Taylor & Francis
Taylor & Francis Group

Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology

Official Journal of the Societa Botanica Italiana

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tplb20

Controlled mycorrhization of the endemic Chilean orchid *Chloraea gavilu* (Orchidaceae)

Guillermo Pereira, Héctor Herrera, César Arriagada, Hertes Cid, José Leonardo García & Cristian Atala

To cite this article: Guillermo Pereira, Héctor Herrera, César Arriagada, Hertes Cid, José Leonardo García & Cristian Atala (2021) Controlled mycorrhization of the endemic Chilean orchid *Chloraea gavilu* (Orchidaceae), Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, 155:4, 848-855, DOI: 10.1080/11263504.2020.1801875

To link to this article: https://doi.org/10.1080/11263504.2020.1801875

Published online: 24 Aug 2020.

PLANT BIOSYSTEMS - AN INTERNATIONAL JOURNAL DEALING WITH ALL ASPECTS OF PLANT BIOLOGY
2021, VOL. 155, NO. 4, 848–855
https://doi.org/10.1080/11263504.2020.1801875

Controlled mycorrhization of the endemic Chilean orchid *Chloraea gavilu* (Orchidaceae)

Guillermo Pereira[a], Héctor Herrera[b], César Arriagada[b], Hertes Cid[a], José Leonardo García[c] and Cristian Atala[d]

[a]Departamento de Ciencias y Tecnología Vegetal, Laboratorio Biotecnología de Hongos, Universidad de Concepción, Los Ángeles, Chile; [b]Departamento de Ciencias Forestales, Laboratorio de Biorremediación, Facultad de Ciencias Agropecuarias y Forestales, Universidad de La Frontera, Temuco, Chile; [c]Campo Experimental Valle del Guadiana, Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP), Durango, México; [d]Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile

ABSTRACT

Orchids require mycorrhizal fungi (OMF) for their germination and growth. Propagation and re-introduction initiatives would likely require inoculation with such fungi. All Chilean Orchidaceae species are terrestrial and likely associate with OMF. We collected adult individuals of the endemic Chilean orchid *Chloraea gavilu* and transported them to a glasshouse where we obtained mature capsules through manual auto-pollination. We asymbiotically germinated seeds in vitro using Malmgren Modified Terrestrial Orchid-Medium (MM). Embryos were put in glass flasks with MM where roots and leaves developed for 16 weeks. Plants were then transplanted to 165 mL pots and randomly separated into three groups; plants inoculated separately with *Ceratobasidium* OMFs isolated from two Chilean orchid species (*Chloraea virescens* and *Codonorchis lessonii*), and uninoculated (control) plants. Plants were then put in a growth chamber. Three months later, inoculated individuals showed pelotons inside parenchyma cells in the roots. Four months after inoculation, mycorrhizal plants had higher root and shoot biomass compared to control plants. At the end of the experiment, the highest mycorrhization was achieved with the *Ceratobasidium* strain isolated from *C. lessonii*. The artificial mycorrhization of endemic orchids could be a key strategy for future conservation and propagation initiatives, especially for endangered or commercially interesting species.

ARTICLE HISTORY
Received 21 November 2019
Accepted 22 June 2020

KEYWORDS
Terrestrial orchid; orchid fungi; mycelial inoculum; mycorrhizal synthesis; orchid propagation

Introduction

Orchids in their natural habitat usually reproduce through sexually produced seeds (Heywood et al. 2007). Once released from the capsule, they strongly depend on mycorrhizal fungi present in the soil that induce germination and embryo growth (Bernard 1899, Burgeff 1959; Smith 1966; Pereira et al. 2005; Murguía and Lee 2007; Smith and Read 2008; Valadares et al. 2012; Herrera et al. 2017). These fungi are called orchid mycorrhizal fungi or OMF, but non-mycorrhizal endophytic fungi can also be found inside orchid roots (Herrera et al. 2017). The association with OMF is especially relevant since orchid seeds lack storage tissues required for seed germination and seedling development (Bernard 1899; Paudel et al. 2013). In the early stages, orchids are mycoheterotrophic, obtaining carbon from the OMF (Smith 1966; Arditti and Ghani 2000; Rasmussen 2002; Smith and Read 2008).

Asymbiotic seed germination (i.e., without fungal inoculation) has been shown to be a proper tool for the production of plantlets of several orchid species for commercial and conservation purposes (Yamazaki and Miyoshi 2006; Kauth et al. 2006, 2008; Dutra et al. 2008; Stewart and Kane 2010; Pereira et al. 2015, 2017). It has been recognized as a time- and cost-efficient method because it can achieve high success rates in some cases, and it does not require previous isolation of fungal species (Johnson et al. 2007; Aggarwal and Zettler 2010; Abraham et al. 2012, Pereira et al. 2017). Knudson (1922, 1946) achieved the first successful in vitro orchid reproduction through seed germination. This method has been used for successful reproduction of commercially important tropical orchids such as *Cattleya* Lindl. and *Laelia* Lindl. species (Arditti et al. 1982; Damon et al. 2004). However, the establishment of protocols for in vitro asymbiotic germination of orchid seeds is species-specific and depends on several factors such as; capsule maturity, components of culture media, light and temperature (Arditti 1992). Compared with their tropical counterparts, there are fewer studies on asymbiotic germination of terrestrial temperate orchids (but see Dixon 1991; Pereira et al. 2015, 2017).

In Chile, the Orchidaceae is represented by 8 genera and 72 species, 27 of which are endemic to the country (Novoa et al. 2015). The genus *Chloraea* Lindl. is the most diverse in Chile and include many endemic, and potentially ornamental, terrestrial orchid species (Novoa et al. 2015). Among *Chloraea* species, *Chloraea gavilu* Lindl. is one of the most attractive due to its large flowers of intense yellow color,

CONTACT Cristian Atala cristian.atala@pucv.cl Laboratorio de Anatomía y Ecología Funcional de Plantas, Facultad de Ciencias, Instituto de Biología, Pontificia Universidad Católica de Valparaíso, Campus Curauma, Curauma, Chile

Published online 24 Aug 2020

and relatively high number of flowers per spike (Novoa et al. 2015). This species is found in Chile from Valparaíso (~32°S) to Valdivia (~40°S) (Novoa et al. 2015), being far less common in the northern parts of its distribution.

