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Associated fungi, symbiotic germination and *in vitro* seedling development of the rare Andean terrestrial orchid *Chloraea riojana*

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ABSTRACT

Chloraea riojana is a rare terrestrial orchid from the Andean region of Northwest Argentina. Here we evaluate the mycorrhizal status, symbiotic germination and protocorm development of the species. Plant material including capsules, roots and whole plant were collected from two natural populations. Symbiotic germination was tested using one fungal endophyte isolated from *C. riojana* and four fungal endophytes previously isolated from the sympatric orchid species *Aa achalensis*. Three isolates were able to promote seed germination and further development into plantlets with 1–2 leaves after 16 weeks of *in vitro* dual culture. Moreover, the strain isolated from *C. riojana* developed intense melanized coilings and pelotons inside protocorms, a rare feature not observed before. These findings contribute to the general knowledge about the orchid mycorrhiza in the subtribe Chloraeinae, and open an opportunity for the conservation not only of this particular species, but also of other rare and poorly known Andean terrestrial orchids.

1. Introduction

Orchids are well distributed along tropical and temperate climates, with an estimated number of 25,000 species (Dressler, 1993). Among them, terrestrial orchids account for about one third of all known species. They predominate in temperate climates, and represent the exclusive life-form in which orchids are able to thrive at high latitudes (Rasmussen, 1995). In South America, the southernmost distribution limit of epiphytic orchids is around the parallel 30°, as beyond this latitude only terrestrial orchids are found. Species belonging to six genera (*Chloraea, Gavilea*, *Codonorchis, Brachystele, Habenaria* and *Bipinnula*) are well adapted to temperate and cold environments at both sides of the Andean range (Correa, 1969; Buzatto et al., 2014; Novoa et al., 2015). Their abundance and distribution is highly variable, with some species restricted to small areas (*i.e. Gavilea australis* is found only in Tierra del Fuego and Malvinas Islands) and others with a broad range of more than 2000 km along the Andean Mountains (*i.e. Chloraea magellanica*) (Schinini et al., 2008; Correa, 1969).

The genus *Chloraea,* with near 46 terrestrial species, has its center of diversity in southern South America (Correa, 1996; Correa and Sánchez, 2003). The genus is not well delimited, and there are still controversies about its monophyletic status (Chemisquy and Morrone, 2010; Cisternas et al., 2012). The *Chloraea* species were historically separated into three major groups according to their geographical distribution: the tropical Andean group (Peru, Bolivia and Northwest Argentina), the Patagonian group (Chile and South Argentina) and the Eastern South American group (Northeast Argentina, Uruguay and South Brasil) (Correa, 1969).

In the Argentinian Northwest Andean Range and the associated Sierras Pampeanas (Famatina, Velasco and Ambato mountain chains), a number of terrestrial orchid species are still waiting to be described. *Chloraea riojana* is a recently discovered species (Sobral and Novoa, 2013) that belongs to the tropical-Andean group. The plant is 40–70 cm tall and has fragrant yellowish-green flowers that bloom in a racemose single spike during November–December (spring in the southern hemisphere). The species grows among perennial grasses and low shrubs in the slopes of the Velasco Mountains, at altitudes between 1500 and 3000 m a.s.l. To date only five small populations of *C. riojana* have been recorded in the same location, and it was not detected in neighboring mountains, which could indicate a highly restricted distribution.

The study of mycorrhizae in orchid species is fundamental as a first approach to elucidate not only the symbiotic fungal identity and plant symbiotic propagation, but also to understand the role of the symbiosis as a potential driver of the species geograph-

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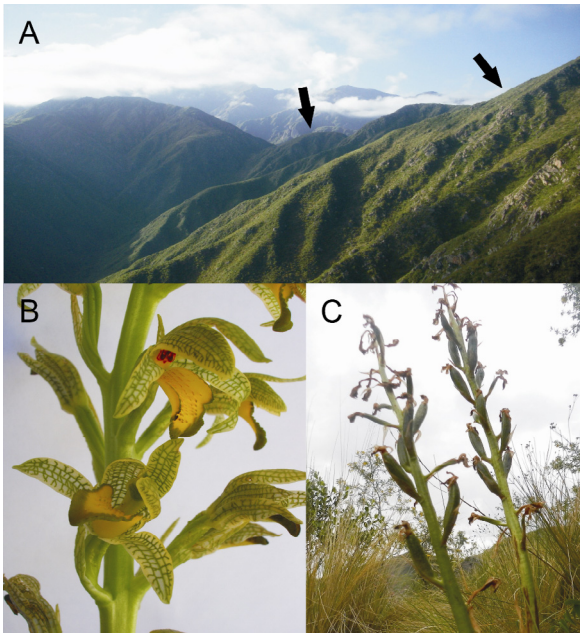

