and the different ecosystems in which orchids grow (Ogura-Tsujita *et al.*, 2012). To be classified as compatible, the fungus must be able to promote seed germination or form peloton structures in root tissues of the associated orchid species, and must show some morphological characteristics conserved in the accepted orchid mycorrhizal fungi (uninucleate, binucleate or multinucleate hyphal cells, septum with 90º branching, no spore formation) (Valadares *et al.*, 2012). Many of these fungi are usually saprotrophs living on the organic matter in soils, whereas some groups form ectomycorrhizae with trees, and still others are plant parasites (Yamato *et al.*, 2005; Roy *et al.*, 2009). Several orchid mycorrhizal fungi can be cultivated axenically, which provides an interesting opportunity to study the mycorrhizal processes (Valadares, 2014; Herrera *et al.*, 2017). In addition, some orchid mycorrhizal fungi cannot

develop their habitual infection processes during the interaction with the orchid seeds, which reflects the powerful metabolism of the tiny seeds when addressing the pathogenic nature of some strains of *Thanatephorus cucumeris*, which has been associated with several orchids (Cowden and Shefferson, 2013; Pereira *et al.*, 2014b; Fracchia *et al.*, 2016; Pecoraro *et al.*, 2018). Orchid mycorrhizal fungi live in root tissues, forming symbiotic structures called "pelotons". The pelotons are symbiotic organelles created to store hyphal coils of mycorrhizal fungi and certainly play a crucial role in the exchange of metabolic and nutritional compounds (Peterson *et al.*, 1996; Dearnaley *et al.*, 2016). Pelotons can exchange nutrients between symbionts and are often digested and absorbed by the plant cell, providing carbon as well as mineral nutrients and water (Valadares, 2014). The presence of intact and degraded pelotons is typical in mycorrhizal roots and both are a source of nutrients for the associated orchid; therefore, pelotons are mycoheterotrophic organs with a key role in orchid nutrition (Smith and Read, 2010; Kuga *et al.*, 2014). To isolate and cultivate mycorrhizal fungi, peloton-like structures must be identified and cultured in an appropriate medium with only a carbon source for the mycorrhizal fungi, as all the nutrition of the orchid embryo requires of the mineral nutrients supplied by the mycorrhizal fungi, such as the oatmeal agar (OMA) medium, generally used for orchid seed germination (Zettler, 1997; Valadares *et al.*, 2012)).

Despite orchid mycorrhizae being specific to the Orchidaceae, this is one of the most numerous plant families, comprising about 899 genera and more than 27,801 species (The Plant List, 2013). Thus, Orchidaceae must be considered a widely distributed interaction, showing orchid individuals present in almost all terrestrial ecosystems, with the exception of the extremely hot and cold ones (Pridgeon *et al.*, 1999; Roberts and Dixon, 2008). This wide distribution also holds true for Chilean orchids, with orchid individuals from the Atacama Desert to southern Patagonia (Novoa *et al.*, 2015). However, as reported for several orchids, most Chilean species are under constant threat because of several man-made ecosystem alterations such as deforestation, due to logging, fire, road construction and the expansion of cities, forest plantations and agriculture, and over-collection for the ornamental, medicinal and food plant industries (Cozzolino *et al.*, 2006; Gale *et al.*, 2018). Worldwide, orchid populations are under constant threat because of climate change, segregation of orchid populations mainly to protected areas, specificity of mycorrhizal associations, and the economic potential that several orchids have (Schödelbauerová *et al.*, 2009; Kottke *et al.*, 2010). Global IUCN Red List (2018) assessments have classified only 948 (3.3%) of the

estimated 27,801 orchid species (Govaerts *et al.*, 2016; Gale *et al.*, 2018), considering over half (56.5%) of these orchids as threatened with extinction. The situation is similar in Chile: of the estimated 72 orchid species, only 14% have been classified with conservation problems (**Figure 1**), and the rest do not have sufficient data to be classified in one of the Global IUCN Red List categories: LC (Least concern), NT (near threatened), VU (vulnerable), EN (endangered), CR (critically endangered), EW (extinct in the wild), EX (extinct) (IUCN, 2018)

COMPLEXITY OF ORCHID SEED DEVELOPMENT

As mentioned above, orchid seeds lack the necessary nutritional reserves to sustain seed germination, which is the basis for the orchid mycorrhizal processes (Rasmussen and Rasmussen, 2009; Jacquemyn *et al.*, 2015; Dearnaley *et al.*, 2016). Orchid seed germination requires compatible mycorrhizal fungi, which are the main nutrient source for the embryo (Athipunyakom *et al.*, 2004; Yamato *et al.*, 2005; Khamchatra *et al.*, 2016a). Under natural conditions, orchids produce a higher number of tiny dust-like seeds (**Figure 2**), which after the fruit stage are dispersed into the environment by multiple mechanisms (Rasmussen and Whigham, 1993; Jersáková and Malinová, 2007; Herrera *et al.*, 2017). At this point, compatible mycorrhizal fungi must associate with orchid seeds, a strategy developed to compensate the undeveloped endosperm, which does not have sufficient reserves for the embryo to advance to the protocorm, and the further plantlet growth and establishment in the field (Brundrett, 2002; Bonnardeaux *et al.*, 2007). At the seedling stage, mycoheterotrophy is essential for orchids and it is certainly an ecological advantage that orchids have evolved to improve their nutritional demands (Leake, 1994; Yam and Arditti, 2009). After the initial colonization by their compatible mycorrhizal fungus, orchids may vary their mycorrhizal interactions, and fungal switches have been reported for several orchid species, which denote different mycorrhizal fungi for seed germination and for protocorm growth to plantlet (Bidartondo *et al.*, 2004; McCormick *et al.*, 2006). Therefore, orchids may remain parasites on fungi throughout their life, exploiting carbon and other nutrient resources directly from the fungal hyphae (Merckx *et al.*, 2009; Motomura *et al.*, 2010). These parasitic interactions are broken-up when the first green leaf emerges and the orchid turns partially autotrophic (Barrett *et al.*, 2010; Gebauer *et al.*, 2016).