Recently, Pereira et al. (2017) asymbiotically germinated seeds of *Chloraea gavilu* Lindl., and two other *Chloraea* species, using Malmgren Modified Terrestrial Orchid Medium (MM) with close to 90% germination. However, when plants obtained in vitro are transplanted to non-sterile media or to pots with soil, most individuals die and/or rot (personal observation, data not shown). Adult orchid plants also seems to require mycorrhizal fungi to grow and survive in natural conditions (Dearnaley et al. 2012). In fact, the roots of some Chilean species from different genera have been found associated with OMF from the Ceratobasidiaceae and Tulasnellaceae (Pereira et al. 2014; Atala et al. 2015; Herrera et al. 2017, 2019) along some other non-mycorrhizal endophytic fungi (Herrera et al. 2017).

In recent years, many Chilean orchids have shown a constant decay in their populations due to an increase in anthropic alterations of their natural habitats (Atala et al. 2017; Herrera et al. 2019). Given their close association with soil fungi, conservation programs oriented toward the reintroduction of orchids in the field, especially endangered or rare orchids, will likely require re-inoculation of asymbiotically-germinated individuals with specific mycorrhizal fungi. The current study aims to evaluate the effect of the inoculation with two strains of OMF on the developments and growth of asymbiotically-obtained *C. gavilu* plants. As mentioned above, this information could be relevant for developing conservation strategies and for possible propagation initiatives for the flower industry.

Materials and methods

Plant material

Individuals of *Chloraea gavilu* Lindl. were collected from the field and were transplanted to a glasshouse (Figure 1A). At flowering, we conducted manual auto-pollination of four flowers per plant with a total sample size of five plants. Thirty-five days after pollination, capsules were harvested before dehiscence and were left to dry for 2 days at 24 ± 1 °C. We harvested capsules at that time following the visual development of the capsule and previous experience with this and other *Chloraea* species. Seeds were obtained from the capsules and stored in a sealed air-tight flask at 4 °C following a previously published protocol (Pereira et al. 2015). For the asymbiotic germination, we used MM medium (Malmgren, 1996), previously proven to achieve close to 90% germination in this species (Pereira et al. 2017). The culture medium was previously sterilized at 121 °C and 1 atm for 20 min and put in 50 mm diameter Petri dishes in a horizontal flux chamber. Culture medium pH was set to 5.8. Seeds of *C. gavilu* were superficially disinfected before germination as previously described (Batty and Brundett 2001; Pereira et al. 2017). Seeds were then put in petri dishes containing culture media described below under a laminar flux chamber using a sterile dropper. When plants reached the shoot stage (Figure 1b, Yamazaki and Miyoshi 2006), they were transplanted to glass flasks containing MM medium where leaf and root development occurred for 16 weeks (Figure 1C).

Fungal material

The used OMF corresponds to two strains from the microorganism collection of the Laboratorio de Biotecnología de Hongos de la Universidad de Concepción (Fungi Biotechnology Lab of University of Concepción). These fungal strains were previously isolated from naturally growing orchids and identified using standard molecular techniques. The strains correspond to GeneBank accessions KT003599, isolated from *Codonorchis lessonni* (Brongn.) Lindl. (hereafter *Ceratobasidium Cl*) and MN199626, isolated from *Chloraea virescens* (Willd.) Lindl. (hereafter *Ceratobasidium Cv*) (Pereira, unpublished data). Both strains belonged to genus *Ceratobasidium* D.P. Rogers. *C. lessonii* individuals were collected from a natural population in Los Guindos sector, road to Antuco Volcano, Biobío Región (37° 21′ 31.26″ S, 71° 51′ 33.28″ W) and *C. virescens* plants were collected in Pucón aerodrome, Araucanía Region (39°17′ 40.34″ S, 71° 54′ 25.49″ W). We selected these strains due to their high radial growth of the mycelium in solid culture (Pereira et al., unpublished data). From actively growing colonies (Figure 1D), three disks of agar and mycelium 5 mm in diameter were cut and separately transferred to 100 mL Erlenmeyer flasks containing 60 mL of liquid potato dextrose agar (PDA) medium at a pH of 5.8. The PDA medium was previously sterilized as described above for the MM medium. Flasks were incubated in the dark at 24 ± 1° C in a stove for 8 days in static conditions. Produced biomass (Figure 1E) was harvested and liquated using a blender. The resulting liquid (mycelial suspension) was used as a source of inoculum for the controlled mycorrhization.

Plant inoculation

After 16 weeks in glass flasks with MM (as described earlier), plants were put in individual 165 mL pots (Figure 1F) filled with previously autoclaved 1:1 vermiculite/peat substrate. Plants were then randomly assigned to one of three treatments. 1) Control plants (i.e., no inoculation). 2) Plants inoculated with strain *Ceratobasidium Cv*, and 3) plants inoculated with strain *Ceratobasidium Cl*. To each inoculated plants we added 10 mL of the corresponding mycelial suspension 1:10 v/v (1 mL of suspension in 10 mL of distilled water). Control plants received the same volume but of distilled water only. Orchids were then left to grow for 12 weeks in a growth chamber, watering once a week indirectly with a constant volume with distilled water. We used eight plants per treatment (24 orchid plants in total).

Evaluation of growth and mycorrhization

We measured number of leaves, number of roots, and shoot and root biomass as a proxy for plant growth. At the end of the experiment, we counted leaves and roots of each plant.