Fig. 1. *Chloraea riojana*: (a) typical habitat of *C. riojana*, showing both sampled populations on the Velasco mountains (La Rioja Province, Argentina), (b) inflorescence with open flowers, (c) spike with capsules.

ical distribution, as well as to find out whether sympatric orchid species can share fungal symbionts in early stages of development (Bonnardeaux et al., 2007; Swarts et al., 2010). Regarding the terrestrial orchids of Chile and Argentina, to date only a few studies have been carried out in relation to their symbiotic partners and plant interactions (Steinfort et al., 2010; Fracchia et al., 2013, 2014; Pereira et al., 2014). In order to fill this gap, we set up as objectives of this research to: (1) assess the mycorrhizal status of the species, (2) isolate protocorm and root endophytic fungi, and (3) assess the germination success of *C. riojana* using one isolated fungus from their roots and four orchid mycorrhizal fungi previously isolated from the sympatric orchid species *Aa achalensis*.

2. Materials and methods

2.1. Orchid populations

Whole plants (aerial and subterranean parts) were collected in November 2013 from two closely located natural populations in the Velasco Mountains, La Rioja Province of Northwest Argentina (2700 m a.s.l., 28° 46′ S, 66° 59′ W; Fig. 1a–c). These mountains correspond to the Sierras Pampeanas system, with mountain tops over 4000 m a.s.l. The semi-arid eastern slopes are covered with low shrubs and perennial grasses, at high altitudes the landscape is characterized by a rocky terrain covered with grasslands (Cabrera, 1976). The rainfall is strongly seasonal, concentrated almost exclusively to the summer months of December and January. The average annual rainfall is about 300 mm and the annual average temperature is approximately 12 °C at 2000 m a.s.l.

Three plant individuals from two populations were collected, stored in plastic bags and transported to the laboratory within 24 h. The roots were used to assess the mycorrhizal status and to isolate the fungal strains. Rhizospheric soil samples were also collected from the sampled plants, following Rasmussen and Whigham (1993) for an alternative fungal isolation procedure from protocorms. In January 2014 we collected mature capsules (n = 36) from the same populations, and stored them in glass vials with silica gel. The viability of the seeds was determined within 48 h using the tetrazolium test (Van Waes and Bebergh, 1986). The remaining seeds were kept in silica gel for 5 weeks at 5 °C in the dark until used in the germination assay and the buried slide method described below.

2.2. Fungal colonization assessment

To asses root colonization, orchid roots were rinsed in tap water and cut into transverse segments of near 1 mm section. These segments were cleared using 10% KOH solution, washed in 0.2 N HCl and stained in 0.05% (w/v) trypan blue in lactic acid overnight, following an adaptation of Phillips and Hayman (1970). Stained root segments were observed under microscope (Leica DMLB) at ×400–1000 magnification to assess fungal colonization, discriminating between dark septate endophytes (DSE, brown) and *Rhizoctonia*-like fungi (stained blue). A total of 15 random segments were assessed for each plant sample (n = 3 for each population). Colonization level of target fungi was obtained with the colonization frequency formula F% (Fracchia et al., 2013).

2.3. Fungal isolation

To isolate *C. riojana* fungal endophytes we followed two methods: (1) hypochlorite surface sterilized root fragments (1–2 mm, 15 fragments per plant) were plated in agar media (potato dextrose agar, PDA) and antibiotics as described elsewhere (Fracchia et al., 2013), and (2) the procedure described by Rasmussen and Whigham (1993), consisting of a double mesh (90 μm) containing the orchid seeds in a slide, which were buried in pots with the collected rhizospheric soil. We buried two slides per pot, each pot containing soil from one sampled plant individual (n = 6). The slides were removed periodically and checked for protocorm formation. When the development of protocorms was evident under a binocular microscope, they were cleaned carefully with sterile water and transferred to agar plates with PDA and antibiotics to allow fungi to grow. Purified fungal strains were stored at 5 °C and included with a strain number in the fungal collection at the CRILAR (Centro Regional de Investigaciones Científicas y Transferencia Tecnológica, La Rioja, Argentina).