Orchid embryos are extremely simple, and are not as well developed as in other plant species (Gale *et al.*, 2018), which is caused by the numerous seeds produced in each capsule

(200,000 to 1,000,000), showing a variable degree of viability (Chen *et al.*, 2012; Valadares *et al.*, 2012; Herrera *et al.*, 2017). Seed germination in orchids promoted by mycorrhizal fungi is necessary to achieve one of the most crucial steps is the orchid life cycle: to generate a primary root and shoot meristem able to start the transition to photoautotrophy (Shefferson *et al.*, 2005; Bidartondo and Read, 2008; Yeung, 2017). Recently, Yeung (2017) listed some details of the orchid life cycle related to embryos, seeds and protocorms: i) orchid embryos are characterized by a small embryo proper, lack of a functional endosperm, a suspensor (in most orchids) with a key role as a nutrient uptake site for water and water soluble substances, embryos with a structural polarity in which cells from the apical zone form meristems and the cells from the basal zone form symbiotic interactions, cells with storage products that are not used unless mycorrhizal establishment has begun, and a final acquisition of desiccation tolerance; ii) importance and involvement of the phytohormones auxin, cytokinins, gibberellins and abscisic acid in the fruit and seed development; iii) presence of a carapace to improve seed survival to enhance their chances of establishing the mycorrhizal association; iv) formation of a protocorm as a further developmental stage to promote plantlet development after mycorrhizal colonization; v) mycorrhizal fungi entrance by suspensor or rhizoid according to the orchid species; and vi) end of the protocorm stage after the shoot apical meristems appear and the plantlet stage begins. All these processes are examples of the complexity of the tiny orchid seeds, and refer to the metabolic and anatomic changes that occur in the first two weeks when orchid seeds are dispersed into the environment. After that, mycorrhizal interactions are extremely dynamic and depend on the trophic nature of the orchids, but all changes point to complex metabolic regulations at both the genomic and symbiotic levels (Perotto *et al.*, 2014; Valadares *et al.*, 2014; Fochi *et al.*, 2017).

THE CHILEAN ORCHIDACEAE

Mycorrhizal interaction in Chilean ecosystems has focused on the study of arbuscular and ectomycorrhizal symbioses, contributing to the knowledge of functionality, distribution and presence in native flora, stress responses, and improvement of yield production of diverse agronomic species (Palfner, 2001; Castillo *et al.*, 2006; Aguilera *et al.*, 2014; Aguilera *et al.*, 2015; Marín *et al.*, 2017). However, there is little research related to orchid mycorrhizal associations, although recently the number of published studies analyzing fungal associations, ecology and germination of orchid seeds has increased (**Table 1**).

Orchids belong to the numerous and cosmopolitan Orchidaceae family. This plant family accounts for roughly 10% of angiosperms, of which 70% are epiphytes. According to Tsai *et al.* (2017), the Orchidaceae family can be divided into 5 subfamilies: Apostasioideae, Vanilloideae, Cypripedioideae, Orchidoideae, and Epidendroideae. Chilean Orchidaceae includes different genera of the subfamily Orchidoideae, tribe Cranichideae [subtribes Cranichidinae (*Myrosmodes* Rchb. f.), Spiranthinae (*Brachystele* Schltr.), Orchidinae (*Habenaria* Willd.), and Chloraeinae (*Bipinnula* Comm. ex Juss., *Chloraea* Lindl., and *Gavilea* Poepp.)] and Codonorchideae (*Codonorchis* Lindl.) (Novoa *et al.* 2015). Phylogenetic relationships between different species of Chilean Orchidaceae have been performed by Cisternas *et al.* (2012), who have indicated that a new classification in the subtribe Chloraeinae is necessary.

Chile is a country with contrasting climatic and ecosystem conditions, which varies from the extremely hot and dry desert in the north to the very cold and wet pristine ecosystems in the south (Armesto *et al.*, 2010; Herrera *et al.*, 2017). These different climatic conditions shape the high diversity of orchid populations. Chile has 72 orchid species distributed throughout the national territory and the Alejandro Selkirk Island (Novoa *et al.*, 2015). They are distributed in 8 genera: *Chloraea*, which include 43 species; *Gavilea*, with 17 species; *Bipinnula*, with 6 species; *Correorchis*, with two species; and the genera *Brachystele*, *Codonorchis*, *Habenaria*, *Myrosmodes*, accounting for 1 species each (**Figure 1**). Furthermore, several native orchid species can form hybrids. Considering all Chilean orchids, 42 are endemic to the country, and 30 are native mainly to Chile and Argentina (**Figure 1**) (Pereira *et al.*, 2014a; Herrera *et al.*, 2017). Only eight Chilean orchid species are considered to be threatened, with the conservation state of most Chilean orchids not having been evaluated (Novoa *et al*., 2015). In the field, we have shown that several orchid populations are decreasing rapidly, which will cause various conservation problems if the scientific community, government and private companies do not undertake responsible natural resource use and/or support the development of conservation programs for threatened species.

Chilean orchids are terrestrial with a strong rhizome that grows in the first centimeters of the soil, living in organic matter, on rocks and in the cortex of big trees (**Figure 2**). They usually lose the shoot biomass in the cold season, which makes orchids dependent on mycoheterotrophic processes, especially in the period when orchids defoliate (Herrera *et al*., 2017). In the warm season, orchids develop photosynthetic tissues, with a shoot ranging from

~20 to ~100 cm, with a floral escape with 1 to ~12 floral capsules, which is relative for the different orchid species (Novoa *et al.*, 2015). However, the mycorrhizal fungi associated with Chilean orchids are almost unexplored (Steinfort *et al.*, 2010; Pereira *et al.*, 2014a; Herrera *et al.*, 2017). Only six published studies have been carried out identifying mycorrhizal fungi associated with native and endemic orchids, three assessing asymbiotic germination media, and only three analyzing the symbiotic potential to promote orchid seed development of several mycorrhizal fungi belonging to the *Rhizoctonia*-like complex (**Table 1**). With respect to the nutritional mode of endemic orchids, there are no published studies analyzing the isotopic fingerprint of orchids and the surrounding plant species, processes that have been described as key to classifying orchids as fully autotrophic, partially mycoheterotrophic or fully mycoheterotrophic (Zimmer *et al.*, 2008; Girlanda *et al.*, 2011; Bougoure *et al.*, 2014). However, we expect partial mycoheterotrophy as the main carbon source for Chilean orchids. This indicates that orchids must obtain carbon first photosynthetically and second heterotrophically, processes that have been reported for other chlorophyllous orchids (Gebauer *et al.*, 2016). Obtaining carbon and other mineral nutrients from their mycorrhizal fungi is essential in several "green" orchids and is a key factor to determining the survival of different orchid species under stress conditions, such as in winter, where most Andean Chilean orchids live as underground organs with no presence of aerial biomass (3-5 months) (Jurkiewicz *et al.*, 2001; Shefferson *et al.*, 2008; Herrera *et al.*, 2017). Despite Chilean orchid habitats being mainly protected areas, there are some orchids that colonize hostile substrates such as sand, rocks, and understory forest of introduced exotic species (Herrera *et al.*, 2017). It is believed that some of these tolerant orchids activate symbiotic mechanisms to obtain nutritional benefits of their fungal partners, but more research is needed to characterize these resistance mechanisms as well as the Chilean orchid population hot-spot in Cordillera de Nahuelbuta, a bedrock soil where almost 40% of Chilean orchid species are found (Kennedy *et al.*, 2002; Romero, 2012).

