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ORIGINAL ARTICLE

Mycorrhizal compatibility and symbiotic seed germination of orchids from the Coastal Range and Andes in south central Chile

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Abstract Little is known about *Orchidaceae* plants in Chile and their mycorrhizal associations, a key issue for designing protective actions for endangered species. We investigated root fungi from seven terrestrial orchid species to identify potential mycorrhizal fungi. The main characteristics of *Rhizoctonia*-like fungi were observed under light microscopy, and isolates were identified through PCR-ITS sequencing. Molecular identification of fungal sequences showed a high diversity of fungi colonizing roots. Fungal ability to germinate seeds of different orchids was determined in symbiotic germination tests; 24 fungal groups were isolated, belonging to the genera *Tulasnella*, *Ceratobasidium*, and *Thanatephorus*. Furthermore, dark septate and other endophytic fungi were identified. The high number of *Rhizoctonia*-like fungi obtained from adult orchids from the Coastal mountain range suggests that, after germination, these orchids may complement their nutritional demands through mycoheterotrophy. Nonetheless, beneficial associations with other endophytic fungi may also co-exist. In this study, isolated mycorrhizal fungi had the ability to induce seed germination at different efficiencies and with low specificity. Germin ation rates were low, but protocorms continued to develop for 60 days. A *Tulasnella* sp. isolated from *Chloraea gavilu* was most effective to induce seed germination of different species. The dark septate endophytic (DSE) fungi did not show any effect on seed development; however, their widespread occurrence in some orchids suggests a putative role in plant establishment.

Keywords Orchid mycorrhiza · *Rhizoctonia*-like fungi · Root fungal endophytes · Symbiotic germination

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Introduction

Orchid mycorrhiza are specific orchid-fungal interactions where orchids establish symbiosis with different fungal clades to sustain seed germination and establishment of the orchid (Gebauer and Meyer 2003; Selosse et al. 2004). Waterman and Bidartondo (2008) listed some remarkable facts about orchid biology: (1) the *Orchidaceae* family is one of the largest plant families, with over 700 genera and an estimated 30,000–35,000 species in different habitats (about one-tenth of flowering plants (Dressler 1993; Schultes 1990); (2) orchids depend on mycorrhizal fungi for organic nutrients to germinate their seeds (Smith and Read 2008); and (3) orchids obtain nutrients from these fungi, although little is known about how fungi benefit from orchids (Cameron et al. 2007; Rasmussen 1995).

The ability of orchids to establish a relationship with an appropriate mycorrhizal fungus is necessary to complete their life cycle and enable growth during the early stages of their reproduction and development (Rasmussen and Rasmussen 2009; Smith and Read 2008). Several fungi participate and contribute to various vital processes in orchids: (1) the *Rhizoctonia*-like fungi (RLF) complex, which are the mycorrhizal fungi that are commonly associated with orchids; (2) ectomycorrhizas, which have been associated with green-leaved orchids (putatively autotrophic species) and also with fully mycoheterotrophic forest orchids; (3) wood- or litter-decomposing fungi have been identified as mycorrhizal fungi in a range of fully mycoheterotrophic orchids; and (4) a broad spectrum of other different endophytic fungi without a specific role (Bayman and Otero 2006; Roy et al. 2009; Selosse et al. 2004). Orchid seeds are minuscule and produced in large numbers, each with minimal nutritional reserves (Rasmussen and Rasmussen 2009; Smith and Read 2008). This renders orchids dependent on mycorrhizal fungi for organic nutrient acquisition during the mycoheterotrophic stage, known as protocorm (Sathiyadash et al. 2012). Under natural conditions, the orchid life cycle starts when dust-size seeds are dispersed by several mechanisms. During germination, a compatible mycorrhizal fungus provides carbohydrates to promote seed development (Bonnardeaux et al. 2007; Brundrett 2002). All orchids have pelotons in mycorrhizal roots; these contain hyphae of mycorrhizal fungi that are separated from the root cell cytoplasm by the plasma membrane (Peterson et al. 1996). Pelotons can exchange nutrients between symbionts and are often digested and absorbed by the plant cell, providing mineral nutrients and water as well as carbon (Valadares 2014). The presence of intact and degraded pelotons are 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 (Kuga et al. 2014; Smith and Read 2008). Moreover, mycorrhizal colonization in later plant developmental stages may also include fungal species outside the RLF complex (Bayman and Otero 2006; Hou and Guo 2009).

Orchids grow in various habitats and possess different trophic strategies ranging from epiphytic to terrestrial and from autotrophic to fully mycoheterotrophic, comprising variable degrees of dependence on specific fungal and bacterial endophytes (McCormick et al. 2004; Puente and Bashan 1994). Under natural conditions, all orchids are fully dependent on mycorrhizal fungi for germination (initial mycoheterotrophic stage), a feature that is retained for life in fully mycoheterotrophic orchids (Leake 1994). In partially mycoheterotrophic orchids, the mycobiont supplies essential minerals and organic nutrients until the first green leaf emerges, when the plant can acquire carbon from two sources, i.e., photosynthesis and fungal-derived carbon (Gebauer and Meyer 2003; Merckx 2013). Autotrophic orchids may become less dependent on mycorrhizal associations after the rapid development of the autotrophic apparatus in leaves and photosynthetic roots of the seedlings (McCormick et al. 2004; Shefferson et al. 2008).

In Chile, the family Orchidaceae includes at least 63 species within seven genera (Pereira et al. 2014). All of these orchids are terrestrial and have high root tissue density. We expected that partial mycoheterotrophism would be a key factor to compensate for any deficit in energy metabolism when orchids defoliate. Recently, some fungal strains associated with the orchids *Bipinnula fimbriata*, *Chloraea gavilu*, and *Chloraea collicensis* in Chile have been described (Pereira et al. 2014; Steinfort et al. 2010). However, given the large number of Chilean orchids and the lack of knowledge of their mycorrhizal associations under different environmental conditions, comprehensive studies regarding mycorrhizal status are important for safeguarding endangered species in symbiotic propagation strategies, since propagation of symbiotic plantlets is essential for orchids with narrow mycorrhizal associations (Bidartondo and Read 2008; Swarts et al. 2010; Vendramin et al. 2010). Knowledge of diverse mycorrhizal fungi in Chile will be helpful for orchid propagation and to understand the symbiotic mechanisms underlying the colonization of different ecological niches in coastal mountains and Andean habitats.