2.4. Molecular characterization of fungi

DNA isolation, amplification, and sequencing – Total genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Valencia, California) and used as template for the PCR amplification of the intergenic spacer region from the nuclear ribosomal DNA (ITS hereafter), including ITS1, the 5.8S subunit and ITS2. Amplification and sequencing were carried out using the primers ITS4 and ITS5 (White et al., 1990). PCR reactions were performed in 25 μl final volume with 50–100 ng of template DNA, 0.2 μM of each primer, 25 μM of DNTPs, 4 mM $MgCl_2$, 1X buffer and 1.5 units of Taq polymerase provided by Invitrogen. The reaction conditions were: a first period of denaturation at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 1 min, and extension at 72 °C for 1 min 30 s. Final extension at 72 °C for 7 min terminated the reactions. Automated sequencing was performed by Macrogen, Inc. (Korea). Sequence from fungal isolate CC38 (Table 1) was deposited at GenBank.

2.4.1. Phylogenetic analysis

The new sequence was edited using BioEdit version 5.0.9 (Hall, 1999) and it was submitted to a BLAST search for a first identification (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ITS representative sequences available in GenBank were selected from those 98% identical to CC38. The alignment was performed using MAFFT program version 6 (Katoh and Toh, 2008) available on-line (http://mafft.

Table 1
Fungi used in the symbiotic germination of *Chloraea riojana*.

Isolate	Host	Close relative	GenBank accession
CC8	*A. achalensis*	*Gaeumannomyces cylindrosporus*	KF151198
CC26	*A. achalensis*	Pezizaceae	KF151200
CC28	*A. achalensis*	*Thanatephorus cucumeris*	KF151201
CC29	*A. achalensis*	*Thanatephorus cucumeris*	KF151202
CC38	*C. riojana*	*Rhizoctonia solani*	KX267766

Table 2
Developmental stages of *in vitro* symbioticlally cultured *Chloraea riojana*.

Stage	Description
0	Ungerminated seed, testa intact
1	Enlarged embryo, testa ruptured
2	Appearance of rhizoids (=germination)
3	Appearance of protomeristem
4	Emergence of first leaf (seedling)

Adapted from Stewart and Kane (2006).

cbrc.jp/alignment/server/). We performed a phylogenetic analysis to assign the isolate to a specific fungal group. Species of *Ceratobasidium/Ceratobasidiaceae* were used as outgroup. *Ceratobasidium* sp. (GenBank Acc. number AF354089) was used to root the tree.

The phylogenetic analysis was performed under the parsimony criterion using TNT version 1.1 (Goloboff et al., 2008). Parsimony-uninformative characters were excluded and gaps were considered as missing data. Search strategy consisted of heuristic searches performed using 1000 series of random addition sequences (RAS) followed by TBR branch rearrangements, retaining 10 trees per series. Trees found were saved in memory and additionally TBR swapped retaining a maximum 10,000 total trees. A strict consensus tree was generated from the most parsimonious trees. Branch support was calculated by bootstrapping, performing 1000 resampling iterations and a heuristic search strategy of 5 addition sequences swapped with TBR with 2 trees saved per replicate.

The ITS sequence from isolate CC38 was deposited at GenBank (Acc. Number KX267766).

2.5. Symbiotic seed germination

Seeds were surface sterilized following Dutra et al. (2009). Once sterilized, seeds (200–250) were plated in 9-cm Petri dishes containing 20 ml of sterile oat meal agar (OMA) medium (2 g l^{-1} rolled oats, agar 0.8%, pH 6.5 measured prior to autoclaving). The plates were previously (4–7 d) inoculated with a 1 cm × 1 cm plug of each fungal strain taken from fresh cultures on PDA. The fungi selected for germination assay are listed in Table 1. We selected a *Rhizoctonia*-like strain (CC38), together with four previously mycorrhizal fungi from the sympatric orchid species *Aa achalensis* (Fracchia et al., 2013). Each treatment consisted of eight replicates inoculated with a single fungal strain. Uninoculated plates served as a control. Petri plates were sealed with Parafilm® M and stored in the dark at 25 ± 2 °C for three weeks. After this period the plates were exposed to a 14 h cool white fluorescent light photoperiod (22 ± 2 °C, 80 μmol m^{-2} s^{-1} at the plate surface). Seed germination and protocorm development were monitored weekly and scored on a scale of 0–5 (Table 2). Percentage seed germination and protocorm development was calculated by dividing the number of seeds in each developmental stage by the total number of viable seeds. Visualization of the fungal structures inside protocorms (n = 10 for each treatment) was evaluated at week 6, after staining them with trypan blue (0.05% tripan blue-water solution) overnight and observed under microscope.