In each natural distribution environment, orchids have developed a particular symbiotic mechanism to achieve fitness to the particular ecosystem conditions, such as limited light exposure, seasonal cold and hot climatic conditions and extremely anthropogenically altered ecosystems (McCormick *et al.*, 2006; Kottke *et al.*, 2010). These adaptations are often related to symbiotic associations with compatible mycorrhizal fungi adapted to the particular nutritional and climatic conditions that allow orchids to complete their life cycle, promoting the transition from seed to protocorm and finally to a partially mycoheterotrophic plantlet,

which will ultimately be able to colonize a determined ecosystem (Dearnaley, 2007; Bidartondo and Read, 2008; Rasmussen and Rasmussen, 2009).

MYCORRHIZAL FUNGI ASSOCIATED WITH CHILEAN ORCHIDS

Research into mycorrhizal fungi associated with Chilean orchids has characterized diverse fungal partners associated with different endemic and native orchids (**Table 1**). Chilean orchids preferably associate with Tulasnellales; however, other mycorrhizal fungi (*Thanatephorus*, *Ceratobasidium*) may also be isolated from Chilean orchids (Steinfort *et al.*, 2010; Pereira *et al.*, 2014a; Atala *et al.*, 2015; Mujica *et al.*, 2016; Herrera *et al.*, 2017). **Table 1** shows a complete analysis and description of the published studies related to symbiotic and asymbiotic strategies applied to Chilean orchids. To date, all the studies have focused on characterizing orchid mycorrhizal fungi associated with orchids and determining the ecological and potential specificity established between the orchids and the isolated mycorrhizal fungi (Steinfort *et al.*, 2010; Herrera *et al.*, 2017), identifying several Tulasnellales as the main mycorrhizal fungi associated with endemic orchids. Tulasnellales seems to be the main genus associated with terrestrial orchids (Ogura-Tsujita *et al.*, 2012). They are often fungal associates of several orchids and live in the photosynthetic roots of some epiphytic orchids or in the underground organs of terrestrial orchids (Jacquemyn *et al.*, 2017; Oberwinkler *et al.*, 2017).

The common technique to isolate mycorrhizal fungi is the peloton isolation method, in which the mycorrhizal fungi are directly isolated from the mycorrhizal tissues (pelotons) (Otero *et al.*, 2002, 2004; Valadares *et al.*, 2012). These techniques are widely used to study orchid-mycorrhizal interactions, but in some cases mycorrhizal fungi isolated from mature roots are not effective in promoting seed germination (Hou and Guo, 2009; Herrera *et al.*, 2017; Fay *et al.*, 2018; Zeng *et al.*, 2018). This is the main technique used to study orchid mycorrhizal interactions, and certainly it is crucial to promoting the seed germination of several terrestrial orchids (Tešitelová *et al.*, 2012; Chen *et al.*, 2017; Zeng *et al.*, 2018). However, not all mycorrhizal fungi can grow in *in vitro* conditions (Egidi *et al.*, 2018). Therefore, identifying orchid mycorrhizal fungi under axenic conditions is a limited methodology to study the ecology of orchid mycorrhizae, and this may explain the low germination rates obtained in some orchids. Hence, any study of orchid-mycorrhizal interactions must include the identification of mycorrhizal fungi through non-culture-dependent methods.

Mycorrhization in orchids is complex and influenced by several ecological mechanisms. Recently published studies have reported various mycorrhizal associations with several mycorrhizal fungi (Rasmussen, 1995; Girlanda *et al.*, 2011; Cowden and Shefferson, 2013; Xing *et al.*, 2013; Xing *et al.*, 2015). The specificity of orchid-mycorrhizal associations are influenced by the environmental conditions associated with the orchid plants, neighboring plant species associated with the orchid, and the abundance and distribution of mycorrhizal fungi in the soil (Waterman and Bidartondo, 2008; Waud *et al.*, 2016a; Waud *et al.*, 2016b; Mehra *et al.*, 2017; Pecoraro *et al.*, 2018). Mycorrhizal associations are extremely variable, even at the individual level, because several orchid species have more than one mycorrhizal fungus in their roots (McCormick *et al*., 2004; Wang *et al*., 2017; Xing *et al*., 2015).Multiple mycorrhizal partners will likely allow orchids to improve nutritional benefits and symbiotic benefits under different natural and stressed conditions (Gebauer and Meyer, 2003; Bidartondo *et al.*, 2004; McCormick *et al.*, 2016). The existence of a fungal switch has been characterized in the life cycle of some orchid species, which indicates that the mycorrhizal fungi necessary to promote seed germination are not functional at further developmental stages (McCormick *et al.*, 2006; Xu *et al.*, 2015). These aspects must be considered in the design of restoration strategies of endemic flora in order to understand the ecology of the mycorrhizal symbioses of endemic orchids.

Eventually, symbiotic seed germination has been achieved in endemic orchids, promoting seed germination to the protocorm stage, and the initial stage of the plantlet (presence of rhizoids and foliar primordia) (Herrera *et al*., 2017; Steinfort *et al*., 2010).These mycorrhizal fungi are usually obtained through the peloton isolation method and are effective in improving protocorm growth to differing degrees (Bonnardeaux *et al.*, 2007; Valadares *et al.*, 2012; Pereira *et al.*, 2014b). These mycorrhizal fungi are functional despite being isolated from mature roots; it is probably an intrinsic characteristic of the orchid seeds, because some studies have shown that orchid seeds cultured in a symbiotic medium without fungal inoculation can also generate orchid protocorm with rhizoids (Fracchia *et al.*, 2014a; Fracchia *et al.*, 2014b; Herrera *et al.*, 2017). We expect that these mycorrhizal fungi isolated from mature roots can enter the orchid protocorm through rhizoids and promote seed development, but the low germination rates must be related to the low generation of protocorms reaching rhizoid production. However, in order to improve germination rates and the opportunity for plantlet establishment, it might be necessary to consider different mycorrhizal fungi for seed germination and further mycorrhizal fungi for plantlet establishment. Hence, to achieve a

complete understanding of the orchid life cycle of the different endemic genera, other "friendly" methodologies should be used to focus the research in the wild, obtaining more plantlets established in their natural distribution areas (Batty *et al.*, 2006a).

