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ORIGINAL PAPER

Symbiotic seed germination and protocorm development of *Aa achalensis* Schltr., a terrestrial orchid endemic from Argentina

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Abstract *Aa achalensis* is an endangered terrestrial orchid endemic from Argentina. In vitro symbiotic seed germination was evaluated for its propagation. Five different fungal strains were isolated from this species: two *Rhizoctonia*-like related to *Thanatephorus cucumeris* and three ascomicetaceous fungi belonging to *Phialophora graminicola* and one to an uncultured *Pezizaceae*. All five isolates promoted seed germination being one *T. cucumeris* strain the most effective. After 16 weeks of growth, 30 % of *A. achalensis* protocorms developed until seedlings with two/four leaves in this treatment. These findings open an opportunity to the knowledge and preservation of this species.

Keywords Orchid mycorrhiza · Seed germination · Dark septate endophytes · Conservation

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Introduction

Aa achalensis Schltr. is a terrestrial orchid that belongs to the subtribe Prescottiinae (Dressler 1993). The genus has 25 described species endemic from mountain environments of South America and five species were cited in Argentina: *A. achalensis*, *Aa fiebrigii*, *Aa hieronymi*, *Aa paludosa*, and *Aa weddelliana* (Schinini et al. 2008). *A. achalensis* is 20–30 cm high and its small white flowers bloom in raceme from September to December (spring southern hemisphere). The habitats of *A. achalensis* include the Chaco Serrano woodlands and the highland grasses up to 3,000 m with relative low temperatures and rocky soils in West and Central Argentina (Bianco and Cantero 1985; Sérsic et al. 2006; Sobral and Fracchia 2010).

The species was previously categorized as vulnerable and included in the red list of the International Union for Conservation of Nature (Vischi et al. 2004). However, new populations of *A. achalensis* were recently found in the slopes of the Velasco mountains in La Rioja Province (Argentina), near 500 km from the previously known populations (Sobral and Fracchia 2010). Although these new findings require a modification in the conservation status of the species, the former and new populations are not included in a national protected area and are thus subjected to grazing, forest fires, illegal extractions, land conversions to agriculture, and the invasion of exotic species among others (Marco and Páez 2000; Cagnolo et al. 2006).

Asymbiotic seed germination has been shown to be a proper tool for the production of plantlets of several orchid species for commercial and conservation purposes (Flachsland et al. 1996; Yamazaki and Miyoshi 2006). Nonetheless, in nature orchid seeds cannot germinate unless they are colonized with compatible mycorrhizal fungi which supply seeds and young plants with carbon and inorganic nutrients (Rasmussen 1995). The isolation, identification, and culture of effective symbiotic

fungi that promote seed germination and/or plant growth of threatened orchid species is essential and can be determinant to the success of conservation programs (Stewart and Kane 2006). It has been emphasized on many occasions that reintroduction programs must include promoting fungal strains, considering the conservation of the fungi as important as the plant species (Porras-Alfaro and Bayman 2007).

Although nearly 280 orchid species are cited for Argentina (Schinini et al. 2008), scarce information about their mycorrhizal status is available (Urcelay et al. 2005; Fracchia et al. 2008), and there is no literature that reports successful symbiotic germination assays. This study aimed to isolate root-associated fungi from the species *A. achalensis* and to determine their role in seed germination and protocorm development. The data obtained from this study will help not only to the propagation and conservation of this species but also to collect information for future research on eight other terrestrial orchid species sympatric with *A. achalensis* in Central and West Argentina.

Materials and methods

Orchid source

Whole plants of *A. achalensis* (n=10) at various developmental stages were collected from natural habitat (Anillaco, La Rioja Province-West Argentina) in November–December 2010 (Fig. 1). The orchid plants were stored in plastic bags and transported to the laboratory within 24 h for fungal isolation and colonization measurement. Seeds were collected from 18 individuals from the same population in December 2010–January 2011. Since the species has acropetal maturation, basal mature capsules (n=60–70) were pooled together in glass vials with silica gel. Viability of seeds was determined within 48 h using the tetrazolium test (Van Waes and Bebergh 1986). A subsample was kept in silica gel for 3 weeks at 5 °C in the dark until used in germination assay. The remainder stored at the same conditions for future investigations.