2.6. Statistical analysis

Seed germination values were subjected to one-way analysis of variance (ANOVA) and the means compared by Duncan's multiple range test ($p = 0.05$). Mean percent root fungal colonization values were expressed with the corresponding standard error.

3. Results

3.1. Root colonization patterns

Pelotons and hyphal coils were observed in all *C. riojana* sampling individuals, with a colonization mean of 21.8 ± 7.3% and 30.1 ± 14.8% for each population. Melanized hyphae were scarcely observed in root samples from a few individuals (3.0 ± 1.1% and 2.6 ± 0.8%). These fungi colonized the first cell layers of the root parenchyma without any necrotic tissue symptom; microsclerotia were not detected.

3.2. Fungal isolation

Twenty-one fungal isolates were recovered from the roots of *C. reticulata*, but none of them were related to taxa belonging to known orchid mycorrhizal fungi. The prevailing genera were *Alternaria*-like (6 isolates), *Penicillium* (3), *Trichoderma* (3), sterile septate fungi not related to *Rhizoctonia*-like morphology (3), and six unidentified ascomycetous septate and hyaline isolates. In the seed baits assay we collected a few protocorms (stage 2, rhizoids) in two slides from two different pots, after 8 weeks of incubation. From only one set of protocorms (n = 3) we could recover a *Rhizoctonia*-like fungus. Also several ascomycetous fungi grew associated to the protocorms when plated in the Petri dishes, but were discarded for the symbiotic germination assay as they were not related to known mycorrhizal fungi.

3.3. Molecular characterization of fungi

BLAST analysis revealed that sequence from isolate CC38 was similar (98–99%) to *Rhizoctonia solani/Thanatephorus cucumeris*. The ITS data set comprises 26 taxa and from a total of 748 characters, 126 were phylogenetically informative. The analysis of the aligned matrix resulted in 38 trees (Length = 258; Consistency Index (CI) = 0.65; Retention Index (RI) = 0.78). The strict consensus is shown in Fig. 2.

The phylogenetic analysis revealed a highly supported group (Bootstrap value (BS) = 100) which gathered isolates of *Rhizoctonia solani/Thanatephorus cucumeris*. Isolate CC38 was placed in a basal polytomy within a subclade (BS = 73) (Fig. 2).

3.4. Symbiotic seed germination

The tetrazolium test revealed a viability of 21% for the harvested *C. reticulata* seeds. In all treatments, control and symbiotic, the embryos swelled breaking the testa within 15 days after sowing. After five weeks, careful examination of protocorms after trypan blue staining revealed intracellular pelotons in those treatments inoculated with strains CC38, CC28 and CC29. The mycelium of the strain CC38 became melanized when the protocorms reached stage 2, allowing us to observe dark pelotons and hyphal coilings without staining (Fig. 3c). In the CC8 and CC26 treatments pelotons did not develop and the protocorms (stage 1) decayed turning brown after four weeks. Differential shapes in protocorm development were observed between the promoting fungal strains. Notably, in stage 3 higher rhizoid density and a globose shape were evident in the treatment inoculated with the CC38 strain (Fig. 3d), compared