Mycorrhizal associations in seed germination and later developmental stages of terrestrial orchids may also include fungal species outside the *Rhizoctonia*-like complex (Hou and Guo, 2009; Ogura-Tsujita *et al.*, 2012). Furthermore, various fungal and bacterial endophytes can also be found inhabiting the roots of several orchids, and a putative beneficial role cannot be ruled out (Bayman and Otero, 2006; Novotná *et al.*, 2018). In Chilean orchids, various fungal endophytes have been reported inhabiting the root tissues (*Phomopsis columnaris*, *Leptodontidium* spp., *Cadophora* sp., *Chaetomium* sp., *Chaetomium globosum*, *Peziza* sp., *Phomopsis* sp., *Hypocrea* sp. *Fusarium* sp. *Neonectria* sp. *Piromyces* sp., *Cylindrocarpon* sp., *Acremonium* sp., and *Pythium* sp.) (Mujica *et al.*, 2016; Herrera *et al.*, 2017). These fungi do not have a functional role in the orchid metabolism, but their isolation from various orchid species merits further investigation to identify potential ecological advantages for orchids to host a broad spectrum of non-mycorrhizal endophytic fungi. Some of the ecological functions likely include stress response, nutrient interchange, resistance to pathogen attack and beneficial metabolic changes.

CONSERVATION STATE OF CHILEAN ORCHIDS

Nowadays, conservation of flora is a worldwide problem, mainly due to alterations in natural ecosystems (McCarty, 2001; Robbirt *et al.*, 2011). This is the case of orchids, which undergo crucial reproduction steps in their life cycle, and are often related to the specific interactions with their mycorrhizal fungi (Cozzolino *et al.*, 2006; Egidi *et al.*, 2018). There are many constraints to orchid development and conservation, and these are often related to human-induced ecosystem alteration as well as depredation (animals for food and humans for ornamental plants and urbanization) (Kottke *et al.*, 2010; Herrera *et al.*, 2017). An examination of the specificity and dynamics of mycorrhizal associations is the starting point to designing safeguarding strategies for endangered species and it is certainly essential to designing restoration programs.

To date, none of the taxa included in Chilean Orchidaceae has been assessed for the IUCN Red List, but the Chilean Ministry of the Environment has reported 8 Chilean species as vulnerable (IUCN, 2018; MMA, 2011; Novoa *et al.*, 2015), but we believe that several

endemic orchids must be classified as near threatened according to the IUCN Red List IUCN/SSC, in light of their constant decline in distribution and populations observed in the wild (IUCN/SSC Orchid Specialist Group, 1996). An appropriate classification system must be developed to accurately define the conservation of endemic orchids in order to define critically threatened orchids to study and implement recovery programs to prevent extinction.

Mycorrhizal fungi in orchids are essential and certainly have a more complex role in orchid metabolism than simple seed germinators. We encourage focusing research on the ecosystem, promoting seed germination in the field in order to generate more effective reproduction strategies for critically endangered and vulnerable species, such as *Bipinnula apinnula*, *Bipinnula taltalensis*, *Bipinnula volckmannii*, *Bipinnula gabriel*, *Chloraea cristata*, *Chloraea cuneata*, *Chloraea disioides*, *Chloraea heteroglossa*, *Chloraea prodigiosa*, *Gavilea insularis* and *Gavilea kingii* (Novoa *et al.*, 2015)

To date, there have been no published studies reporting a successful reintroduction methodology for terrestrial endemic orchids. However, there are some remarkable studies addressing symbiotic and asymbiotic reproduction (**Table 1**). Focusing on symbiotic reproduction, it would be extremely necessary to germinate orchid seeds with mycorrhizal fungi useful for seed germination and design strategies to reintroduce orchids to the wild. These fungi may be obtained by sowing seed packets in order to isolate fungal partners directly from naturally germinated protocorms, a technique known as seed-baiting (Batty *et al.*, 2006a; Khamchatra *et al.*, 2016a). Mycorrhizal fungi for further developmental stages can be obtained from the typical peloton isolation method and are candidates for subsequent inoculations. With respect to asymbiotic seed germination, there have been some reports of endemic orchid testing in different asymbiotic media, but further inoculation with other mycorrhizal fungi is not considered. There are reports of successful orchid plantlet reintroductions obtained from asymbiotic germination media, but the presence of mycorrhizal fungi in mature plants is ecologically important to different stress conditions and improves the final establishment in the wild (Hou and Guo, 2009; Oja *et al.*, 2015; Khamchatra *et al.*, 2016a; Waud *et al.*, 2016b).

Despite no published information being available with regard to successful Chilean orchid reintroductions, several efforts are being made on behalf of threatened orchid populations; for example, the different restoration programs developed by Jardin Botanico Nacional,

EXPLORA projects, and PROENTA-UFRO, who have developed some methodologies for the reintroduction, reproduction or transplant of threatened orchids to natural ecosystems.

The design of a restoration program for endemic orchids must include a full understanding of the mycorrhizal fungi associated with orchids, the ecological conditions in which orchids are established (climate and presence of other plant species), distribution of mycorrhizal fungi in the soil, presence of pollinators and viability of the generated orchid seeds, and the conservation state of the endemic orchids (Bonnardeaux *et al.*, 2007; Dearnaley, 2007; Selosse, 2014; Dearnaley *et al.*, 2016). This last point is crucial to designing restoration programs and should include initial work with an endemic orchid not under conservation threat. This is essential to preventing the extinction of endemic orchids, particularly of species with limited individuals such as *B. Gabriel* (Bravo-Monasterio *et al.*, 2014). Designing an appropriate methodology will reduce the risk of extinction and damage to the orchid individuals. Therefore, we propose a working methodology that considers all the symbiotic aspects of the endemic orchids, starting with the recognition of appropriate mycorrhizal fungi for seed germination so as to identify mycorrhizal fungi for plantlet establishment (candidate for inoculation in symbiotic and asymbiotic-germinated protocorm), and the use of bioaugmentation technologies of mycorrhizal fungi and other root endophytes in the wild because orchid mycorrhizal fungi distribution has been characterized as one of the most crucial steps related to the distribution of several orchid species (**Figure 3**). All these aspects point to improving the number of mycorrhizal plantlets for successful establishment, growth and phenotypic characteristics of the flowers in the wild.