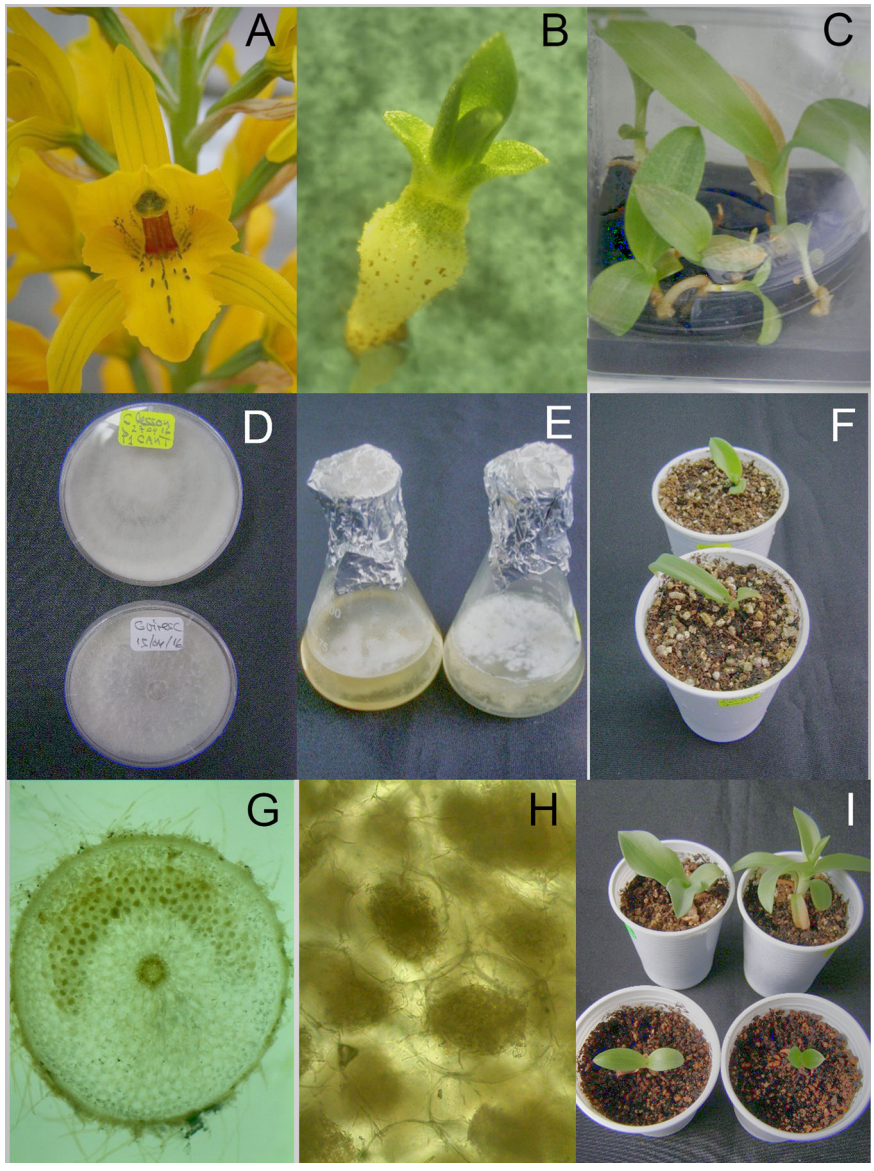

Figure 1. (A) *Chloraea gavilu* flowers. (B) Seed in shoot stage phase. (C) Seedlings of *C. gavilu* in an in vitro culture. (D) OMF colonies. (E) Mycelial growth in liquid culture medium. (F) *C. gavilu* seedlings at transplant to pots and inoculation. (G) Transversal section of a mycorrhized root of *C. gavilu*. (H) Hyphae pelotons inside parenchyma cells. (I) Plants of *C. gavilu* 4 months after inoculation. Control plants correspond to the two pots at the bottom and inoculated plants (with *Ceratobasidium CI*) correspond to the two upper pots.

Then plants were cut and roots and shoots were separated and dried in a stove (Memmert, model BE-400) at 60 °C for 48 h. Plants roots and shoots were then weighted with an analytical scale (RADWAG®, USA) and the dry weight of aerial and subterranean portions was determined. In all cases, constant weight was achieved after 48 h, thus assuring correct measurements of dry weight.

We also determined the mycorrhization level in each orchid. This was done by randomly sampling three roots from each individual. Roots were transversally cut at the middle and base and checked for presence of pelotons inside parenchyma cells (Rasmussen and Whigham 2002; Bertolini et al. 2012). We also measured the area percentage of the cross section with fungal presence inside plant cells. Both parameters (presence/absence of pelotons and mycorrhizated area) were estimated using a high-resolution scope (Olympus SZ2-ILST).

Data analysis

The software Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analyses. Mycorrhizal percentage was arcsine-transformed to normalize the data. A one-way ANOVA with inoculation as a fixed factor was used to determine differences in mycorrhization percentage between treatments followed by a posteriori Tukey test.

Results

Three months after inoculation of *C. gavilu* individuals, pelotons inside root parenchyma cells were observed in plants inoculated with both OMF strains (Figure 1G,H). No pelotons were evidenced in the control treatment. Four months after inoculation plants from inoculated treatments were larger compared to control plants (Figure 2). Plants inoculated with