The purpose of this study was to isolate and identify mycorrhizal fungi associated with seven Chilean orchids and to test the ability of the isolated fungi to induce seed germination by examining roots of orchids growing in the Maule Region of central-southern Chile, six from the Andes and two from the Coastal Range.

Materials and methods

Study sites and sampling

Samples were collected during the flowering stage (November 2014) in the Maule Region of Chile. Eight locations with high orchid occurrence were selected for this study (Fig. 1). Different populations colonize different areas in the Andes and Coastal Range. At each site, the soil rhizosphere samples were collected (0–20 cm deep) and brought to the laboratory where they were sieved at 2 mm and kept at 4 °C for further analysis. Table 1 shows their main characteristics. Orchid roots occupied the top 10 cm of soil or grew in organic matter accumulated above rocks. To avoid unnecessary damage to orchids, only a 5-cm segment of the active root system of each plant was excised and placed in paper bags.

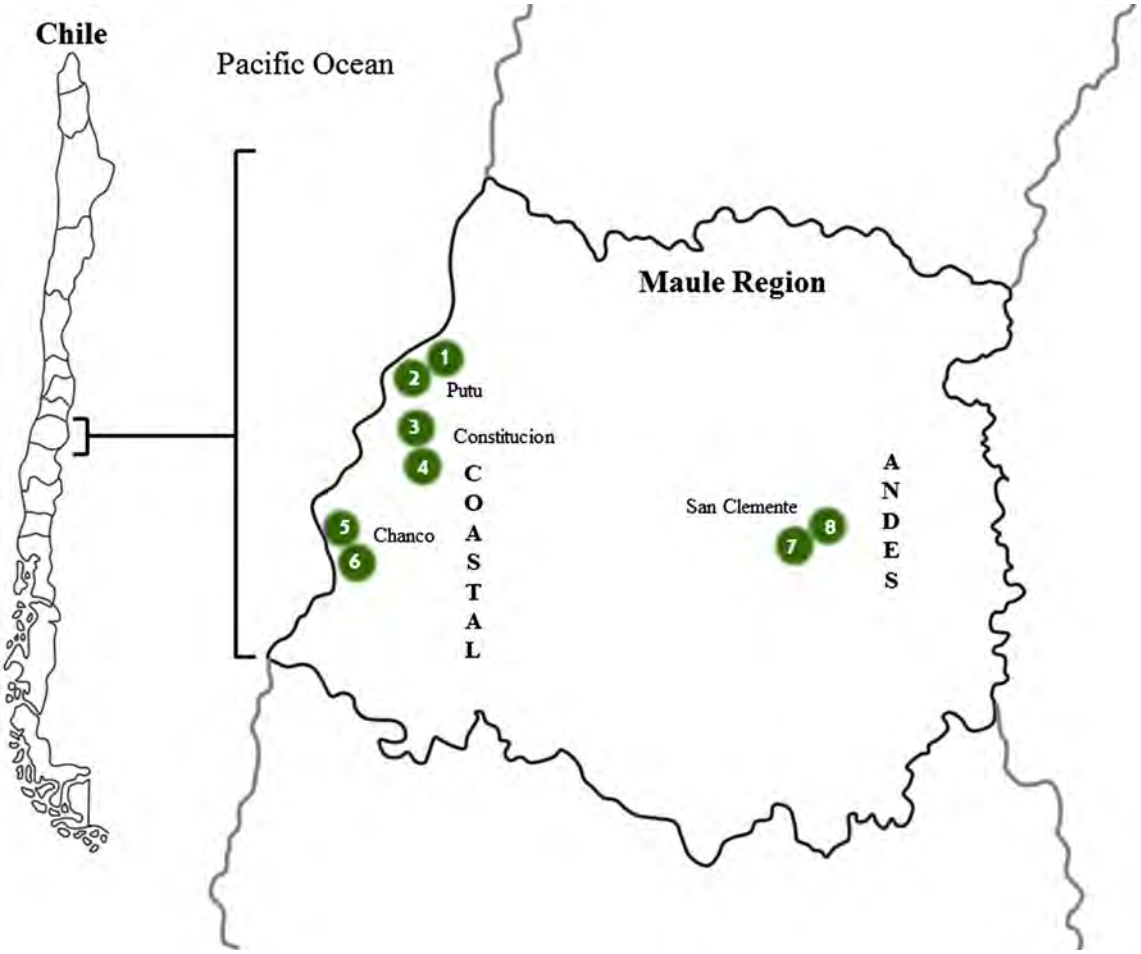


Fig. 1 Sampling sites in the Maule Region in south central Chile. Orchids 1 to 6 were found in the Coastal range, and orchids 7 and 8 were found in the Andes. *Chloraea chrysantha* = *O1*; *Chloraea gavilu* = *O2*; *Chloraea bletioides* = *O3*; *Bipinnula fimbriata* = *O4*; *Chloraea crispa* = *O5*; *Chloraea longipetala* = *O6*; *Chloraea grandiflora* = *O7*; and *Chloraea gavilu* = *O8*

Biochemical characterization of soil rhizosphere

Enzymatic assays were conducted in order to evaluate biochemical microsite differences between sampling locations. Acid phosphatase activity was measured using p-nitrophenyl phosphate (PNPP) as a substrate. β-Glucosidase activity was determined by detection of p-nitrophenol (PNP) released from p-nitrophenyl-β-D-glucopyranoside (PNG). In both assays, the p-nitrophenol formed was determined in a spectrophotometer at 398 nm (Tabatabai and Bremner 1969). Fluorescein diacetate (FDA) hydrolysis was assessed and expressed as micrograms fluorescein released per gram of dry soil (Adam and Duncan 2001). Final FDA concentration was measured as absorbance at 490 nm.

Fungal isolation and characterization

Isolation of fungi was performed according to Valadares et al. (2011), with minor modifications. One day after sampling, root samples were washed in distilled water and cut into 5-cm segments. Fragment surfaces were disinfected by washing for 1 min in 70 % ethanol and 4 min in 20 % sodium hypochlorite solution (0.5 % active chlorine), followed by five washings with sterile water. Fragments were sliced transversely and observed for the presence of pelotons (hyphal coils) under a stereoscopic microscope (×40). Root slices containing pelotons had the velamen (outer cell layer of the root cortex of orchids) removed; the remaining cortical fragments were washed in sterile water. Eight to ten fragments were placed on Petri dishes and incubated at 25 ± 1 °C in potato dextrose agar (PDA) supplemented with streptomycin (100 mg L^{-1}) to isolate orchid mycorrhizal fungi. Roots not containing pelotons were also incubated in PDA to isolate other endophytes unrelated to pelotons.