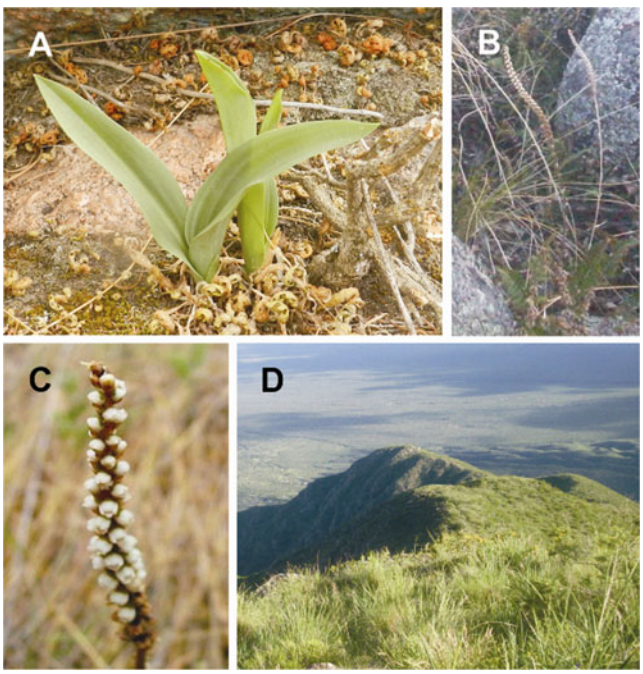

Fig. 1 **a** *A. achalensis* plants. **b** Plants with flowers. **c** Inflorescence. **d** Typical habitat at the Velasco mountains (La Rioja Province, Argentina)

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 ClH, and stained in 0.05 % (w/v) Trypan Blue in lactic acid overnight, following an adaptation of the procedure of Phillips and Hayman (1970). Stained root segments were observed under microscope (Leica DMLB) at ×400–1,000 magnification to assess fungal colonization, discriminating between dark septate endophytes (DSE, brown) and *Rhizoctonia*-like fungi (stained blue). Thirty segments were assessed for each plant sample (n=10). Colonization level of target fungi was obtained with the colonization frequency formula (F%):

$$F\% = \frac{\text{number of segment colonized by target fungi}}{\text{total number of segments examined}} \times 100$$

Fungal isolation and culture

Healthy orchid roots were detached from plants (n=6) and rinsed with tap water to remove debris. Roots were cut in 2 cm segments and surface-sterilized in 70 % ethanol for 2 min, following 3 min in a 10 % NaOCl solution and finally rinsed in sterile distilled water five times. Transversal slices of near 1 mm were transferred to Petri plates with potato dextrose agar (PDA, Britania, Argentina) medium supplemented with antibiotics (streptomycin 25 mg/l, tetracycline 100 mg/l). Plates were sealed with Parafilm® M (American National Can™), incubated at 22 °C in the dark, and observed periodically until fungal colonies were observed emerging from the root disks. Mycelium from these colonies were subcultured onto fresh PDA for purification and finally transferred to glass tubes with the same growth medium. Purified fungal strains were stored at 5 °C and included with a strain number in the fungal collection at the Centro Regional de Investigaciones Científicas, La Rioja, Argentina.

Fungal morphological characterization

Fungal isolates were grown in Petri plates with 20 ml PDA at 22 °C for 7–21 days, and colony color and growth rate

measured. Replicates of each strain were left for at least 7 weeks to allow development of sclerotia and monilioid cells (*Rhizoctonia*-like) and sporulation (DSE, septate hyaline endophyte). The soil–agar method of Stretton et al. (1964) was used to induce teleomorph formation of *Rhizoctonia*-like isolates. Each set of measurements was repeated in three different subcultures.

Fungal molecular characterization

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 $MgCl_2$, 1× buffer, and 1.5 units of Taq polymerase provided by Invitrogen. The reaction conditions were as follows: 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. PCR products were run out on a 1 % TAE agarose gel stained with ethidium bromide. Purification of PCR products and automated sequencing were performed by Macrogen, Inc. (Korea). Sequences from isolates CC8, CC10, CC26, CC28, and CC29 were deposited at GenBank (account numbers KF151198, KF151199, KF151200, KF151201, KF151202, respectively).