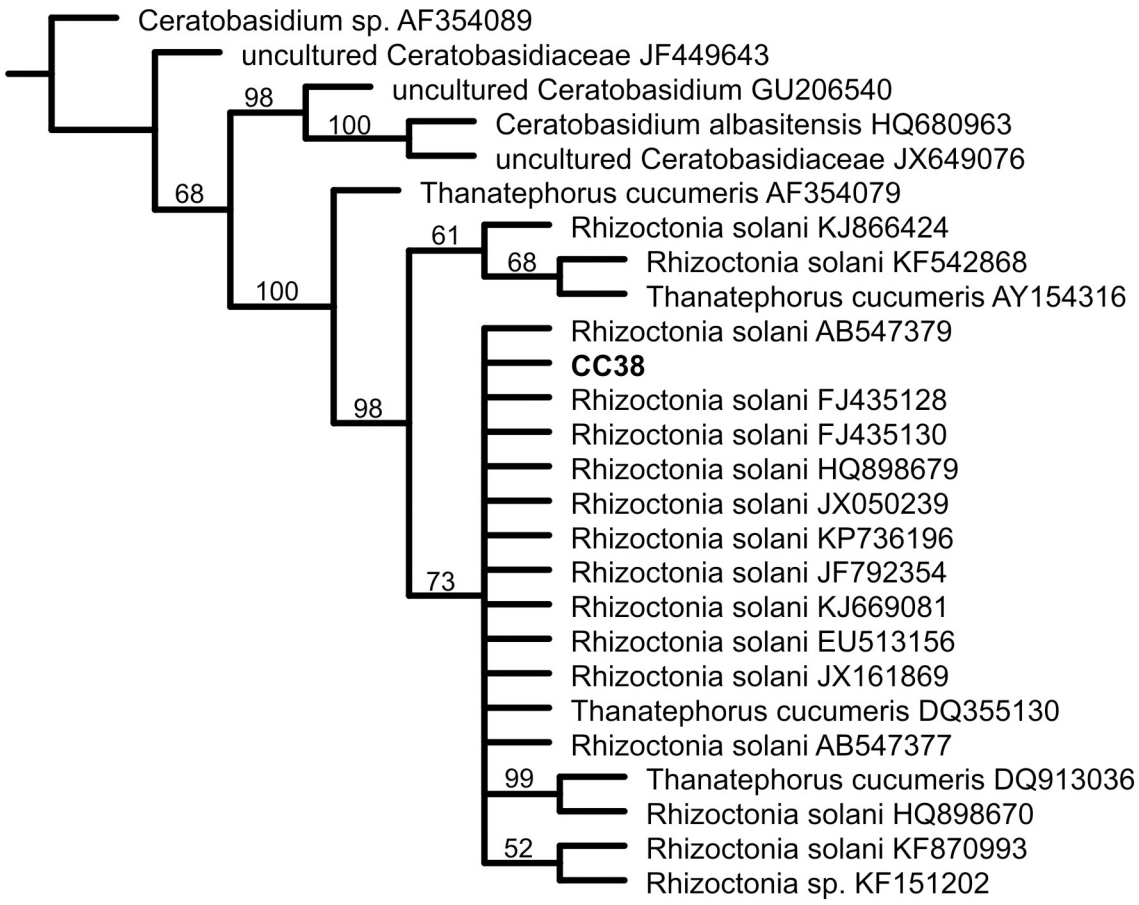

Fig. 2. Strict consensus of the nine most parsimonious trees (length = 259, CI = 0.97, RI = 0.99) resulting from the ITS data matrix analysis. Numbers above branches refer to bootstrap values.

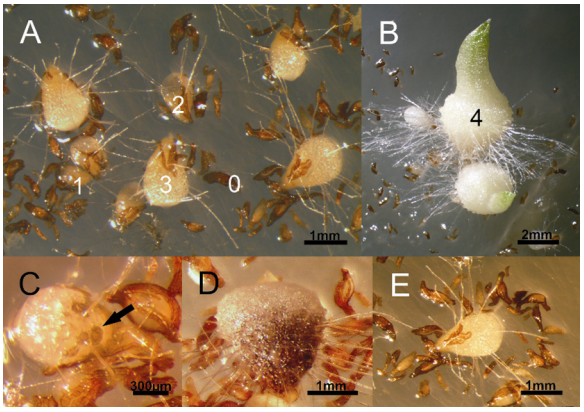

Fig. 3. Germinated seed and protocorm developmental stages: (a) 0–3 stages with the CC28 strain, (b) stage 4 in the same treatment, (c) melanized pelotons inside stage 2 protocorm inoculated with the CC38 strain, (d) globose protocorms inoculated with the strain CC38, (e) stage 3 protocorm of the CC28 treatment.

with lower hair density and an angular shape in the CC28 and CC29 treatments (Fig. 3a and e).

Seed germination and protocorm development until seedlings with two leaves (stage 5) were observed in the treatments inoculated with the *Thanatephorus* related strains (CC28, CC29, CC38) (Figs. 4–5). The most effective strain (CC28) allowed the development of nearly 13% of viable seeds into plantlets with leaves after 16 weeks of *in vitro* culture (Fig. 5). Seedlings with 2–3 leaves from all promoting strains were transplanted axenically (week 18) to new flasks with fresh oat-agar medium for further growth (data not shown). Less than 20% of these seedlings survived after 20 weeks of culture.