Despite mycorrhizal fungi associated with orchids being greater in number than those cultured in vitro, these isolates are the main candidates for use in restoration strategies (Swarts *et al.*, 2010; Khamchatra *et al.*, 2016b). Therefore, the starting point to design reintroduction methodologies is to consider a pool of mycorrhizal fungi isolated from endemic orchids. To do that, we suggest designing a standard methodology to isolate and deposit mycorrhizal fungi in culture collection centers to preserve mycorrhizal fungi isolated from endemic orchids, as well as to collect orchid seeds to deposit in a seed bank of endemic orchids.

CURRENT METHODOLOGIES FOR WORKING WITH ENDEMIC ORCHIDS

According to the literature review, we point to different methodologies and strategies that can be applied to the study of orchid mycorrhizae for safeguarding endemic flora, which are mainly detailed in **Figure 3**.

Asymbiotic seed germination

Asymbiotic seed germination has been applied to reproduce several orchid species and seeks to emulate the nutrition obtained by the mycorrhizal fungi under *in vitro* conditions (Dutra *et al.*, 2008; Dutra *et al.*, 2009). Several culture media have been tested and usually vary in the chemical composition and concentration of several micronutrients and natural compound extracts (Fay, 1992; Li *et al.*, 2018). Furthermore, the use of phytohormones as growth stimulators has been reported (Romero *et al*., 2017). However, plantlet establishment has been low when plantlets are transplanted out the flask, where there are different atmospheric and nutritional conditions, and where the pathogen attack and climate conditions cause low plant establishment in the wild (Clements *et al.*, 1986; Ramsay and Dixon, 2003).

Asymbiotic seed germination has been tested in the native orchids *Chloraea virescens*, *Chloraea crispa*, *Chloraea gavilu*, and *Bipinnula fimbriata*, analyzing the protocorm growth promotion of seeds sown in asymbiotic media, such as agar water, culture medium banana, culture medium tomato, Knudson C, and Malmgren modified, BM-2 terrestrial orchids, and Vacin and Went (Pereira *et al.*, 2015; Pereira *et al.*, 2017; Romero *et al.*, 2017). Working with asymbiotic methodologies requires the the phenolic carapace to be present in endemic orchids and the high presence of abscisic acid as a common characteristic of the orchid seed development, which adversely affects asymbiotic seed germination (Yeung, 2017). An evaluation of the asymbiotic potential must be done by applying different methods to remove the carapace or selection of 'green' capsules (immature seeds) to increase the asymbiotic seed germination rate of endemic orchids (Yamazaki and Miyoshi, 2006; Mweetwa *et al.*, 2008; Hosomi *et al.*, 2011; Hosomi *et al.*, 2012). Immature capsules can also produce viable seeds, whose embryos have completed the histodifferentiation program for protocorm formation; and the low ABA levels and the thin cuticle around the embryo proper limits the physical barriers of the orchid seeds, which are not yet fully developed, facilitating the germination of immature seeds (Lee *et al.*, 2007). Furthermore, physical barriers such as the thick seed coat and the carapace are not well developed (Yeung, 2017). All these features facilitate the asymbiotic germination of immature seeds. These aspects have been recently considered by Romero *et al.* (2017), who studied the symbiotic germination of the endemic orchid *C.*

gavilu, achieving plantlet production in several germinated seeds. In order to improve survival and establishment in the wild, we recommend that further inoculation with mycorrhizal fungi isolated from mature orchids be considered, because these fungi act as important nutritional and metabolic sources for the orchid, even under stress conditions (Jurkiewicz *et al.*, 2001; Kuga *et al.*, 2014; Fay *et al.*, 2018). This subsequent inoculation of mycorrhizal fungi must decrease the negative impacts on the germinated orchids after transplantation, because in several cases the protocorm development of asymbiotic and symbiotic germinated seeds show a different growth rate (Yeung, 2017).

Symbiotic seed germination

Although different orchid species can be reproduced by asymbiotic germination methods, symbiotic germination of the same orchid can generate more developed embryos and the growth is better in the mycorrhizal ones (Johnson *et al.*, 2007). Hence, obtaining mycorrhizal protocorms is extremely necessary to the reintroduction of orchid populations to their natural distribution environments. To perform symbiotic germination, the mycorrhizal fungi associated with orchids must be isolated and identified (Otero *et al.*, 2002; Valadares *et al.*, 2012). The use of OMA has been described as the main inoculation medium to promote the *in vitro* symbiotic seed germination of several orchids (Clements *et al.*, 1986; Zettler, 1997; Tešitelová *et al.*, 2012). In Petri dishes, the symbiotic potential of mycorrhizal fungi to promote seed germination can be tested on the same plate (Herrera *et al.*, 2017). Mycorrhizal association in orchids are extremely variable; some orchids have shown narrow fungal clades associated with their roots, whereas others have shown wider fungal mycobionts (Tešitelová *et al.*, 2012; Valadares *et al.*, 2012). This is commonly related to the distribution of orchid species, with the orchid being associated with several widely distributed mycorrhizal fungi compared to orchids with specific mycorrhizal associations (Swarts *et al.*, 2010). In symbiotic seed germination trials the identification of the ecological specificity (fungal associations established under natural conditions) and the potential specificity (mycorrhizal fungi able to promote seed germination that can be isolated from different orchid species) must be defined (Steinfort *et al.*, 2010). A mycorrhizal fungus with high potential specificity would be a very interesting candidate to incorporate in restoration programs of endemic orchids.

Symbiotic seed germination has been tested in endemic orchids promoting seed development to differing degrees (**Table 1**). These studies have identified several mycorrhizal fungi with a

potential use in reintroduction strategies for the orchids studied. However, the diversity of the mycorrhizal associations seems to be high and related to the different climatic and ecosystem conditions. This reveals the need to study mycorrhizal associations of endemic orchids individually, and to consider the design of preservation strategies for the different isolated mycorrhizal fungi.