Sequence alignment and phylogenetic analyses Assembly and editing of sequences were performed with BioEdit version 5.0.9 (Hall 1999). All sequences were submitted to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments were performed using MAFFT program version 6 (Katoh and Toh 2008) available on line (http://mafft.cbrc.jp/alignment/server/). We performed phylogenetic analyses to assign isolates to a specific fungal group using ITS representative sequences available in GenBank. Sequences with at least 97 % similarity were chosen. One of the analysis included sequences from *Ceratobasidiales*, *Tulasnellales*, *Sebacinae*, and one from *Multiclavula corynoides* to the root of the tree (GenBank account number U66440). The second analysis included sequences from *Pezizaceae*, *Gaeumannomyces*, *Phialophora graminicola*, and one from *Epacris pulchella* to root the tree (GenBank account number AY627805).

The phylogenetic analyses were 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 1,000 series of random addition sequences followed by tree bisection and reconnection (TBR) branch rearrangements, retaining ten 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 1,000 resampling iterations and a heuristic search strategy of five addition sequences swapped with TBR with two trees saved per replicate.

Symbiotic seed germination

A. achalensis seeds were surface-sterilized following the procedure of Dutra et al. (2009). After sterilization, seeds (150–200) were plated in 9-cm-diameter Petri plates containing 20 ml of sterile oat meal agar medium (2 g l^{-1} rolled oats, agar 0.7 %, pH 6.5 measured prior to autoclaving). The plates were inoculated with a 1×1-cm plug of each fungal inoculum taken from the hyphal edge after culturing on PDA. Each treatment consisted of eight replicates inoculated with a single fungal strain. Although contaminated plates were discarded, the number of replicates for a given treatment was never less than five. Uninoculated plates served as a control treatment. Petri plates were sealed with Parafilm® M (American National Can™) and stored in the dark at 22±2 °C for 4 weeks. After this period, plates were exposed to a 14-h white light photoperiod with cool white fluorescent tubes at 80 μmol m^{-2} s^{-1} measured at the plate surface. Seed germination and protocorm development were monitored weekly and scored on a scale of 0–5 (Table 1, Fig. 2f, g). Percent 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 mycobiont structures inside protocroms (n=10) was evaluated at week 4, after staining them with Trypan Blue (0.05 % Trypan Blue–water solution) overnight and observed under the microscope. The symbiotic assay was performed three times.

Table 1 Seed germination and protocorm developmental stages of symbiotic in vitro culture of *A. achalensis*. Adapted from Stewart and Kane (2006)

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
5	Presence of second leaf (=seedling)

Statistical analysis

Seed germination percentages 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 (DSE and *Rhizoctonia*-like) were expressed with the corresponding standard deviation.

Results

Colonization patterns

Pelotons and hyphal coils of *Rhizoctonia*-like mycorrhizal fungi were observed in all *A. achalensis* sampling individuals (Fig. 2a), with a mean percentage of 32.3±12.0 %. Melanized hyphae were also observed in root samples from all individuals (9.4±6.7 %). These fungi colonized the first cells layers of the root parenchyma without any necrotic tissue symptom. Globose to subglobose microsclerotia (Fig. 2b) were detected in 40 % of the sampled individuals.

Fungal isolation and morphological characterization

Five endophytic fungal isolates (two DSE, two *Rhizoctonia*-like, one septate hyaline endophyte) were recovered from the roots of *A. achalensis*. Data in Table 2 show colonial appearances, morphological features, and growth rates of isolates. These characteristics were ineffective at allowing us to determine the taxonomic identity of any fungal strains. No sporulation or teleomorphic stages were observed. Sclerotial masses were developed in both *Rhizoctonia*-like fungi and septate hyaline endophyte. Colonies general appearance of strains CC8 and CC28 are shown in Fig. 2d, e.

Molecular identification of fungal isolates

Isolates CC8, CC10, and CC26 BLAST analyses revealed that the sequences from isolates CC8 and CC10 were similar (99 %) to *Gaeumannomyces cylindrica*/*P. graminicola*. Isolate CC26 resulted similar (98 %) to uncultured *Pezizaceae* sequences. The ITS data set comprises 18 taxa, and from a total of 612 characters, 211 were phylogenetically informative. The