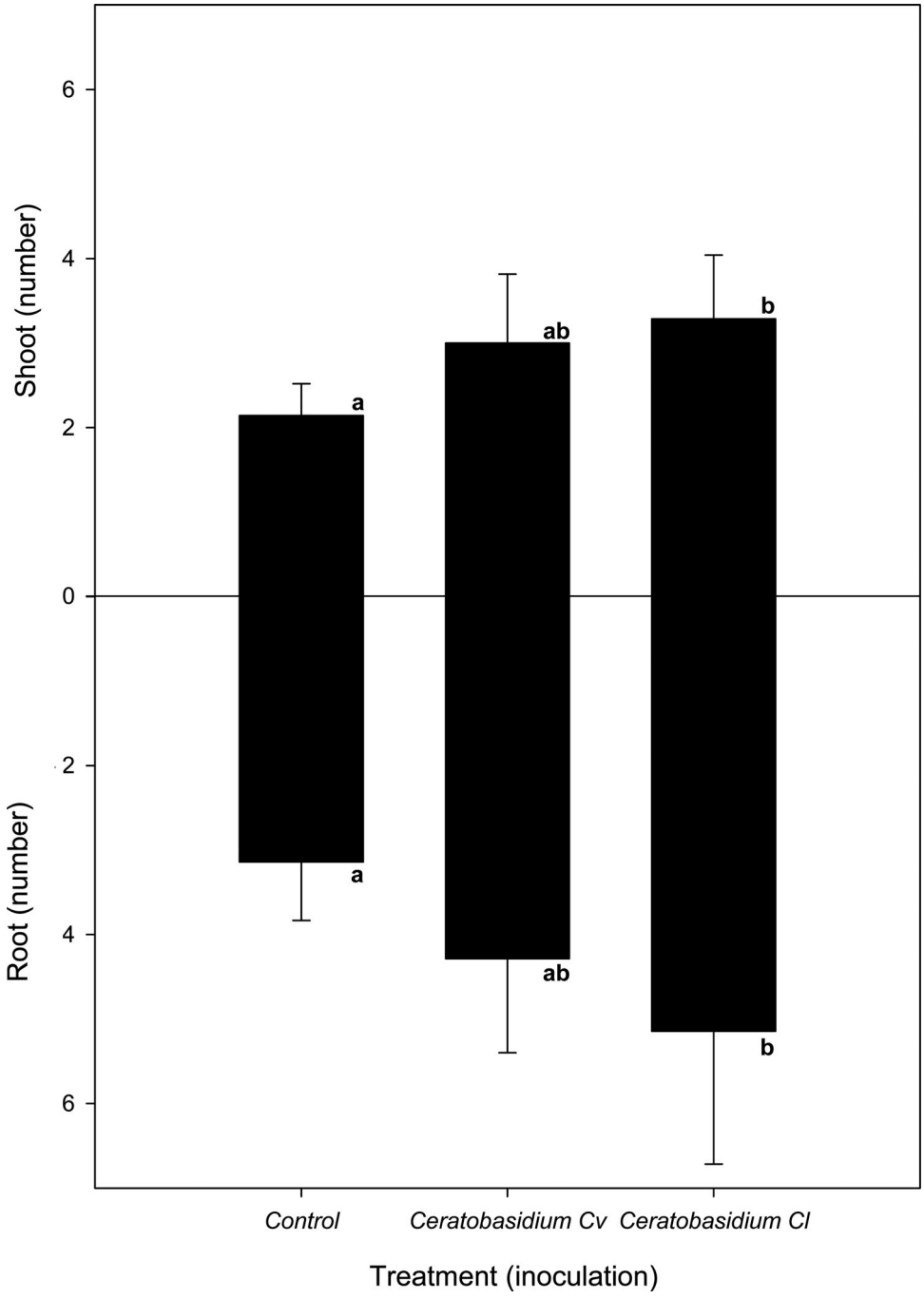

Figure 2. Number of leaves and roots in *C. gavilu* plants four months after inoculation with two OMF and in not-inoculated (control) plants. Different letters correspond to statistical differences (*t*-test, $p \leq 0.05$). Data shown correspond to average ± standard error. Sample size = 7 plants per treatment.

the *Ceratobasidium Cl* strain had more leaves and roots compared to control plants (Figure 2, Tukey test, $p < 0.05$). Plants inoculated with the *Ceratobasidium Cv* strain did not statistically differ in leaf and root number with the other two treatments (Figure 2, Tukey test, $p > 0.05$). Survival was unaffected by inoculation and reached 100% in all treatments. Additionally, inoculated plants (both strains) had higher root and shoot biomass compared to control plants (Figure 3, Tukey test, $p < 0.05$), but the root/shoot ratio did not statistically differ between treatments (Mann-Whitney test, $p > 0.05$). There were no differences in biomass between both inoculated treatments (Figure 3, Tukey test, $p > 0.05$). Mycorrhization was higher in plants inoculated with strain *Ceratobasidium Cl* compared to plants inoculated with strain *Ceratobasidium Cv* (Figure 4, Tukey test, $p < 0.05$). No mycorrhization was evident in control plants (Figure 4).

Discussion

Our study describes for the first time a successful inoculation of an endemic Chilean orchid, obtained by asymbiotic seed germination, providing new evidences about the beneficial effect of the mycorrhization by the improvement in growth, survival, and fitness of plants during ex vitro acclimation. A better understanding of germination and seedling establishment is needed for conservation of orchid populations