Critical steps in this isolation technique are the detection of features of orchid mycorrhiza inside living roots by observing pelotons inside brownish zones of roots followed by the observation of RLF characteristics on Petri plates. Fungi with 90° hyphal branching, constrictions close to the branching point, and no spore formation (on PDA media) were classified

Table 1 List of species and locations of orchids sampled in the experiments

Plant species/common name	Label	Location	Growth habitat	Number of sampled plants
Chloraea chrysantha Poepp.[a]/Tulipán del monte (mountain tulip)	O1	Constitucion (35° 49′ 57″ S 72° 18′ 87″ W)	Soil (ultisol)	4
Chloraea gavilu Lindl./ gavilu	O2	Constitucion (35° 49′ 57″ S 72° 18′ 87″ W)	Soil (ultisol)	4
Chloraea bletioides Lindl.[a]/Pico de loro (parrot beak)	O3	Putu (35° 24′ 40″ S 72° 36′ 20″ W)	Organic matter over rocks	4
Bipinnula fimbriata Phil. I. M. Johnst.[a]/ Flor del Bigote (flower mustache)	O4	Putu (35° 24′ 40″ S 72° 36′ 20″ W)	Sand and organic matter over rocks	4
Chloraea crispa Lindl.[a]/no common name	O5	Chanco (35° 70′ 39″ S 72° 44′ 05″ W)	Dune sand	4
Chloraea longipetala Phil.[a]/no common name	O6	Chanco (35° 72′ 48″ S 72° 54′ 49″ W)	Dune sand	4
Chloraea grandiflora Poepp. [b]/gavilu	O7	San Clemente (35° 58′ 77″ S 71° 02′ 79″ W)	Soil (andisol)	4
Chloraea gavilu Lindl.[b]/gavilu	O8	San Clemente (35° 58′ 77″ S 71° 02′ 79″ W)	Soil (andisol)	4

[a] Orchids sampled in the Coastal range

[b] Orchids sampled in the Andean range

as RLF (Otero et al. 2002; Valadares et al. 2011). Colonies were observed daily, and radial growth was measured and expressed in millimeters per day. Each selected fungus was transferred to Petri dishes containing fresh PDA plates and modified oatmeal agar (OMA medium) (4 g oats L^{-1}, 10 g agar, pH 5.6, supplemented with streptomycin 100 mg L^{-1}) for confirmation of RLF (Warcup 1981). Fungi that did not meet these criteria were classified as endophytes.

Plugs of each fungal colony were placed in the center of Petri dishes containing OMA and PDA and incubated at 25 ± 1 °C. The diameter of the growing colony was measured daily. Fungal characteristics were observed at 10 and 20 days of incubation, and color and growth rate were recorded. Growth rate was calculated as the mean difference between daily diameter measurements, expressed in millimeters per day. Fungal species richness was estimated using the Margalef index, $Ri = (S-1)/\ln N$, where S is the number of species in the assemblage, N is the number of isolates, and ln is the natural logarithm (Suryanarayanan and Kumaresan 2000; Yuan et al. 2009). To compare endophyte assemblages inside orchid roots, the Jaccard similarity coefficient was calculated as follows: $Sc = C\,(A + B - C)^{-1}$, where A and B are the total number of fungal species isolated from two orchid species, and C is the number of fungal species common to both orchids; results were expressed as a percentage (Yuan et al. 2009).

Morphological features of isolated fungi were used to describe and classify the fungal diversity and also to select fungal groups to obtain DNA sequences. Microscope slides were placed on a paper towel inside Petri dishes and autoclaved. The paper towel was then moistened with 1 mL of sterilized deionized water and 300 μL of filtered and sterile (0.45 μm) liquid OMA medium spread across the slide. Mycelia plugs of each isolate were incubated on the slides at 28 ± 1 °C for 7 days. Samples were viewed under a fluorescence microscope. Each fungus was treated with 0.01 % lactophenol cotton blue stain (LPCB) (61335, Sigma-Aldrich, St. Louis, MO) as the staining agent and Hoechst dye 1 % solution (33342, Sigma-Aldrich) for 10 min to observe hyphal features. Mycorrhizal infections were estimated (Schatz et al. 2010) using the LPCB staining agent.

Molecular identification of fungal isolates

DNA extraction was performed from liquid cultures of the different isolates, using the DNeasy Plant Mini Kit (69104, Qiagen, Hilden, Germany). Oligonucleotide primers were used to amplify the internal transcribed spacers of the ITS1 region (5′-TCCGTAGGTGAACCTGCGG) and ITS4 region (5′-TCCTCCGCTTATTGATATGC) of genomic rDNA (White et al. 1990). The PCR cycle was initial denaturing at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 1 min each, annealing at 55.5 °C for 1 min, extension at 72 °C for 1 min, and final extension for 5 min at 72 °C. PCR products were purified using the QIAquick PCR purification kit (28104, Qiagen) prior to sequencing. Sequencing was performed by Macrogen (Seoul, South Korea).

Sequence analysis

A BLAST search was conducted to find the closest known sequences with the ITS region of nuclear rDNA. All sequences were deposited in the GenBank (accession numbers KP278146 to KP278169). Multiple sequence alignment was performed and visually checked with the following modifications: fungal sequences were aligned, using ClustalX with default conditions for gap opening and gap extension penalty (Larkin et al. 2007). All positions containing gaps and deletion

data were eliminated from the dataset, using BioEdit software (Hall 1999). Phylogenetic trees were constructed with the neighbor-joining method, using the MEGA 6 software (Chen et al. 2011; Tamura et al. 2013).