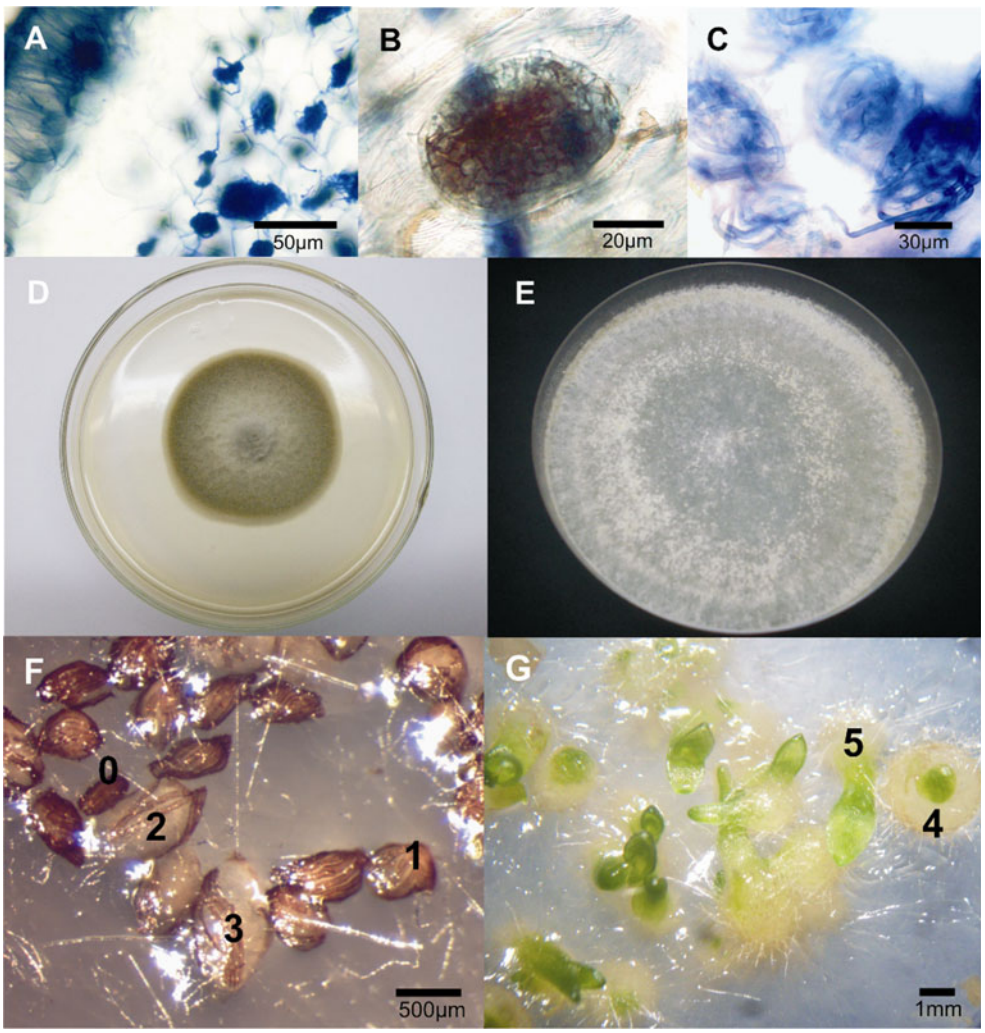

Fig. 2 **a**–**c** Intrarradical fungal structures: **a** *Rhizoctonia*-like pelotons; **b** DSE microscleortium; **c** pelotons from strain CC28 inside protocorms. **d**, **e** Fungal colonies: **d** CC8; **e** CC28. **f**, **g** protocorm developmental stages

Table 2 Cultural and morphometric characteristics of fungal isolates

Fungal isolate	Accession number	Fungal type	Colony appearance	Colony growth rate (mm/h)	Sclerotial masses (color, size)	Sporulation/ teleomorph	Monilioid cells
CC8	KF151198	Dark septate endophyte	Flat, dark brown	0.04–0.06	Absent	Absent	Absent
CC10	KF151199	Dark septate endophyte	Flat, dark brown	0.03–0.06	Absent	Absent	Absent
CC26	KF151200	Septate hyaline endophyte	Glabrous, yellowish	0.50–0.53	Beige (2.2–4.5)	Absent	Absent
CC28	KF151201	*Rhizoctonia*-like	Cottony, white	0.40–0.49	Brown (0.4–0.8)	Absent	Ellipsoid to subglobose
CC29	KF151202	*Rhizoctonia*-like	Cottony, pale yellow	0.32–0.41	Brown (0.3–0.5)	Absent	Irregularly ellipsoidal

analysis of the aligned matrix resulted in nine trees (length=259, consistency index (CI)=0.97, retention index (RI)=0.99). The strict consensus is shown in Fig. 3. The phylogenetic analysis revealed two highly supported groups where isolates were placed: group 1 including sequences of uncultured *Pezizaceae* (bootstrap value (BS)=100) and group 2 gathering together sequences of *G. cylindrica*/*P. graminicola* (BS=100). Within group 1, isolate CC26 was recovered as sister of the reference sequence FJ788779, identified as uncultured *Pezizaceae*. Isolates CC10 and CC8 are sister to each other (BS=98) and they are included in a polytomy with *G. cylindrica*/*P. graminicola* (group 2) with high support (BS=100).