4. Discussion

In this study we present novel and valuable information directed towards the biological knowledge and conservation of a rare and poorly known terrestrial orchid from the Andean range. We contribute also to the general knowledge of the *Chloraea* genus, mainly related to fruit formation, mycorrhizal symbiotic fungi, specificity and early development stages.

All collected individuals of *C. riojana* harbor pelotons in the roots, but only a very few DSE hyphae in the outer cortical cells. We have already observed this pattern in other terrestrial orchids of central Argentina, as well as in the sympatric species *Aa achalensis* (Fracchia et al., 2008). Remarkably, we could not isolate any *Rhizoctonia*-like fungi from the roots, despite they were colonized and fresh pelotons were evident. We observed similar behavior in other orchid species from central Argentina (*Bipinnula pennicillata, B. biplumata*), where it was not possible to isolate fungal strains from root fragments or excised pelotons (Pers. obs.). Possible causes could be that recalcitrant isolates could not be adapted to grow in the agarized media we utilized, or alternatively, that fast growing saprotrophic fungi could inhibit the development of mycorrhizal fungi in the plates. Numerous studies have shown remarkable differences in the relative frequencies of genera of orchid mycorrhizal fungi when using culture isolation methods and those detected by direct amplification of DNA, suggesting that only a fraction of the

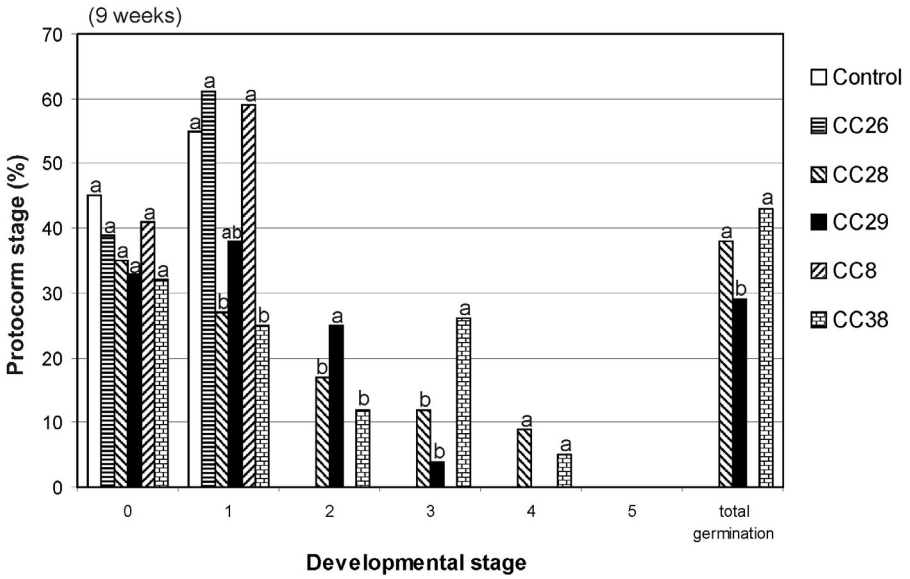

Fig. 4. Effect of fungal isolates on protocorm development of *Chloraea riojana* 9 weeks after sowing. Histobars with the same letter in each graph are not significantly different (ANOVA and means compared by Duncan's multiple range test, p = 0.05).