Symbiotic germination tests

In order to understand the effect of different isolated mycorrhizal fungi on seed development, a multiple germination test was performed. Seeds were sowed in OMA, and potential specificity was tested for different fungal strains. Prior to the symbiotic seed germination tests, a tetrazolium test was conducted to assess seed viability (Lakon 1949). The surface of 200 μg of seeds from one mature fruit capsule was disinfected according to Dutra et al. (2009), with minor modifications. Seeds were immersed in an 8:1:1 solution of 8 mL of sterile deionized water, 1 mL of sodium hypochlorite (5 % chlorine), and 1 mL of 100 % alcohol for 3 min, followed by five washes in sterile deionized water. The seeds were placed in a suspension of 50 mL of deionized water. Then, 500 mL of this suspension was placed in Petri dishes containing 20 mL of modified OMA medium (4 g oats L^{-1} and 10 g agar at pH 5.6, supplemented with 100 mg L^{-1} streptomycin) and a plug of mycelia for fungal inoculation. A randomized design was used, with each fungal isolate as a treatment, with ten replicates per treatment and a control of ten uninoculated plates. The percentage of germination per plate was measured (Steinfort et al. 2010). Plates were incubated in the dark at 25 ± 1 °C for 8 weeks. Each Petri dish was then analyzed under a microscope (×20–40), and germination stages of 100 seeds per plate were recorded (Stewart and Kane 2006). The growth index was calculated (Valadares et al. 2011) as follows: $GI = (N_1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4)/(N_0 + N_1 + N_2 + N_3 + N_4)$, where N_0 is the number of seeds in stage 0, N_1 is the number of seeds in stage 1, and so on.

Statistical analyses

All data were analyzed using one-way ANOVA to compare each treatment according to measurements of enzyme activity. Prior to statistical analysis, data were tested for normality and homogeneous variances. For the symbiotic germination test, ANOVA was performed between germination stages, where stages 2 and 3 were likely to produce protocorms with the potential to become adult plants, as defined by Mitchell (1989). All statistical tests were conducted using R software (Ihaka and Gentleman 1996). Results were compared using the SD of means and Tukey's multiple range test. Statistical significance was set at $p < 0.05$.

Results

Sampling

Seven orchid species were identified colonizing different soil types (Table 1). Two species came from the Andes and six from the Coastal Range. Specimens were found growing at the ground surface, with their roots penetrating the first few centimeters of soil, sand, or organic matter derived from herbaceous plants on rocks. The chemical and general enzymatic characterizations of the soils are summarized in Supplementary Table S1. In general, soil substrates were very similar, with the exception of phosphorus content, with the highest content in "Constitucion" and "San Clemente" soils (~4-fold compared to the lowest content). Soil enzyme activity was statistically different between soils. The San Clemente soils had the highest enzyme activity and organic matter content. Soil biochemical properties were also statistically different between soils sampled. The San Clemente soils had the highest microbial activity, as reflected in higher rates of FDA hydrolysis, acid phosphatase, and β-glucosidase activity ($p < 0.05$).

Fungal isolation and morphological characterization

All orchid roots evaluated in this study were colonized by mycorrhizal fungi. Estimates of mycorrhizal colonization were dependent on orchid habitat; *Chloraea bletioides* and *B. fimbriata* were found growing on rocky substrates, showing the highest mycorrhizal infection (50 ± 8 %), with higher frequencies of degraded pelotons throughout the cortical region. There were fewer pelotons and few brownish root zones (a sign of mycorrhization) in orchid fragments growing in loose soil. In both cases, colonized root fragments were easily distinguished from non-colonized fragments (Fig. 2). We made a preliminary classification of isolated fungi as RLF (mycorrhizal fungi) and endophytes, mainly based on morphological features, such as colony and hyphal characteristics and absence of spore formation, as seen in Fig. 2 (Valadares et al. 2011). At least one RLF was isolated from each orchid, and a variable number of endophytes were found (Table 2). In total, 224 mycobionts were isolated, of which 96 isolates correspond to the RLF complex (isolates ORK1 to ORK13), whereas 128 were classified as endophytes and other unidentified fungi (isolates ORK14 to ORK24). Margalef's diversity index of endophytic fungi in the eight orchids was significantly greater in *Chloraea longipetala* (1.55) and *C. gavilu* (1.41) from the San Clemente soil and was lowest in *Chloraea chrysantha* (0.69) and *C. gavilu* (0.62) from the Constitucion soil (Table 2). Based on morphological characteristics, orchid source, and growth rate on PDA and OMA media, 24 groups of fungal isolates were formed (see

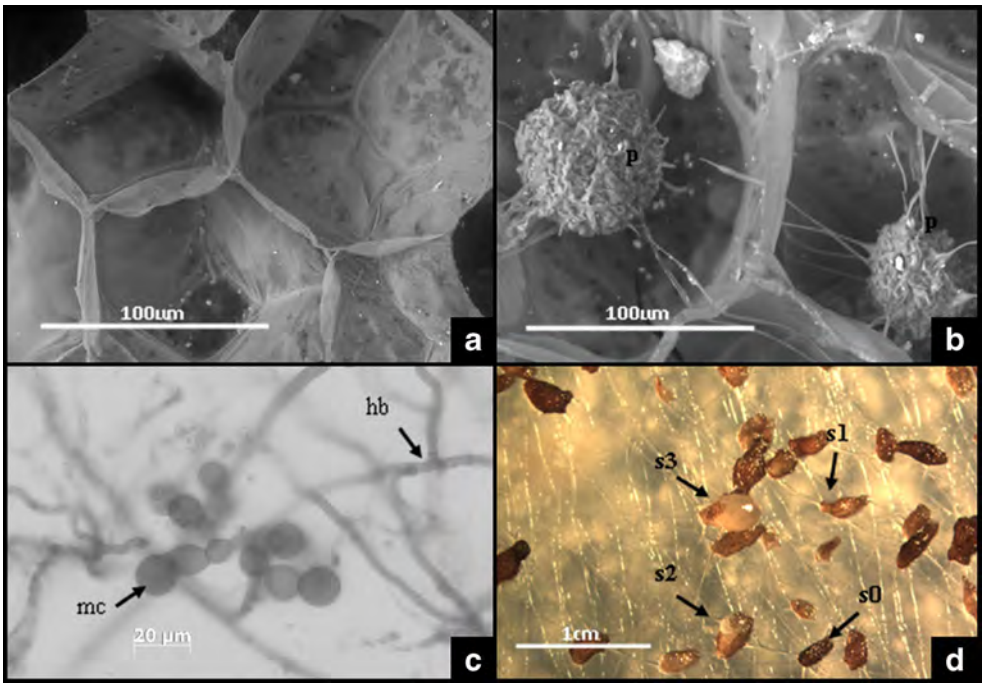


Fig. 2 **a** *Chloraea grandiflora* roots not colonized; **b** colonized by mycorrhizal fungi, showing pelotons (*p*); **c** monilioid cells of *Tulasnella* sp. growing in PDA medium (*hb* 90° hyphal branching, *mc* monilioid cells); and **d** symbiotic seed germination of *Chloraea chrysantha* seeds showing different development stages after 6 weeks in OMA medium (*s0* = stage 0, *s1* = stage 1, *s2* = stage 2, *s3* = stage 3)

Supplementary Table S2). After 10 and 20 days of incubation at 25 °C on PDA and OMA media, fungal colonies had either black, white, cream, or brown colors. The technique we used to isolate RLF was very successful. Without prior identification of colonized root segments and pelotons, a relatively large proportion of endophytes may be isolated from the orchid roots. The composition of the endophytic fungal community varied according to the orchid species, with different similarity coefficients even at the same sampling location (see Supplementary Table S3).