Isolates CC28 and CC29 BLAST analyses revealed that the sequences from isolates CC28 and CC29 were similar (98 and 99 %, respectively) to *Thanatephorus cucumeris*/*Rhizoctonia solani*. The ITS data set comprises 28 sequences and from a total of 862 characters, 489 were phylogenetically informative. The analysis of the aligned matrix resulted in two trees (length=1,492, CI=0.63, RI=0.84). The strict consensus is shown in Fig. 4. The phylogenetic analysis revealed two main highly supported groups: group 1 (BS=100) gathers clones CC28 and CC29 together with reference sequences of *T. cucumeris* and *Ceratobasidium* sp. and group 2 (BS=82) composed of sequences from *Epulorhiza*, *Tulasnella*, and *Sebacina*.

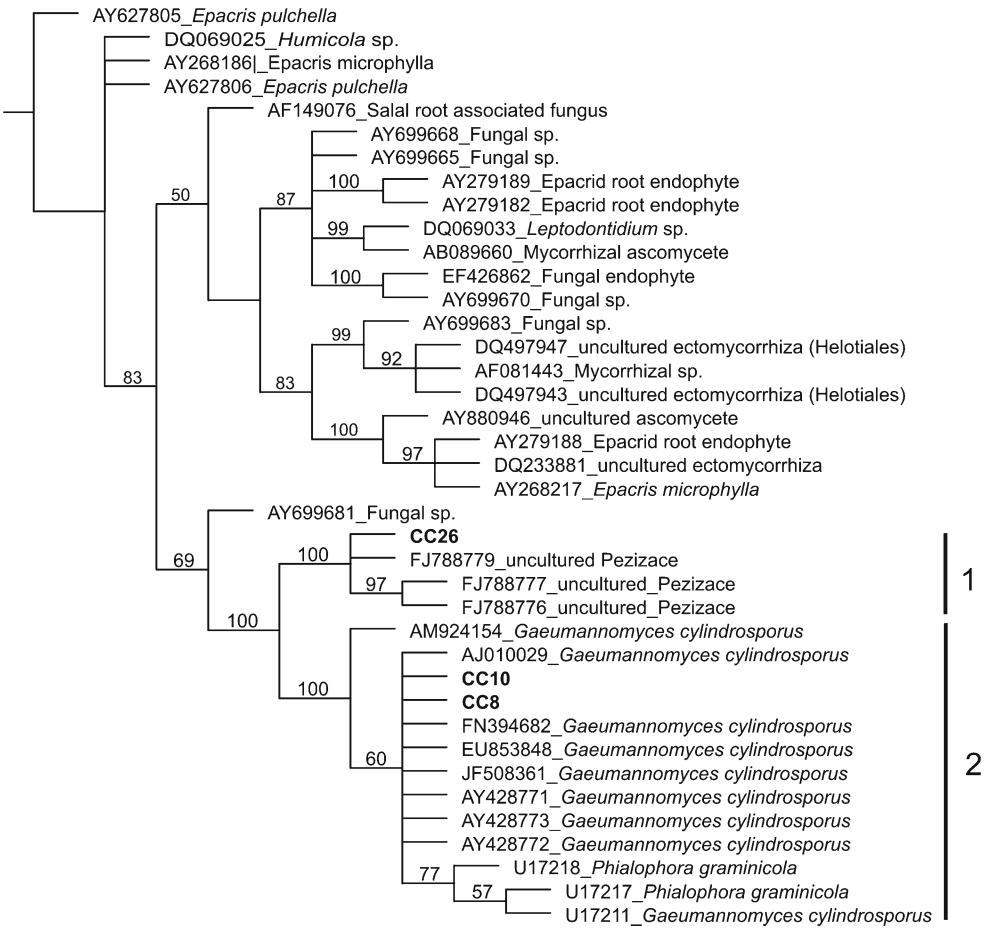

Fig. 3 Strict consensus of the nine most parsimonious trees (length=259, CI=0.97, RI=0.99) resulting from the ITS data matrix analysis. *Numbers above branches* refer to bootstrap values. *Bars* indicate main clades discussed in the text