The seed baiting technique

Isolating mycorrhizal fungi using *in situ* methods by burying seed packets in the field is useful for isolating effective mycorrhizal fungi for seed germination (Rasmussen and Whigham, 1993; Batty *et al.*, 2006a; Khamchatra *et al.*, 2016a). However, this methodology has not yet been applied to study the germination of endemic orchids. Inoculation with mycorrhizal fungi from a mature orchid is also an accepted strategy to germinate orchid seeds, especially for orchids that are able to swell in size to the protocorm stage (Fracchia *et al.*, 2014a; Herrera *et al.*, 2017). However, not all orchids are able to grow initially in the absence of mycorrhizal fungi, needing specific mycorrhizal fungi to germinate.

The seed baiting technique has been applied to study the mycorrhizal interactions of several terrestrial orchids (Wang *et al.*, 2011; Zi *et al.*, 2014). These *in situ* baiting studies buried seed packets in the field during the growth period to study the mycorrhizal fungi able to germinate seeds in the wild, which is similar to other techniques used to quantify the germination of other plants and to detect other plant parasites (Brundrett *et al.*, 2003). In this technique, soil samples are collected from the leaf litter layer and uppermost topsoil layer (*ex situ* seed baiting) or the seed packets are buried directly in the field (*in situ* seed baiting) to promote seed germination. Seed baits can be performed using individual or multiple compartment packets with a nylon mesh (90 µm) to retain the seeds without impeding fungal growth (Brundrett *et al.*, 2003). After the growth period, seeds in the seed packets are examined and mycorrhizal fungi are appropriated for germination can be isolated and identified.

The use of seed baiting techniques is necessary to identify mycorrhizal fungi associated with the first developmental stages of orchids, and it certainly can improve the knowledge of fungal associations of endemic orchids, providing crucial data for the implementation of reintroduction strategies for threatened orchid populations.

Mycorrhizal fungi and seed preservation

Mycorrhizal fungi and seed conservation is considered an effective measure to improve the population numbers of several endangered species. Orchid seeds for banking need to be dried to low moisture (3 to 7%) and stored at different temperatures, whereas the fungal associates are commonly grown on agar-based culture media and stored at room temperature or 4°C (Popova *et al.*, 2016; Schofield *et al.*, 2018). All scientific studies analyzing seed germination of endemic orchids must consider applying *ex situ* conservations methods. Germplasm conservation has been used as a measure to preserve orchids with reproduction problems (Martin, 2003; Sarasan *et al.*, 2006). Different preservation methods have been tested in Batty *et al.* (2001), describing a methodology to store dried orchid seeds in liquid nitrogen, and analyzing the germination rates of freshly collected seeds or those stored at 4, 18 and 22 °C, which were usually better in the treatments with liquid nitrogen. Furthermore, the mycorrhizal fungi that promoted the germination and growth of different orchid species could also be stored in liquid nitrogen and other methods including lyophilization and freezing (Swarts and Dixon, 2009). In the study of the mycorrhizal association of Chilean orchids, we suggest that a collection of native orchid seed and associated fungi be created so as to design appropriate methodologies of reintroduction of endemic orchids to the natural distribution habitats.

Reintroduction of endemic orchids to natural distribution areas

Reintroducing orchid species into their natural ecosystem involves more than reproducing suitable plants, because the decline in the orchid population is related to the absence of compatible mycorrhizal fungi in the soil (Swarts and Dixon, 2009; Swarts *et al.*, 2010; Pecoraro *et al.*, 2018). Therefore, to save endemic orchids, knowledge of compatible mycorrhizal fungi for both seed germination and plant establishment is required. These mycorrhizal fungi can be isolated, identified and stored to develop the restoration methodologies. One of the new methodologies applied to promote the reintroduction of orchids in the wild are related to the use of microencapsulation technologies. Microencapsulation involves isolating compatible mycorrhizal fungi (mainly from *ex situ* seed baiting techniques) and dried seeds, which are encapsulated mainly in sodium alginate beads using various protocols (Crasta and Bopaiah; Martin, 2003; Thammasiri, 2008; Sommerville *et al.*, 2008). According to Gantait *et al.* (2012), the alginate coat of encapsulated explants shields plant tissues from physical and environmental injury, reduces dehydration, and offers mechanical pressure to grip the explants inside the gel matrix during

storage. Usually, an encapsulation methodology disinfects the orchid seeds superficially (usually NaOCl, 0.5% active Cl) and rinses them with sterile deionized water. After that, seeds or protocorm-like bodies must be added to a sterile solution of sodium alginate. Fungal hyphae from OMA cultures need to be scraped and incorporated to the sodium alginate solution. After that, sodium alginate is mixed and the resulting suspensions are pipetted drop by drop into a sterile solution of calcium chloride to form the individual beads. Then, the beads are added to a sucrose solution to be dried and stored according to the different protocols available (Wood *et al.*, 2000; Sommerville *et al.*, 2008; Luo *et al.*, 2009; Gantait *et al.*, 2012).

The development of *ex situ* conservation programs of seeds or other propagules are necessary for orchid conservation, especially for threatened species, and for reintroduction into the wild (Batty *et al.*, 2006b; Li and Pritchard, 2009; Seaton *et al.*, 2010; da Silva, 2013). In orchids, the reintroduction methodologies must consider the different mycorrhizal fungi associated with orchids in order to obtain symbiotic benefits for the plantlet in the field (Cameron *et al.*, 2006; Cameron *et al.*, 2008; Herrera *et al.*, 2017). Usually, symbiotic germinated plants are obtained under laboratory conditions (Clements *et al.*, 1986; Athipunyakom *et al.*, 2004; Batty *et al.*, 2006b). However, when mycorrhizal orchid seedlings are transplanted to the field, the inexistence of compatible mycorrhizal fungi may result in no germination by the subsequent orchid seeds (Stewart *et al.*, 2003; Zettler *et al.*, 2003). Therefore, the encapsulation of mycorrhizal fungi for augmentation strategies, soil microsite for germination of terrestrial orchids, and the simultaneous encapsulation of mycorrhizal fungi and seeds are necessary to design an appropriate methodology for safeguarding endemic orchids (Wood *et al.*, 2000; Sommerville *et al.*, 2008; Ercole *et al.*, 2013).