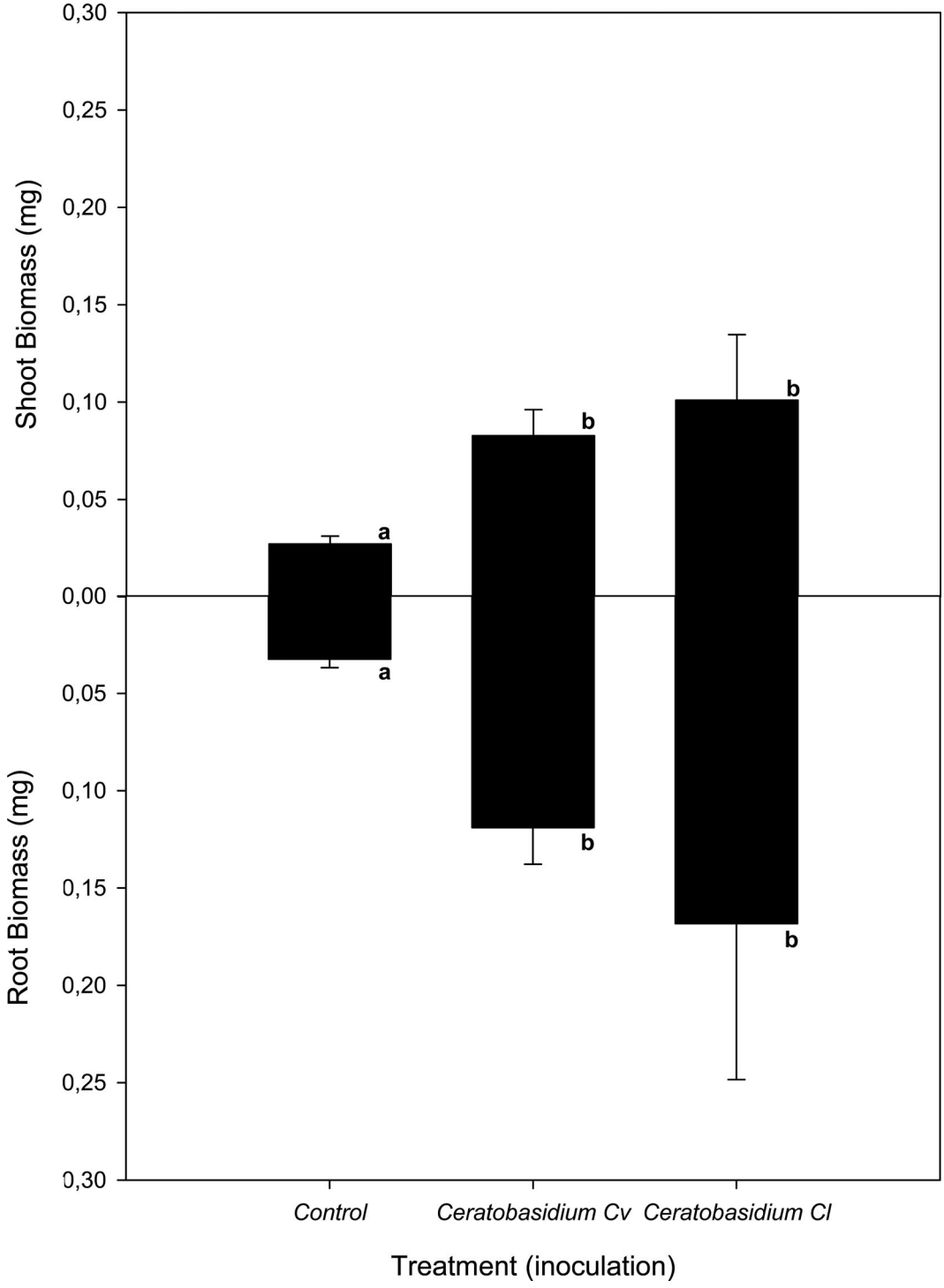

Figure 3. Shoot and root biomass of *C. gavilu* plants four months after inoculation with two OMF and in not-inoculated (control) plants. Different letters correspond to statistical differences (*t*-test, $p \leq 0.05$). Data shown correspond to average ± standard error. Sample size = 7 plants per treatment.

(Swarts and Dixon 2009; Rasmussen et al. 2015). Symbiotic germination of seed occur naturally in the field and has been used in laboratory germination assays (i.e., Herrera et al. 2017). Along with natural substrate modification experiments, in vitro germination can contribute to the understanding of the specific requirements for germination and embryo growth (Thakur and Dongarwar 2013). Asymbiotic seed germination (i.e., without fungal inoculation), as used here, has been shown to be a proper tool for the production of plantlets of several orchid species for commercial and conservation purposes (Yamazaki and Miyoshi 2006; Dutra et al. 2008; Kauth et al. 2006, 2008; Stewart and Kane 2010; Pereira et al. 2015, 2017). However, when reintroduced into the field, seedlings obtained via asymbiotic germination have lower survival rates than seedlings obtained via symbiotic germination (Guimarães et al. 2013), suggesting the need for mycorrhizal symbiosis. 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

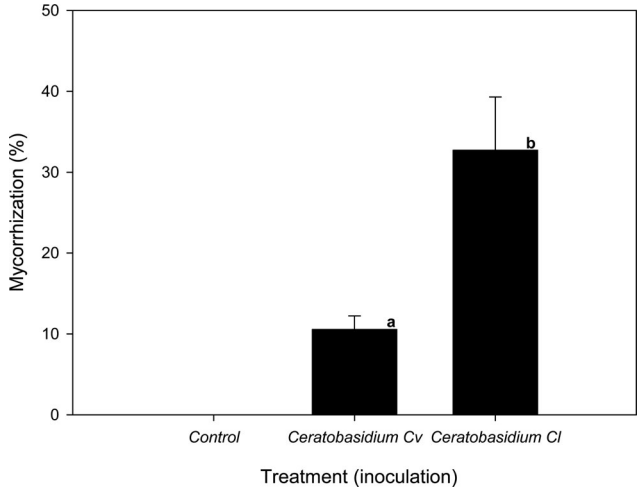

Figure 4. Mycorrhization percentage in *C. gavilu* plants four months after inoculation with two OMF and in not-inoculated (control) plants. Different letters correspond to statistical differences (*t*-test, $p \leq 0.05$). Data shown correspond to average ± standard error. Sample size = 7 plants per treatment.

mycorrhizal fungi to obtain nutritional compounds at early developmental stages (Smith 1966; Herrera et al. 2019). While many species can be propagated asymbiotically in vitro from seeds, or vegetatively from plant explants, the presence of fungal mycorrhizae is likely to enhance orchid plant hardening and establishment success in reintroduction programs in the field (Otero et al. 2013).

Individuals of the terrestrial orchid *Chloraea gavilu,* produced asymbiotically in vitro, were successfully mycorrhized in laboratory condition using OMF strains isolated from other native orchids. Inoculated (mycorrhized) plants were larger compared to uninoculated plants, similar to previous field studies, where inoculated plants were larger and healthier compared to uninoculated plants, but inoculum increased growth only in combination with soil aeration (Smith et al. 2015). The difference in growth between inoculated and uninoculated plants in our study could be explained by increased photosynthesis in inoculated plants (Fukai et al. 1997). This higher growth could also be attributed to carbon transfer from the peat to the orchid through the OMF in inoculated plants as seen in other studies (Cameron et al. 2006; Herrera et al. 2019).