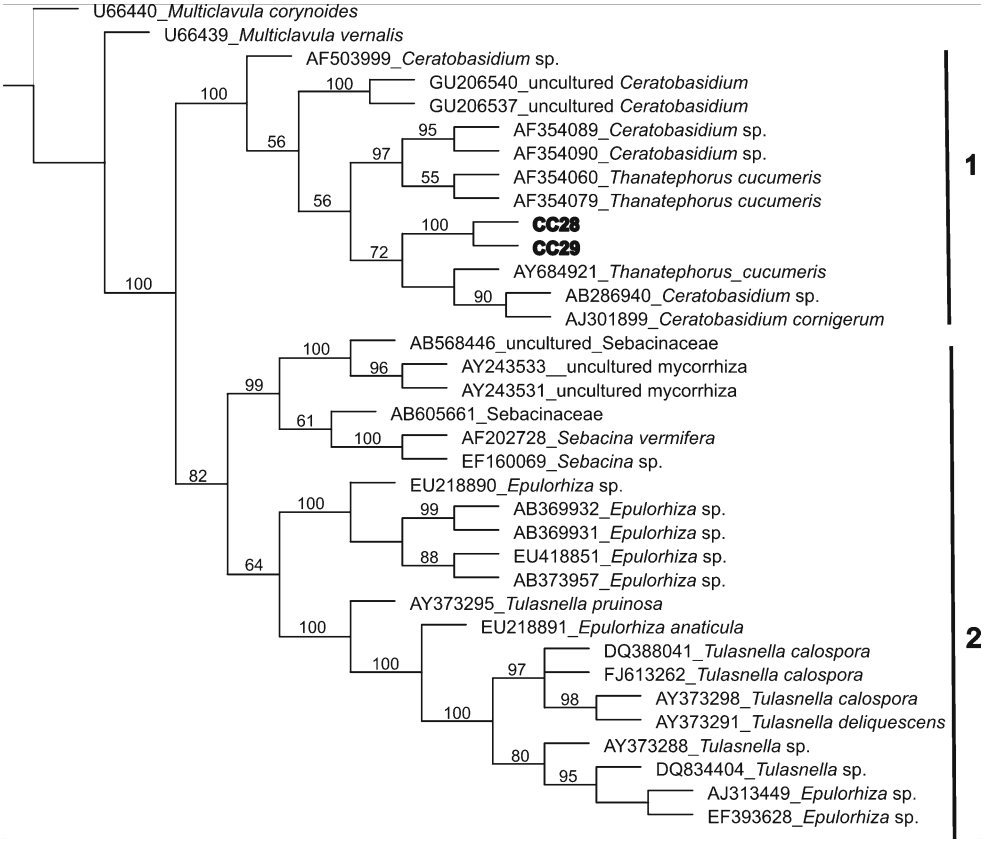

Fig. 4 Strict consensus of the two most parsimonious trees (length=1,492, CI=0.63, RI=0.84) resulting from the ITS data matrix analysis. *Numbers above branches* refer to bootstrap values. *Bars* indicate main clades discussed in the text

Symbiotic seed germination

The tetrazolium test revealed a viability of 42.9 % for the harvested *A. achalensis* seeds. In all treatments, the embryos swelled breaking the testa within 25 days after sowing. At 5 weeks, careful examination of protocorms after Trypan Blue staining revealed typical pelotons in the treatments inoculated with the *Rhizoctonia*-like fungi (strains CC28, CC29) (Fig. 2c) and the sterile hyaline strain (CC26). The DSE fungi colonized the seeds with coiling hyphae inside the protocorm cells but no compact pelotons were observed. After 12 weeks, when no further growth was measured in both DSE treatments, tissue disorganization was evident in some protocorms.

Protocorm developmental stages are shown at week 10 and 16 after sowing (Fig. 5). Total seed germination was significantly higher in all inoculated treatments, being both *Rhizoctonia*-like fungi (CC28, CC29) and the sterile hyaline strain (CC26) the most effective. In the asymbiotic treatment (control), the seeds swelled but we did not observe rhizoids along the assay. The CC28 strain was the only fungal strain that induced protocorm development beyond stage 3 (56 %), and 30 % percent of the viable seeds in this treatment grow until stage 5 after 16 weeks (Fig. 2g).