Various encapsulation-dehydration protocols have been applied to the *ex situ* conservation of orchids, and aim to simplify the procedures for reintroduction or population augmentation of threatened orchid species. The simultaneous encapsulation of orchid seeds (*Dactylorhiza fuchsia* and *Anacamptis morio*) and mycorrhizal fungi (*Ceratobasidium cornigerum*) in sodium alginate beads can be prepared for long storage at 196°C for 30 days, with high levels of seed and fungal viability (Wood *et al.*, 2000). In addition, Sommerville *et al.* (2008) developed an encapsulation-dehydration protocol for conservation of two threatened terrestrial orchids from Australia and their fungal symbionts, which can be used for the restoration of sites slated for population enhancement or from which orchid and fungal

populations have disappeared entirely. In addition, Gantait *et al.* (2012) and Luo *et al.* (2009) tested the microencapsulation of protocorm-like bodies as plantlet restoration strategies for endangered medicinal and commercial orchids.

EXPLORING THE ECONOMIC POTENTIAL OF ENDEMIC ORCHIDS

Certainly, Chilean orchids have unique shapes and colors that are potentially attractive for commercial purposes. Once the conservation issues have been addressed and working with reintroduction programs, we recommend exploring the economic potential of the endemic orchid; safeguarding the endemic orchid flora and knowing the appropriate reproduction traits for reintroduction will achieve a successful commercial reproduction program for Chilean orchids. To date, limited commercial projects have addressed the economic potential of endemic orchids (FIA-PI-C-1998-1-A-022; FIA-PI-C-2003-1-A-81; FIA-PI-C-2007-1-A-003) (FIA, 2018). However, to date we have been unable to show massive native orchids for sale, and the conservation problems persist. To explore the economic potential of endemic orchids, the phenotypic characteristic of the flowers must be improved and the growing time from seed to flowering plant must be reduced. One strategy to improve plant characteristics that have been applied to orchids for commercial purposes is polyploidy induction. According to Chen and Chen (2011), polyploidy can improve floral and growth characteristics, generating protocorms that can be propagated (Chen *et al.*, 2009; Chen and Chen 2011), but these processes are time-consuming and need the economic investment of the government and the interest of the private sector to exploit the endemic orchid flora.

CONCLUSION

Conservation problems in orchids are caused by the particular mechanism of the seed structure, the need for compatible mycorrhizal fungi to begin seed germination and progress to protocorm, changes in mycorrhizal partners for plantlet development and establishment, and associations with several bacterial and fungal endophytes that certainly play an important role in orchid metabolism. Certainly, our knowledge about orchid mycorrhizal interactions in Chilean ecosystems has increased considerably. However, further research into endemic orchids must be encouraged to develop more applicable methodologies for a universal propagation and reintroduction protocol for Chilean orchids in order to prevent the decay and extinction of these orchids with particular and original flowers, colors and reproduction strategies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

TABLE LEGEND

Table 1. Published studies on Chilean orchids identifying main mycorrhizal fungi and promoting orchid seed germination.

FIGURE LEGEND

Figure 1. Schematic representation of the Chilean Orchidaceae family, showing the number of endemic and native orchids (bars), the eight genera and the number of species (parenthesis), and the abundance and state of the conservation of orchid species (circle) according to Novoa et al. (2015).

Figure 2. Shoot development of *Gavilea lutea* after the cold season in the Andean mountains from the Region of La Araucanía (a). Root biomass of *Chloraea bletioides* from the coastal mountain in the Region of Maule (b). Seed capsules showing the dust-like seeds of *Bipinnula fimbriata* from littoral ecosystems in the Region of Valparaíso. Scale (bar = 1 cm).

Figure 3. Proposed working diagram for reintroduction of threatened Chilean orchid species (terrestrial). Concepts in boxes are crucial steps in the work with orchid mycorrhizae. Text in bold are suggestions of the methodologies for further reintroduction programs.

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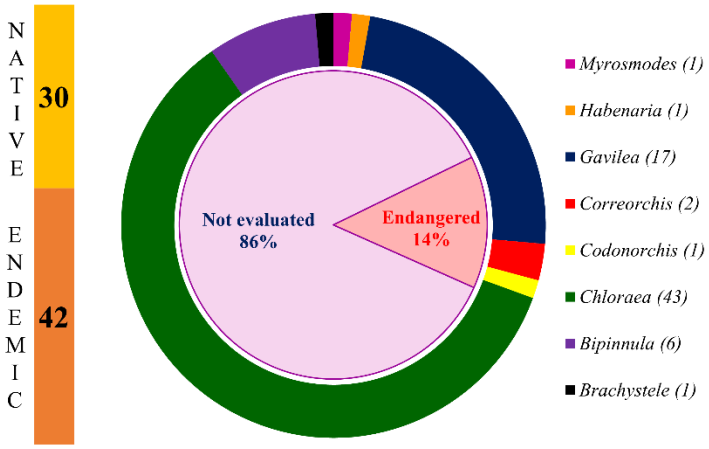
Table 1. Published studies in Chilean orchids identifying main mycorrhizal fungi and promoting orchid seed germination.

Target Orchid	Main mycorrhizal fungi	Aim	Methods	Reference
Bipinnula fimbriata *Chloraea crispa*	*Ceratobasidium* sp. *Tulasnella calospora* Uncultured *Ceratobasidium* *Thanatephorus cucumeris*	To isolate and identify mycorrhizal fungi Symbiotic seed germination.	Isolation and molecular identification of mycorrhizal fungi. Symbiotic germination in OMA media.	Steinfort *et al.* (2010)
B. fimbriata	Not identified	To germinate orchid seed in asymbiotic germination media.	Culture in agar water, culture medium banana, culture medium tomato, Knudson C, and Malmgren modified terrestrial orchid media.	Pereira *et al.* (2015)
B. fimbriata *Bipinnula plumosa*	*T. calospora*, *Tulasnella danica* *Tulasnella asymmetrica* *Ceratobasidium* sp. *C. albasitensis* *Rhizoctonia* sp. *R. butinii*.	To show how soil nutrient availability relates to composition and diversity of mycorrhizal fungi.	Fungal isolations and molecular identification. Estimation of nitrate content and Olsen P.	Mujica *et al.* (2016)
Chloraea chrysantha *Chloraea gavilu* *Chloraea bletioides* *B. fimbriata* *C. crispa* *Chloraea longipetala* *Chloraea grandiflora*	*Ceratobasidium* sp. *Thanatephorus* sp. Uncultured *Tulasnellaceae* *Tulasnella* sp. *Tulasnellaceae* sp.	To isolate and identify orchid mycorrhizal fungi from the Region of Maule. Symbiotic seed germination.	Fungal isolation and molecular identification. Multiple symbiotic germination trials in OMA media.	Herrera *et al.* (2017)
Chloraea virescens *C. crispa* *C. gavilu*	Not identified	To germinate the orchid seeds under different asymbiotic media.	Culture in Agar Water, Knudson C, Banana Culture Media, Tomato Culture Media, Malmgren Modified, Murashige and Skoog, and Murashige and Skoog (50% salt and vitamins).	Pereira *et al.* (2017)
Chloraea cuneata	Not identified	To isolate fungal partners of *C. cuneata.*	Culture in PDA media. Morphological identification.	Atala *et al.* (2015)
Chloraea collicensis *C. gavilu*	*T. calospora* *Tulasnellaceae* sp.	To isolate and identify mycorrhizal fungi.	Fungal isolation and molecular identification.	Pereira *et al.* (2014a)
C. gavilu	Not identified	To test various asymbiotic germination media for *C. gavilu.*	Culture in Murashige and Skoog (50% salt and vitamins); Modified Malmgren; BM-2 Terrestrial Orchids; and Vacin and Went. All culture media supplemented with 1% (w/v) sucrose, 0.05% (w/v) yeast extract, 4.44 μmol^{-1} BAP, 0.7% (w/v) Agar, and pH 5.8.	Romero *et al.* (2017)
C. crispa	*Rhizoctonia* sp.	To characterize morphometrically the symbiotic germination stages of *C. crispa* seeds	Symbiotic culture in OMA media and examination of germination stages using software image analysis	Verdugo *et al.* (2007)