Molecular identification and phylogenetic analyses

BLAST searches are summarized in Table 3. According to phylogenetic analyses, fungi colonizing particular orchids are not clustered (Fig. 3). However, among orchid mycorrhizal fungi, two clades were observed (orders *Tulasnellales* and *Ceratobasidiales*). Isolate ORK6 showed high homology with a *Tulasnellaceae* isolated from *Gavilea australis*, a genus with 17 species of Chilean orchids (Novoa et al. 2015). Sequence identities ranged from 95 to 99 %; the species were accepted when identity between query and match was >99 %, and the genus was accepted when >95 % (Chen et al. 2011; Sánchez Márquez et al. 2008). According to morphological and molecular data, 13 different fungal sequences corresponding to the RLF complex and 11 different endophyte sequences were identified, with three matching with dark septate endophytes (DSE) (*Leptotidium orchidicola*, *Cadophora* sp., and *Phialocephala fortinii*).

For *Rhizoctonia*-like fungi, ITS sequences of isolates ORK1 and ORK2 (from *Chloraea crispa* and *C. longipetala*, respectively) were classified as *Ceratobasidium* spp., based on their closest match in the GenBank database. Similarly, the sequence of isolate ORK3 (from *C. longipetala*) was classified as *Thanatephorus* sp. Sequences from isolates ORK4 to ORK13 (from *C. crispa*, *B. fimbriata*, *C. gavilu*, *C. chrysantha*, *Chloraea grandiflora*, *C. bletioides*, and *C. longipetala*) were of variable homology with different fungi in the order *Tulasnellales* (Table 3, references therein). We also found other fungal endophytes in cortical tissues, especially from the orchids of the Andes (52 isolates). ITS sequences of isolates ORK 14 and ORK 16 (from *C. longipetala* and *C. crispa*, respectively) were classified as *Phomopsis columnaris*. Sequences ORK17 and ORK18 (from *C. gavilu* and *C. grandiflora*) were classified as *Leptodontidium* spp. Sequence ORK19 (from *C. grandiflora*) was classified as *Cadophora* sp. Sequences ORK20 and ORK 21 (from *C. chrysantha* and *C. bletioides*, respectively) were classified as *Chaetomium* sp. and *Chaetomium globosum*, respectively. Sequence ORK24 was classified as *P. fortinii*. Based on their low homology, sequences ORK15, ORK22, and ORK23 were designated as unidentified fungi (Chen et al. 2011). All information regarding molecular identification of fungal isolates is shown in Table 3.

Potential of mycorrhizal fungi to induce seed germination

To test the specificity of the mycorrhizal interactions, a multiple germination test was used over 8 weeks. The tetrazolium test revealed variable seed viability, with *B. fimbriata* having the highest viability (65 %) and *C. longipetala* having the

Table 2 Occurrence of the different fungi inside the roots of orchids in the Coastal Range and Andes in south central Chile

Source/host	*Thanetophorus* sp.	*Ceratobasidium* sp.	*Tulasnella* sp.	*Phomopsis columnaris*	Unidentified 1	*Leptodontidium* sp.	*Cadophora* sp.	*Chaetomium* sp.	Unidentified 2	*Phialocephala fortinii*	Margalef's richness index
Coastal Range											
Chloraea chrysantha	–	–	13	–	–	2	–	3	–	–	0.692
Chloraea gavilu	–	–	15	–	6	4	–	–	–	–	0.621
Chloraea bletioides	1	–	20	–	12	–	2	6	5	–	1.306
Bipinnula fimbriata	2	5	8	–	–	1	–	–	–	–	1.082
Chloraea crispa	–	–	3	15	–	1	–	–	–	4	0.957
Chloraea longipetala	1	3	6	10	–	3	–	–	–	2	1.553
Frequency[a]	0.026	0.052	0.425	0.163	0.118	0.072	0.013	0.059	0.033	0.039	
Andes											
Chloraea gavilu	–	–	11	1	2	9	10	–	–	2	1.406
Chloraea grandiflora	–	–	8	3	–	14	4	–	2	5	1.395
Frequency[a]	0	0	0.268	0.056	0.028	0.324	0.197	0	0.028	0.099	
Number of isolates	4	8	84	29	20	34	16	9	7	13	
									Total		224

[a] Estimated frequency of isolation of each fungal species in the Coastal Range and the Andean range

Table 3 Molecular identification of potential mycorrhizal fungi isolated from orchids in the Coastal Range and Andes in south central Chile based on the closest match in the GenBank database