In our study, the *Ceratobasidium Cl* strain showed the best results in growth and mycorrhization percentage. Previous studies in other orchids such as species of *Vanilla* Plumier ex Mill. have also found positive effects of *Ceratobasidium* inoculation on plant growth (Porras-Alfaro and Bayman 2007; Ordóñez et al. 2012). Other *Ceratobasidium* strains have also been found in other Chilean species such as *Bipinnula* Comm. ex Juss. and *Chloraea* species (Herrera et al. 2017, 2019; Pereira et al. 2018). These fungi seems to be important both to induce symbiotic germination of seeds (Herrera et al., 2017) and as mycorrhizal partners for adults orchids (Pereira et al. 2014). They are found, along some Tulasnellaceae fungi, inside the roots of adult individuals of several Chilean terrestrial orchids (Pereira et al. 2014, 2015; Atala et al. 2015; Herrera et al. 2017, 2019) suggesting a functional role possible in stress tolerance or partial mycoheterotrophy. Terrestrial orchids are particularly vulnerable because of their extreme dependence on co-associated organisms, namely insect pollinators and mycorrhizal fungi, and this may explain—in part—why these plants are often the first organisms to disappear from ecosystems undergoing change (Dixon et al. 2003; Swarts and Dixon 2009). In Chile, the main threat for native terrestrial orchids is habitat loss due to deforestation and clearing for cattle, agriculture, cattle herbivory, and urban development (i.e., Atala et al. 2017). Moreover, many species are endemic to Central Chile (Novoa et al. 2015), the most densely populated area of the country and with higher anthropic impact (Schulz et al. 2011).

The orchid-fungi association of a given orchid species can be characterized by its specificity. A high specificity means a single orchid species associates only with closely related fungi, or even a single fungal species/strain (Otero and Bayman 2009). Examples of high specificity are found in some terrestrial orchids from Australia (Swarts and Dixon 2009; Phillips et al. 2011). On the other hand, a more generalist orchid species (i.e., with low specificity), associate with a phyllogenetically wide range of fungal partners or mycobionts (Otero and Bayman 2009). Many tropical orchid species are somewhat generalists in their mycorrhizal associations (Otero et al. 2002; Otero and Bayman 2009), although some temperate species have also been found with the same strategy (Herrera et al. 2017). However, there is a distinction between ecological and potential specificity (Masuhara and Katsuya 1994; Rasmussen 2002). The first refers to the in situ associations (i.e., in the field), and the latter refers to in vitro associations (i.e., in laboratory conditions). The potential specificity means that the in vitro association with a given fungal species can results in increased seed germination or plant growth (Otero and Bayman 2009). This fungal species may be absent from the orchids' habitat, but it could help in their in vitro propagation nevertheless, as was the case of this study.

The in vitro germination and controlled mycorrhization of orchid species could be a solution for orchid conservation, especially for endangered species. In Chile, some critically endangered species are located in unprotected and highly disturbed sites that have been subjected to pollution and erosion (Atala et al. 2017). It is unknown if the conservation status of these species is partially due to a decline in their mycobionts. Evidence from Australian orchids suggests that other factors, such as pollinators, could be more relevant in determining species' rarity (Phillips et al., 2011). Nevertheless, for any successful conservation or restoration program of orchid species, it is vital to ascertain that the requirements of the focal species are met, not least the factors that limit seed germination and seedling establishment (Rasmussen et al. 2015). If these requirements are unknown, a program involving changes in management or plant relocation may not achieve lasting conservation benefits. Clearly, orchid species that are currently endangered, either from unsustainable extraction for commercialization or from habitat degradation should be prioritized for orchid mycorrhizal studies (Otero et al. 2013). However, effective application of OMF for conservation and commercial purposes requires a considerable amount of a priori study in order to determine which

combination of plant species and fungal partner is most effective (Otero et al. 2013). *C. gavilu*, seems to have low specificity in their mycorrhizal associations since it can successfully associate with fungi isolated from other orchid species (even an orchid from a different genus and tribe), and belonging to two families of fungi, namely Ceratobasidiaceae and Tulasnellaceae. This information can be essential for future propagation initiatives both for conservation and for production of species with a potential economic interest.

Lastly, the mycorrhization and production of adult orchid plants was possible because stock cultures of previously isolated fungi were maintained and kept in the microorganism collection at Universidad de Concepción, Los Ángeles Campus. These type of biological collection can be essential for the future development of biotechnological and industrial applications (Hawksworth 1985; Malik and Claus 1987), as well as in biological conservation and systematics (Hawksworth 1985, 2003). In fact, some fungi isolated from Chilean orchid species have a biocontroller effect on pathogen fungal species like *Rhizoctonia solani* J.G. Kühn (Pereira et al., unpublished data, but see Mosquera-Espinosa et al. 2013; Otero et al. 2013). We show here, that these fungal species maintained in pure cultures could be also used in conservation and propagation of orchid species, possibly also in endangered species of the same genus like *Chloraea disoides* Lindl., critically endangered and endemic to Chile (Novoa et al. 2015). Thus, it is very important to support and maintain biological collections, such as fungal and bacterial collections, and to ensure resources for initiatives oriented at understanding fungal biodiversity.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The present work was funded by VRID, Universidad de Concepción [Project No. 214.418.006-1.0IN]; and Dirección de Investigación de la Pontificia Universidad Católica de Valparaíso [DI-regular 037.446/2015 – DI regular 039.333/2016].

ORCID

Héctor Herrera http://orcid.org/0000-0002-2862-4726
Cristian Atala http://orcid.org/0000-0003-1337-9534

References

Abraham S, Augustine J, Thomas TD. 2012. Asymbiotic seed germination and in vitro conservation of *Coelogyne nervosa* A. Rich. an endemic orchid to Western Ghats. Physiol Mol Biol Plants 18(3):245–251.

Aggarwal S, Zettler LW. 2010. Reintroduction of an endangered terrestrial orchid *Dactylorhiza hatagirea* (D. Don) Soo assisted by symbiotic seed germination: first report from the Indian subcontinent. Nat Sci. 8(10):139–145.

Arditti J. 1992. Fundamentals of orchid biology. New York: John Wiley.