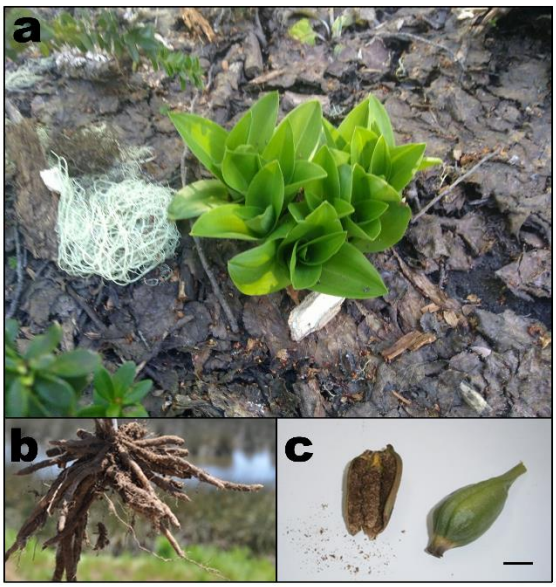
1091

1092 **Figure 1.**

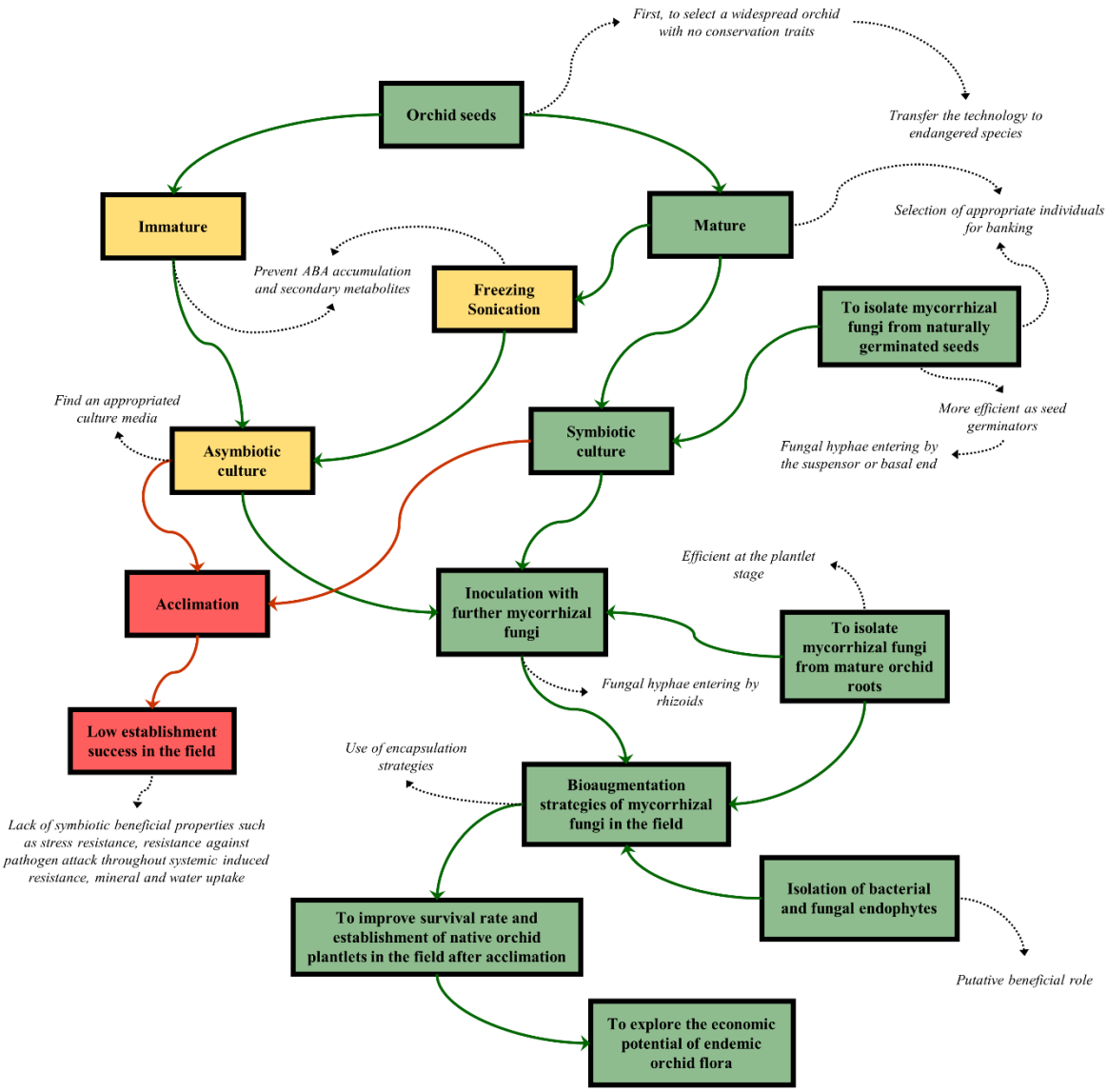


1093

Figure 2



1096 **Figure 3.**



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