Fungal isolate	GenBank Accession number	Host	Close relatives (accession number)	% identity	Source	Reference
Rhizoctonia-like fungi						
ORK1	KP278168	*Chloraea crispa*	*Ceratobasidium* sp. (HQ914092)	99	*Sarcochilus parviflorus*	(Gowland et al. 2013)
ORK2	KP278169	*Chloraea longipetala*	*Ceratobasidium* sp. (HQ914091)	99	*Sarcochilus hillii*	(Gowland et al. 2013)
ORK3	KP278146	*Chloraea longipetala*	*Thanetophorus* sp. (KJ777649)	97	Unspecified	Genbank
ORK4	KP278147	*Chloraea longipetala*	Uncultured *Tulasnellaceae* (JX545220)	99	*Dendrobium officinale*	(Xing et al. 2013)
ORK5	KP278148	*Bipinnula fimbriata*	Uncultured *Tulasnellaceae* (JX545220)	98	*Dendrobium officinale*	(Xing et al. 2013)
ORK6	KP278149	*Chloraea gavilu*	*Tulasnella* sp. (KJ713701)	99	*Gavilea australis*	(Fracchia et al. 2014a)
ORK7	KP278150	*Chloraea gavilu*	*Uncultured Tulasnellaceae* (JX649080)	99	*Anacamptis morio*	(Bailarote et al. 2012)
ORK8	KP278151	*Chloraea chrysantha*	*Tulasnella* sp. (KJ713701)	99	*Gavilea australis*	(Fracchia et al. 2014a)
ORK9	KP278152	*Chloraea grandiflora*	Uncultured *Tulasnellaceae* (JQ994398)	99	*Piperia yadonii*	(Pandey et al. 2013)
ORK10	KP278153	*Chloraea bletioides*	Uncultured *Tulasnellaceae* (JX649082)	98	*Anacamptis morio*	(Bailarote et al. 2012)
ORK11	KP278154	*Chloraea crispa*	Uncultured *Tulasnellaceae* (JQ994398)	97	*Piperia yadonii*	(Pandey et al. 2013)
ORK12	KP278155	*Chloraea longipetala*	Uncultured *Tulasnellaceae* (JQ994397)	97	*Piperia yadonii*	(Pandey et al. 2013)
ORK13	KP278156	*Chloraea gavilu*	*Tulasnellaceae* sp. (JX138565)	96	*Diuris magnifica*	(Sommer et al. 2012)
Endophytes						
ORK14	KP278157	*Chloraea longipetala*	*Phomopsis columnaris* (KM519653)	99	Olive cultivar *Cobrancosa*	Genbank
ORK15	KP278158	*Chloraea bletioides*	*Catenulostroma germanicum* (EU019253)	95	Stone	(Crous et al. 2007)
ORK16	KP278159	*Chloraea crispa*	*Phomopsis columnaris* (KM519653)	99	Olive cultivar *Cobrancosa*	Genbank
ORK17	KP278160	*Chloraea gavilu*	*Leptodontidium orchidicola* (AF486133)	97	*Platanthera hyperborean*	Genbank
ORK18	KP278161	*Chloraea grandiflora*	*Leptodontidium orchidicola* (GU479910)	97	*Trillium tschonoskii*	Genbank
ORK19	KP278162	*Chloraea grandiflora*	*Cadophora sp.* (JN859252)	99	*Juniperus communis*	(Knapp et al. 2012)
ORK20	KP278163	*Chloraea chrysantha*	*Chaetomium globosum* (JQ964802)	97	Unspecified	Genbank
ORK21	KP278164	*Chloraea bletioides*	*Chaetomium globosum* (JN209870)	99	Unspecified	Genbank
ORK22	KP278165	*Chloraea bletioides*	*Catenulostroma germanicum* (EU019253)	95	Stone	(Crous et al. 2007)
ORK23	KP278166	*Chloraea gavilu*	*Penicillium chrysogenum* (JF834167)	95	Sea sediments	Genbank
ORK24	KP278167	*Chloraea gavilu*	*Phialocephala fortinii* (KJ817297)	99	*Vaccinium vitis*	Genbank

lowest (45 %). Results of the germination test for the seven fungal isolates are shown in Fig. 4. All isolated RLF fungi have the potential to promote seed germination to varying degrees, even when the fungus does not come from the same orchid species. The overall germination index was greatest for the ORK13 strain (see Supplementary Table S4). This fungus