Arditti J, Clements MA, Fast G, Hadley G, Nishimura G, Ernst R. 1982. Orchid seed germination and seedling culture. A manual. In: Arditti J, editor. Orchid biology. Reviews and perspectives II. Ithaca (NY): Cornell University Press; p. 243–370.

Arditti J, Ghani AKA. 2000. Numerical and physical properties of orchid seeds and their biological implications. New Phytol. 145(3):367–421.

Atala C, Muñoz-Tapia L, Pereira G, Romero C, Vargas R, Acuña-Rodríguez IS, Molina-Montenegro MA, Brito E. 2017. The effect of future climate change on the conservation of *Chloraea disoides* Lindl. (Orchidaceae) in Chile. Braz J Bot. 40(1):353–360.

Atala C, Pereira G, Romero C, Muñoz-Tapia L, Vargas R, Suz LM. 2015. Orchidiod fungi of the form-genus *Rhizoctonia* associated with the roots of *Chloraea cuneata* Lindl. From Araucanía, Chile. Gayana Bot. 72(1):145–148.

Batty M, Brundett M. 2001. Orchid conservation techniques manual. In: Canberra, plant science, Kings Park and Botanic Garden. Perth, Western Australia: First International Orchid Conservation Congress Training Course.

Bayman P, Otero JT. 2006. Microbial endophytes of orchid roots. In: Schulz BJE, Boyle C, Sieber TN, editors. Microbial root endophytes. New York (NY): Springer; p. 153–178.

Bernard N. 1899. Sur la germination du *Neottia nidus-avis*. C R Hebd Seances Acad. Sci. 128:1253–1255.

Bertolini V, Damon A, Rojas AN. 2012. Symbiotic germination of three species of epiphytic orchids susceptible to genetic erosion, from Soconusco (Chiapas, Mexico). Eur J Environ Sci. 1(2):60–68.

Burgeff H. 1959. Mycorrhiza of orchids. In: Withner CL, editor. The orchids: A scientific survey. New York (NY): Ronald Press; p. 361–395.

Cameron DD, Leake JR, Read DJ. 2006. Mutualistic mycorrhiza in orchids: Evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. New Phytol. 171(2): 405–416.

Damon A, Aguilar GE, Rivera L, Nikolaeva V, Colegio de la Frontera Sur. Tapachula, Chiapas. 2004. Germinación in vitro de semillas inmaduras de tres especies de orquídeas de la región del Soconusco, Chiapas, México [In vitro germination of immature seeds of three orchid species from the Soconusco region, Chiapas, Mexico]. RCHSH. X(2): 195–203. (in Spanish).

Dearnaley JD, Martos F, Selosse MA. 2012. Orchid mycorrhizas: molecular ecology, physiology, evolution, and conservation aspects. In: Hock B, editor. Fungal associations. 2nd ed. Berlin, Germany: Springer-Verlag; p. 207–230.

Dixon KW. 1991. Seeder/clonal concepts in Western Australian orchids. In: Wells TCE, Willems JH, editors. Population ecology of terrestrial orchids. The Hague, The Netherlands: Academic Publishing; p. 111–123.

Dixon KW, Cribb PJ, Kell SP, Barrett RL. 2003. Orchid conservation. Kota Kinabalu, Sabah: Natural History Publications.

Dutra D, Johnson T, Kauth P, Stewart S, Kane M, Richardson L. 2008. Asymbiotic seed germination, in vitro seedling development, and greenhouse acclimatization of the threatened terrestrial orchid *Bletia purpurea*. Plant Cell Tiss Organ Cult. 94(1):11–21.

Fukai S, Fujiwara K, Okamoto K, Hasegawa A, Goi M. 1997. Effects of red and blue light on germination and protocorm growth of *Calanthe* Satsuma. Lindleyana 12:169–171.

Guimarães FAR, Pereira MC, Da Silva C, Pereira D, Feliciano S, Gomes T, Megumi MC. 2013. Symbiotic propagation of seedlings of *Cyrtopodium glutiniferum* Raddi (Orchidaceae). Acta Bot Bras. 27(3): 590–596.

Hawksworth DL. 1985. Fungus culture collections as a biotechnological resource. Biotechnol Genet Eng Rev. 3:417–453.

Hawksworth DL. 2003. Monitoring and safeguarding fungal resources worldwide: The need for an international collaborative MycoAction Plan. Fungal Divers 13:29–45.

Herrera H, García-Romera I, Meneses C, Pereira G, Arriagada C. 2019. Orchid mycorrhizal interactions on the Pacific Side of the Andes from Chile. A review. J Soil Sci Plant Nutr. 19(1):187–202.

Herrera H, Valadares R, Contreras D, Bashan Y, Arriagada C. 2017. Mycorrhizal compatibility and symbiotic seed germination of orchids from the Coastal Range and Andes in south central Chile. Mycorrhiza 27(3):175–188.

Heywood VH, Brummitt RK, Culham AY, Seberg O. 2007. Flowering plant families of the world. Ontario, Canada: Firefly Books.

Johnson T, Stewart SL, Dutra D, Kane ME, Richardson L. 2007. Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)