Discussion

Symbiotic orchid propagation has been achieved with varying success in some South American native species (epiphytes and terrestrial) from Colombia (Otero Ospina and Bayman 2009), Brazil (Pereira et al. 2005), and Chile (Steinfort et al. 2010). Nonetheless, there is no literature reporting symbiotic propagation assays in Argentina. Thereby, this is the first report of a successful in vitro symbiotic germination assay of a native orchid species from Argentina, as well as for a species belonging to the South American genus *Aa*. In vitro asymbiotic culture was performed with several Argentinean native orchids (Flachsland et al. 1996; Flachsland et al. 2006), but this report did not include *Aa* species. However, considering endangered species, symbiotic germination has become a favored methodology for orchid seed propagation, mainly for conservation and reintroduction programs. Several studies worldwide reported symbiotic propagation of endangered species (Stewart and Kane 2006, 2007; Chutima et al. 2011).

Most terrestrial orchid roots harbor a wide fungal diversity, including mainly species of *Basidiomyetes* and *Ascomycetes* orders (Currah et al. 1987; Selosse et al. 2004; Bidartondo and Read 2008; Yuan et al. 2009). *A. achalensis* and two other sympatric terrestrial orchid species from Central Argentina, *Sacoila lanceolata* (Aubl.) Garay and *Pelexia*