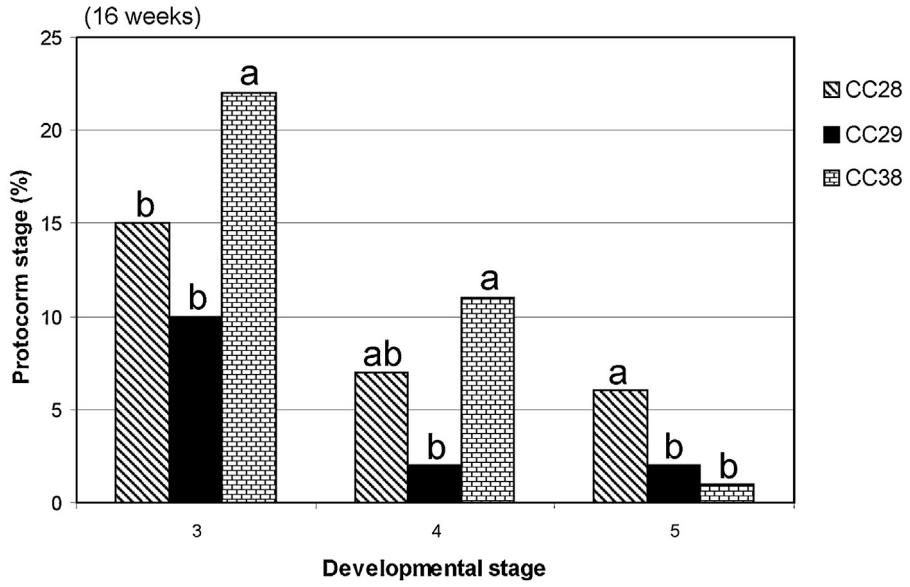

Fig. 5. Effect of fungal isolates on protocorm development of *Chloraea riojana* 16 weeks after sowing. Histobars with the same letter in each graph are not significantly different (ANOVA and means compared by Duncan's multiple range test, p = 0.05).

root endophytes has the ability to grow in the commonly used agar media (Kristiansen et al., 2001; Porras-Alfaro and Bayman, 2007).

Chloraea riojana seeds germinated and developed protocorms with its own fungal isolate (CC38) as well as with those related to *Rhizoctonia* and isolated from *Aa achalensis*. Both ascomycetes (CC8 and CC26) had no effect on protocorm development, and necrosis and tissue disorganization was evident in contrast to what was observed in *A. achalensis* (Fracchia et al., 2013). In both sampled populations plus two other that were detected later in 2015, *A. achalensis* was growing in the same patch. This shared occupation of the niche, at least for this habitat, could be determined by the presence of compatible mycorrhizal fungi in both species. The role of symbiotic fungi as drivers of orchid species distribution and plant recruitment was discussed by several authors in the last years (Bonnardeaux et al., 2007; Swarts et al., 2010). However, it is more likely that other major variables involved in plant establishment and development (edaphic conditions, pollinators) have different weight, depending on the taxonomical identity of the species (Davis et al., 2015). Some authors have also discussed the implication of using exclusively *in vitro* techniques to describe orchid–fungi relationships, considering that it may not always represent the symbiotic germination that occurs in the wild (Phillips et al., 2011).

The isolate strain CC38 became dark and melanized, including the pelotons inside protocorm cells from stage 3. Melanin is a natural component of most fungal walls that increase their rigidity, and it is supposed to reduce permeability and metabolite exchange in endophytic fungi (Money et al., 1998; Henson et al., 1999). In orchids, pelotons and coils are considered the structures where the fungi translocate nutrients to the protocorms in the early stages of plant development (Dearnaley, 2007). Despite this particular behavior, CC38 strain promoted efficiently symbiotic germination

in *C. riojana,* and a few protocorms were viable until stage 5, becoming dark at the base without any necrosis symptom (see Fig. 2d).

The populations of *C. riojana* in the Velasco Mountains of Northwest Argentina described in this work represent the only record of the species to date. There is a conservation risk of this and other sympatric terrestrial orchid species in this and similar regions. Argentinian and Chilean Andean plant communities are currently severely impacted by introduced mammalian herbivores like hares, wild boars, feral goats and cattle (Vázquez, 2002; Novillo and Ojeda, 2008). Thus, we can expect that particularly the terrestrial orchids, which lack mechanical and chemical defenses but have highly palatable leaves and fleshy tuberous roots, could be intensively predated by these herbivores. Indeed, it has been demonstrated that *Chloraea* species are commonly grazed by introduced European boars (*Sus scrofa*) in Chile (Skewes et al., 2007). In our study site, European boars and deers (*Cervus elaphus*) were introduced in the nineties and are now well distributed together with feral cattle, goats and donkeys (Sobral and Fracchia, 2010). This could explain why the few *C. riojana* individuals we found in the valleys at lower altitudes (<1700 m a.s.l.), were well protected by a shell of thorny plants. In contrast, both populations studied in this work are located in steep slopes at higher altitudes, where the access for feral cattle is more difficult. However, in one of these sites we recently observed soil disturbance and plant removal, apparently caused by boars.

In current times, due to anthropogenic activities and climatic change, we are facing what some scientists have called a sixth mass extinction (Barnosky et al., 2011). Therefore, the study of the natural biology of those species with restricted distribution represents a valuable tool for conservation purposes and future restoration programs. We expect that this work, as well as others with focus on Andean terrestrial orchids, contribute to a more comprehensive picture about their ecological requirements before these rare species become unnoticeable extinct.

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