preliminary evidence for the symbiotic culture advantage. Plant Cell Tiss Organ Cult. 90(3):313–323.
Kauth PJ, Kane ME, Vendrame WA, Reinhardt-Adams C. 2008. Asymbiotic germination response to photoperiod and nutritional media in six populations of *Calopogon tuberosus* var. tuberosus (Orchidaceae): evidence for ecotypic differentiation. Ann Bot. 102(5):783–793.
Kauth PJ, Vendrame WA, Kane ME. 2006. In vitro seed culture and seedling development of *Calopogon tuberosus*. Plant Cell Tiss Organ Cult. 85(1):91–102.
Knudson L. 1922. Non-symbiotic germination of orchid seed. Bot Gaz. 73(1):1–25.
Knudson L. 1946. A new nutrient solution for germination of orchid seed. Am Orchid Soc Bull. 15:214–217.
Malik KA, Claus D. 1987. Bacterial culture collections: Their importance to biotechnology and microbiology. Biotechnol Genet Eng Rev. 5: 137–198.
Malmgren S. 1996. Orchid propagation: Theory and practice. In: Allen C, editor. North American native terrestrial orchids: Propagation and production. Maryland: North American Native Terrestrial Orchid Conference; p. 63–71.
Masuhara G, Katsuya K. 1994. *In situ* and in vitro specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon.) Ames. var. amoena (M. Beiberstein) Hara (Orchidaceae). New Phytol. 127(4):711–718.
Mosquera-Espinosa AT, Bayman P, Prado GA, Gomez-Carabali A, Otero JT. 2013. The double life of Ceratobasidium: Orchid mycorrhizal fungi and their potential for biocontrol of *Rhizoctonia solani* sheath blight of rice. Mycologia 105(1):141–150.
Murguía GJ, Lee EH. 2007. Manual de producción de orquídeas [Orchid production manual]. Xalapa, Mexico: Universidad Veracruzana. (in Spanish).
Novoa P, Espejo J, Alarcón D, Cisternas M, Domínguez E. 2015. Guía de campo de las orquídeas chilenas, segunda edición ampliada [Field guide of Chilean orchids, enhanced. 2nd ed.]. Concepción, Chile: Ediciones Corporación Chilena de la Madera. (in Spanish).
Ordóñez N, Otero T, Díez M. 2012. Hongos endófitos de orquídeas y su efecto sobre el crecimiento en *Vanilla planofolia* Andrews [Orchids endophyte fungi and their effects on the growth of *Ceratobasidium*]. Acta Agronom. 61(3):282–290. (in Spanish).
Otero JT, Ackerman J, Bayman P. 2002. Diversity and host specificity of endophytic Rhizoctonia-like fungi from tropical orchids. Am J Bot. 89(11):1852–1858.
Otero JT, Bayman P. 2009. Germinación simbiótica y asimbiótica en semillas de orquídeas epífitas [Symbiotic and asymbiotic seed germination in epiphytic orchids. Acta Agronom. 58:270–276. (in Spanish).
Otero J, Mosquera AT, Flanagan NS. 2013. Tropical orchid mycorrhizae: Potential applications in orchid conservation, commercialization, and beyond. Lankesteriana 13(1–2):57–63.
Paudel M, Pradhan S, Pant B. 2013. In vitro seed germination and seedling development of *Esmeralda clarkei* Rchb.F. (Orchidaceae). Plant Tissue Cult Biotechnol. 22(2):107–111.
Pereira G, Albornoz V, Muñoz-Tapia L, Romero C, Atala C. 2015. Asymbiotic germination of *Bipinnula fimbriata* (Orchidaceae) seeds in different culture media. Seed Sci Technol. 43(3):1–11.
Pereira G, Albornoz V, Romero C, Lara S, Sánchez-Olate M, Ríos D, Atala C. 2017. Asymbiotic germination in three *Chloraea* species (Orchidaceae) from Chile. Gayana Bot. 74:1–78.
Pereira G, Romero C, Suz LM, Atala C. 2014. Essential mycorrhizal partners of the endemic Chilean orchids *Chloraea collicensis* and *C. gavilu*. Flora 209(2):95–99.
Pereira OL, Megumi MC, De Lima C, Montandon G. 2005. Isolamento e identificacao de fungos micorrízicos rizoctonióides asociados a três espécies de orquídeas epífitas neotropicais no Brazil [Isolation and identification of Rhizoctonia-like mycorrhizal fungi associated with three neotropical epiphytic orchid species from Brazil]. Rev Bras Ciênc Solo. 29(2):191–197. (in Portuguese).
Phillips RD, Barrett MD, Dixon KW, Hopper SD. 2011. Do mycorrhizal symbioses cause rarity in orchids? J Ecol. 99(3):858–869.
Porras-Alfaro A, Bayman P. 2007. Mycorrhizal fungi of Vanilla: Diversity, specificity and effects on seed germination and plant growth. Mycologia 99(4):510–525.
Rasmussen HN. 2002. Recent developments in the study of orchid mycorrhiza. Plant Soil. 244(1/2):149–163.
Rasmussen HN, Dixon KW, Jersáková J, Těšitelová T. 2015. Germination and seedling establishment in orchids: A complex of requirements. Ann Bot. 116(3):391–402.
Rasmussen HN, Whigham DF. 2002. Phenology of roots and mycorrhiza in five orchid species differing in phototropic strategy. New Phytol. 154(3):797–807.
Schulz J, Cayuela L, Rey-Benayas JM, Schröder B. 2011. Factors influencing vegetation cover change in Mediterranean Central Chile (1975–2008). Appl Veg Sci. 14(4):571–582.
Smith SE. 1966. Physiology and ecology of orchid mycorrhizal fungi with reference to seedling nutrition. New Phytol. 65(4):488–499.
Smith SE, Read DJ. 2008. Mycorrhizal symbiosis. 2nd ed. San Diego (CA): Academic press.
Smith ZF, James EA, Mclean CB. 2015. Experimental reintroduction of the threatened terrestrial orchid *Diuris fragrantissima*. Lankesteriana 7(1–2):377–380.
Stewart SL, Kane ME. 2010. Effects of carbohydrate source on the in vitro asymbiotic seed germination of the terrestrial orchid *Habenaria macroceratitis*. J Plant Nutr. 33(8):1155–1165.
Swarts ND, Dixon KW. 2009. Terrestrial orchid conservation in the age of extinction. Ann Bot. 104(3):543–556.
Thakur U, Dongarwar N. 2013. A new report of in vitro flowering and multiple shooting in a wild epiphytic orchid *Oberonia recurva* Lindl. from asymbiotically germinated seedlings. Plant Know J. 2(3):113–118.
Valadares R, Pereira M, Otero J, Cardoso E. 2012. Narrow fungal mycorrhizal diversity in a population of the Orchid *Coppensis doniana*. Biotropica 44(1):114–122.
Yamazaki J, Miyoshi K. 2006. In vitro asymbiotic germination of immature seed and formation of protocorm by *Cephalanthera falcata* (Orchidaceae). Ann Bot. 98(6):1197–1206.