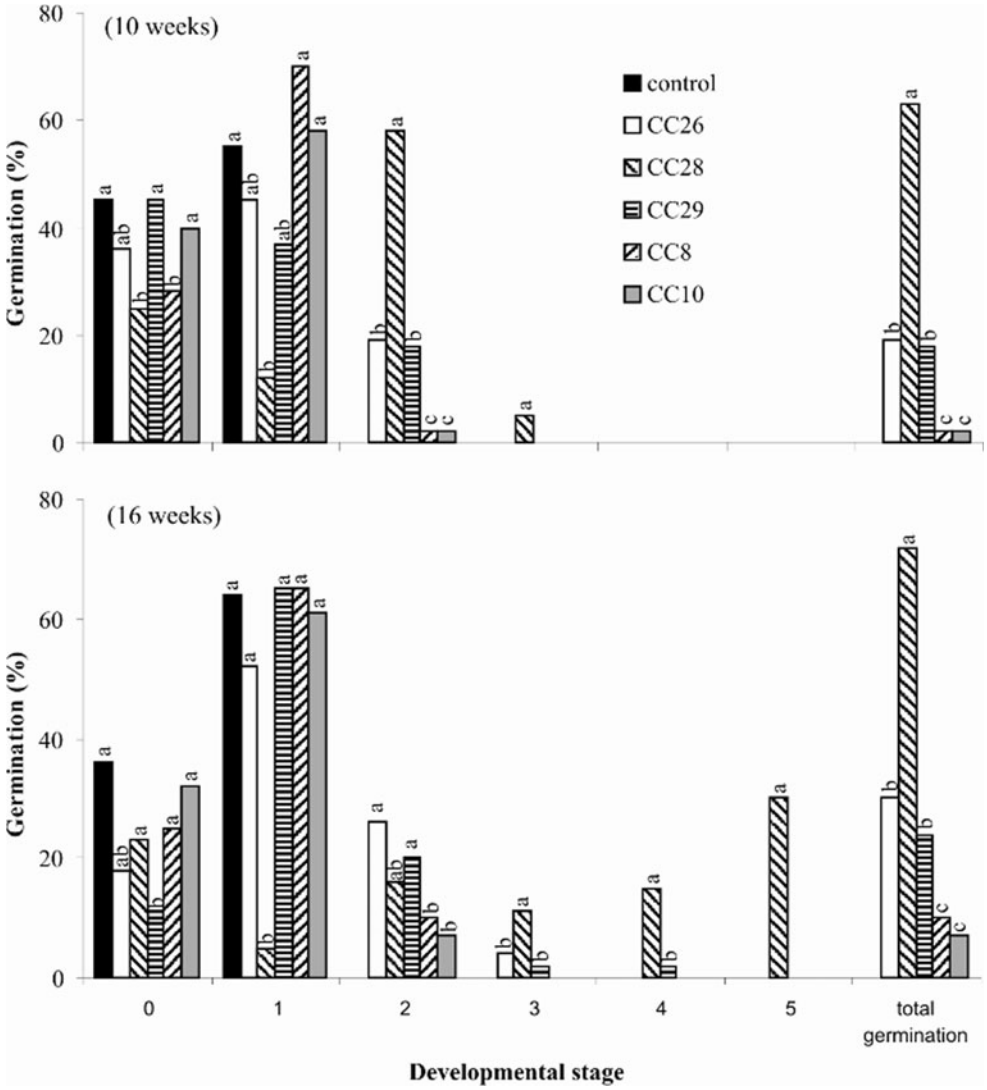

Fig. 5 Effect of five fungal isolates on protocorm development of *A. achalensis* 10 and 16 weeks after sowing. *Values* are means with *bars* indicating standard deviation. *Histobars with the same letter* in each graph are not significantly different (ANOVA and means compared by Duncan's multiple range test ($p = 0.05$))

bonaeriensis (Lindl.) Schltr., commonly harbor *Rhizoctonia*-like but also DSE in the roots, without any necrotic tissue symptom (Fracchia et al. 2008). It is clear that the simple presence of these fungi in orchid roots does not necessarily indicate a functional association. These fungi will need to be isolated and tested in germination and seedling growth assays before they can be designated as orchid mycorrhizal fungi (Dearnaley 2007).

In our work, interestingly, all five isolated fungal strains promote in vitro seed germination, despite the isolates belonging to distant taxonomic groups. We detected a rhizoctonian fungal strain (CC28), closely related to *T. cucumeris* that promoted efficiently in vitro seed germination and further protocorm development until plantlets with two to three leaves. The detection of a strain with these characteristics may be crucial for the target species conservation (Stewart and Kane 2007). Species of basidiomycete genera, mostly belonging to the rhizoctonian group, and isolated from terrestrial orchids roots, were shown to promote seed germination, protocorm development, and/or plant growth of several orchid species (Zettler and Hofer 1998; Batty et al. 2006; Stewart and Kane 2006). The ascomycetaceous isolates also promoted *A. achalensis* seed germination, but none of them allowed protocorm development beyond stage 3. Only the strain CC26, related to an uncultured *Pezizaceae*, promoted a moderate growth until this stage (4 %). It was proposed that there can be a narrow checkpoint for mycorrhizal range during seedling growth relative to the more promiscuous germination and mature stages of orchid life cycle (Bidartondo and Read 2008). In accordance to this assertion, the five isolates tested promoted seed germination but only one, the *Rhizoctonia*-like CC28 strain, was able to further protocorm growth until stage 5. It was demonstrated that some ascomycetaceous fungi can promote seed germination and further biomass growth in orchids. Zimmerman and Peterson (2007) demonstrated the promoting effect of a strain of the DSE fungus *Phialocephala fortinii* on seed germination and growth of the terrestrial orchid *Dactylorhiza praetermissa*. Also a

Phialophora species was detected by molecular tools associated with orchid roots and forming typical pelotons, suggesting these taxa as a potential mycorrhizal fungi in orchids (Bidartondo et al. 2004).

It is interesting to consider the ecological implications of *P. graminicola* as a symbiotic seed germination promoter in *A. achalensis*. This fungus can colonize diverse non-orchidaceae plants forming typical DSE colonization patterns (Newsham 2011; Fracchia, personal communication). Considering that several plant species growing close to *A. achalensis* could harbor these fungi inside the roots, we might expect plant to plant interactions via DSE fungi, as was demonstrated for mixotrophic/mycoheterotrophic orchids and ectomycorrhizal basidiomycetaceous fungi (Dearnaley 2007).

It is important to consider that all tested fungi were isolated from adult plants. However, it was well demonstrated that these plants can still provide strains that promote germination and protocorm development. Nevertheless, fungal isolates from early growth stages could help to understand the symbiosis dynamic in *A. achalensis* and demonstrate whether a fungal switching event occurs, as appear to occur in other orchid species (McCormick et al. 2006; Rasmussen and Rasmussen 2007). Finally, we reported a first approach to the conservation of an endangered native orchid from Argentina. Seedling acclimatization, time required to further plant growth, and an evaluation of the survival rate in nature are the next steps towards a better knowledge of the species and to improve success in future conservation programs of this and other native orchid species.

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