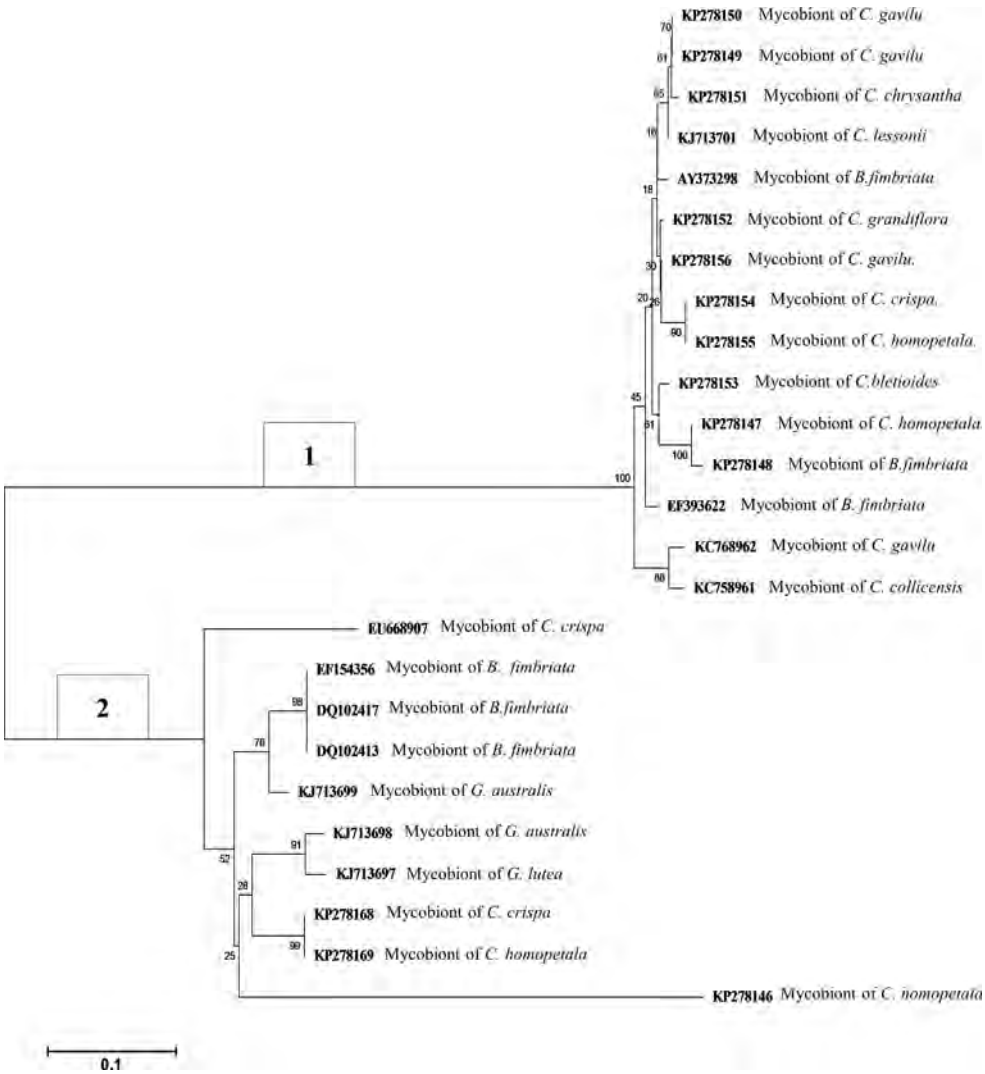
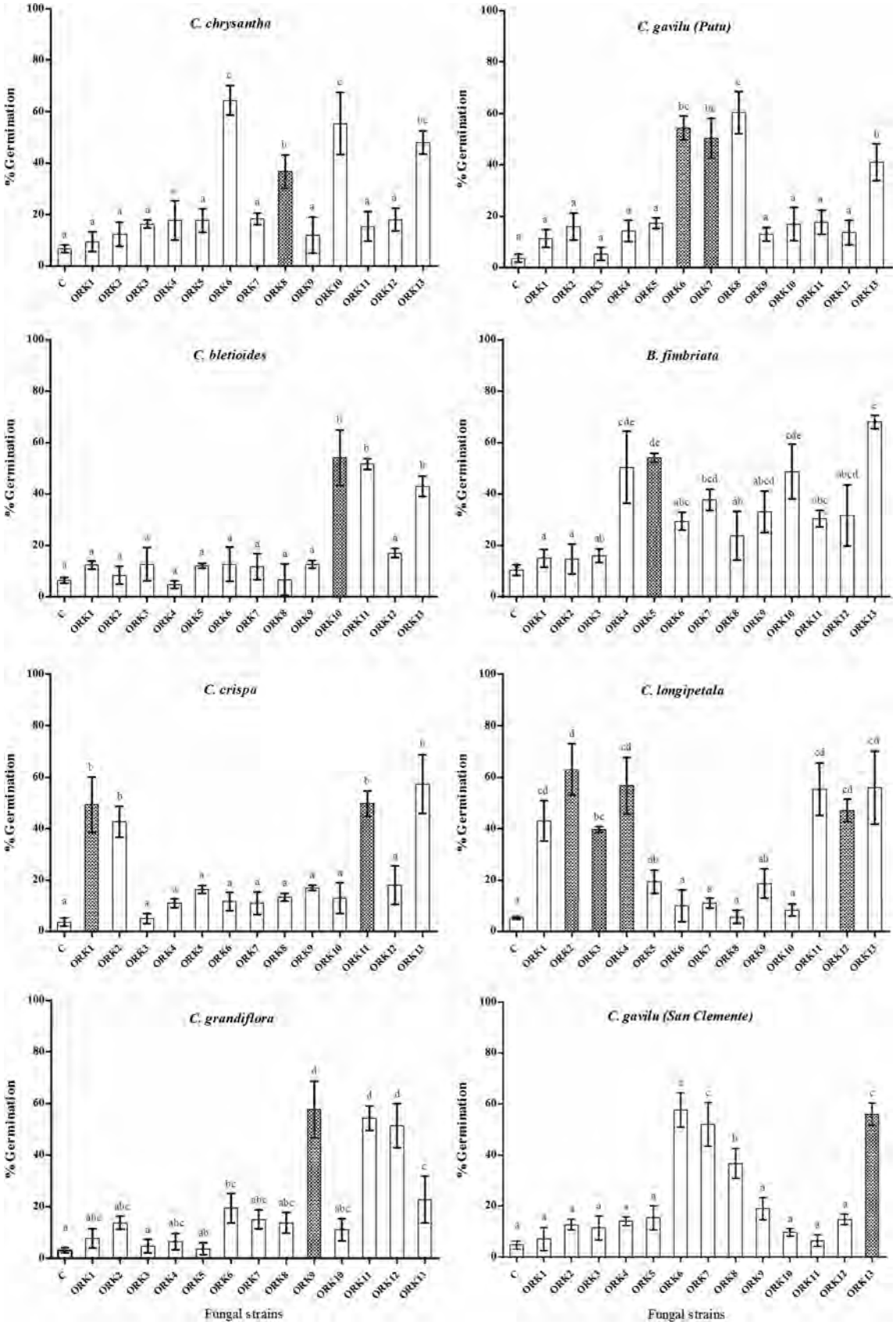


Fig. 3 Maximum likelihood tree of ITS sequences of *Rhizoctonia*-like isolates, showing relationships in terms of distance. *Numbers near branches* refer to bootstrap support values. *Numbers in boxes* indicate principal clades (*1* = order *Tulasnellales*; *2* = order *Ceratobasidiales*). The tree also includes fungal sequences isolated from related orchids

can promote sprouting to further stages of development with very low specificity to orchid species. In the germination test, there were no significant differences among fungal isolates in the first stages of germination ($p < 0.05$). However, when later stages were analyzed, significant differences were found (Fig. 4). The potential specificity (potential of mycorrhizal fungi to induce germination of other orchid species) was highly variable. The seeds of *B. fimbriata* did not exhibit any specificity for mycorrhizal fungi; this is reflected in its high germination index (see Supplementary Table S4). Germination of *C. gavilu* and *C. grandiflora* was more stimulated by specific mycorrhizal fungi (Fig. 4). In control treatments, we observed that some embryos may break the seed coat similar to inoculated treatments; nevertheless, no further development occurred. Additionally, the effect of the different endophytic strains (including dark septate endophytes) was assessed, but no evidence of statistically significant differences were observed, resulting in minimal development and many decayed embryos.

Discussion

Growth of orchids in an ecosystem depends on environmental factors, including temperature, soil type, competition, pollination, and the presence of mycorrhizal fungi (Gregg and Kéry 2006; Huber et al. 2005; Swarts et al. 2010). Mycorrhizal fungi are essential for successful symbiotic development of orchids under natural conditions, to reintroduce endangered species to their natural environment, and to cultivate species of ornamental and medical interest (Chen et al. 2012; Nontachaiyapoom et al. 2010; Pereira et al. 2003). Roots of most green orchids host a variety of fungi, mainly including species of *Tulasnella*, *Ceratobasidium*, *Sebacina*, and *Thanatephorus* (Sneh et al. 1991; Smith and Read 2008). In our study, the most common mycobiont was *Tulasnella* spp., which have been reported as commonly associated with terrestrial orchids (Ogura-Tsujita et al. 2012). Furthermore, we identified strains of *Ceratobasidium* and *Thanatephorus*, described as typical mycorrhizal fungi in terrestrial orchids (Steinfort et al. 2010; Valadares et al. 2011).


C. chrysantha
C. gavilu (Putu)
C. bletioides
B. fimbriata
C. crispa
C. longipetala
C. grandiflora
C. gavilu (San Clemente)
% Germination
Fungal strains
C
ORK1
ORK2
ORK3
ORK4
ORK5
ORK6
ORK7
ORK8
ORK9
ORK10
ORK11
ORK12
ORK13

◀ **Fig. 4** Germination assay showing effects of isolated fungi in protocorm development of orchid seeds in OMA medium. *Same letters* in each subfigure are not significantly different according to Tukey's multiple range test ($p < 0.05$). *Shaded bars* show fungi isolated from the same orchid. ORK1 and ORK2 are *Ceratobasidium* spp., ORK3 is *Thanetophorus* sp., and ORK4 to ORK13 are *Tulasnella* spp.

The degree of specificity between orchids and their mycorrhizal fungi is highly variable and is influenced by environmental conditions and surrounding plant species (Otero et al. 2011; Selosse and Roy 2009; Waterman and Bidartondo 2008). Fungi associated with *C. gavilu* differed in the two sampling areas. This was also determined in other studies that show mycorrhizal preferences according to different habitats and even individual orchids, which can have more than one mycorrhizal fungus inside their roots (Jacquemyn et al. 2014; Jacquemyn et al. 2010; McCormick et al. 2004; Shefferson et al. 2008). The form of fungal associations varies considerably, from broad, with orchids that are compatible with several fungal groups (*C. longipetala*), to narrow, with orchids that are compatible with only a few groups of fungi (*C. gavilu* from the Coastal Range of Chile). Similar results are reported by Steinfort et al. (2010), who identified at least five different mycorrhizal fungi colonizing roots in *B. fimbriata*, and by Pereira et al. (2014), who identified two fungi colonizing roots of *C. collicensis* and *C. gavilu*. It is crucial to perform germination tests in the classification of mycorrhizal fungi, using isolated fungi that promote germination under in vitro conditions (Shimura and Koda 2005; Tešitelová et al. 2012; Valadares et al. 2011).

Symbiotic and asymbiotic seed propagation have been tested in the Chilean orchid *B. fimbriata* in order to find effective laboratory strategies to preserve this endemic orchid. These reports do not include orchids from the *Chloraea* genus (Pereira et al. 2015; Steinfort et al. 2010). Our study demonstrates that all isolated mycorrhizal fungi (RLF) have the ability to induce seed germination and orchid development to different degrees with the presence of protomeristem and rhizoids, especially species of *Tulasnella* and *Ceratobasidium*. Terrestrial orchids associated with fungi from the polyphyletic *Rhizoctonia* group are effective in promoting seed development (Batty et al. 2006; Valadares et al. 2011). In this case, the strain that is closely related to *Thanatephorus* was not able to induce rhizoid formation, but the presence of a protomeristem is an indication of seed development. Although most mycorrhizal fungi were able to induce seed germination up to the third phase of development, *Tulasnella* ORK13 was the only strain with the ability to induce seed development and protocorm growth in order to promote later developmental stages; thus, it is a candidate for conservation and reintroduction programs.

On the other hand, our results have shown that some seeds continue to develop in the control media (OMA without mycorrhizal fungus), a process previously described by Fracchia et al. (2014a). These authors suggest that some embryos have the ability to develop without specific nutrients; however, these seeds are not able to continue protocorm development. Our experiments have shown that all orchids are able to form associations that differ under laboratory conditions, allowing fungi to promote seed germination in orchids that are not associated with the target orchid in nature (Esitken et al. 2005).

In our germination tests, the slow growth rate from seed to protocorm may be attributed to the physiological status of the orchids used as sources of mycorrhizal fungi, since we used colonized roots of adult plants rather than orchids at young seedling stages. Despite the fact that isolating fungi from adult orchids is considered to be a successful strategy to study mycorrhizal diversity in terrestrial orchids (Jiang et al. 2015; Steinfort et al. 2010; Valadares et al. 2011), using fungi from protocorms (young seedling stages) is often more suitable to study seed germination (Rasmussen and Whigham 1993). In our experiments, seeds start to break the testa after 30 days in darkness, a finding that is inconsistent with the shorter times reported for other related orchids (Fracchia et al. 2014a, b). This observation suggests a possible fungal switch under natural conditions, with mycorrhizal fungal lineages varying in different plant development stages (Masuhara and Katsuya 1994; McCormick et al. 2006). However, orchid seed development using fungi isolated from adult roots slowly continues. Mycobionts are candidates to be considered in recovery programs due to their ability to germinate orchid seeds without fungal switch after the protocorm stage, especially the isolate *Tulasnella* ORK13, which was identified as the most effective mycorrhizal fungus due to its ability to promote seed germination nonspecifically until later developmental stages.

Endophytic growth of non-mycorrhizal fungi has often been reported in orchids, but little attention has been given to whether this has a beneficial or detrimental effect on orchid metabolism (Bayman and Otero 2006; Chen et al. 2011; Yuan et al. 2009). We observed that at least five fungal strains belonged to the genera of non-orchid mycorrhizal fungi, particularly fungi isolated from *C. grandiflora* and *C. gavilu* from the Andes. Among terrestrial orchids, different DSE have been reported to promote seed germination or establish pelotons after inoculation of asymbiotic germinated plantlets (Fracchia et al. 2014a; Hou and Guo 2009). The abundance of several plant species in the same habitat as well as the climatic conditions that are characteristic of the Andean region promote the establishment of plant linkages through fungal mycelium, as shown in mycoheterotrophic orchids (Bidartondo et al. 2004; Dearnaley 2007; McKendrick et al. 2000; Simard and Durall 2004). Nonetheless, the role of non-mycorrhizal fungi in orchid development has not been explored.

This is the first study on the Pacific side of the Andes to identify fungi belonging to the polyphyletic *Rhizoctonia* group and root fungal endophytes. We were able to demonstrate that seed germination is promoted by mycorrhizal fungi associated with terrestrial orchids in a section of two mountain ranges in south central Chile.

Conclusions

The terrestrial orchids under study did not have a preference for a specific mycorrhizal fungus, showing associations ranging from typical orchid mycorrhizae to endophytes, with the fungal order *Tulasnellales* demonstrated as the most common mycorrhizal symbionts and *Leptodontidium* sp. as the most common fungal endophyte. It was possible to promote development of orchid seeds with different mycorrhizal fungi which have been shown to contribute to plant fitness. Many of the isolated dark septate fungal clades have been reported, but their widespread occurrence in orchid radical tissues from the Andes deserves more attention.

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