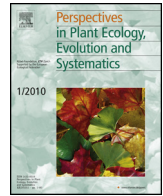




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Research article

Fungal endophytes associated with roots of nurse cushion species have positive effects on native and invasive beneficiary plants in an alpine ecosystem



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ABSTRACT

Facilitation has been proposed to be a fundamental mechanism for plant coexistence, being particularly important in highly stressful environments such as alpine environments. In this type of environment, species called “cushion plants” can ameliorate the stressful conditions, acting as nurses for other plants. Of the several mechanisms proposed in the positive-interactions framework, plant–microorganism interaction may be one of the most common, but least documented. Here we show that the presence of endophytes isolated from the roots of cushion plants *Laretia acaulis* can play a fundamental role in the establishment, performance and survival of both native and exotic plant seedlings that are known to be facilitated by the cushion species.

To test this, we measured survival and growth of two native and one invasive species at 3200 m in the Andes of Central Chile. Plants were grown inside artificial cushions filled with native soil, with or without sterilization, and with or without fungal endophytic inoculation to evaluate the role of fungal endophytes on survival and growth. In addition, we conducted a second experiment in a greenhouse with the invasive species to evaluate the effect of fungal endophytic infection/association on plant ecophysiological performance, dry biomass and seed output.

Overall, our results showed a strong positive effect of fungal endophytes on the survival and growth of both native and invasive species. Moreover, maximum quantum efficiency (Fv/Fm), biomass accumulation and seed production were enhanced in the invasive species when soils were inoculated with endophytes. Thus, facilitation by root endophytic fungi on native and invasive alpine plants could determine survival and establishment in this harsh environment.

Several studies have shown that direct facilitation by cushion plants in alpine environments improves the performance and fitness of both native and exotic plants. Our results suggest that there are indirect effects, mediated by microorganism associations that may also help to explain the successful establishment of native and invasive species in these environments. If indirect plant–plant facilitation through root fungal endophytes proves to be a widespread phenomenon in alpine ecosystems, it could be a key component in the structuring of plant communities in those stressful environments.

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Introduction

Positive interactions have been proposed to be a fundamental mechanism for plant coexistence (Bruno et al., 2003; Callaway, 2007). These positive interactions are especially important in environments where abiotic stress is high, such as alpine environments

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(Callaway et al., 2002; Cavieres et al., 2006, 2013; Anthelme and Dangles, 2012). There is ample evidence that plants in these environments, particularly those with cushion type morphologies, help other species. Cushion plants have been shown to act as nurses for other plants (Callaway et al., 2002; Badano et al., 2002; Cavieres et al., 2008; Reid et al., 2010; Butterfield et al., 2013; Schöb, 2014), having a positive impact on the diversity and productivity of alpine communities (Cavieres and Badano, 2009; Cavieres et al., 2013). Several mechanisms have been proposed to explain the facilitative effect of cushions on the establishment and growth of other plants. Alpine cushion plants can reduce water stress and increase water availability for other plants (Núñez et al., 2009a; Molina-Montenegro et al., 2006; Schöb, 2013), increase nutrient availability (Anthelme and Dangles, 2012; Schöb, 2014), buffer extreme temperatures (Molina-Montenegro et al., 2006; Cavieres et al., 2008) and stabilize otherwise dynamic substrates (Körner, 2003). In general, cushion plants improve the physiological performance of the individuals growing inside of them by developing microsites with better abiotic conditions compared to open areas (Cavieres et al., 2005, 2008; Schöb et al., 2012). However, although these direct mechanisms behind alpine cushion facilitation are well documented, other biotic actors may have powerful effects mediating facilitation in these extreme environments, including ubiquitous soil microorganisms and their interactions with plant species. Soil microorganisms can influence plant performance either positively, e.g., through mycorrhizal associations (van der Heijden and Horton, 2009; Smith and Read, 2010) or root fungal endophyte infection (Rodríguez et al., 2009), or negatively, e.g., through the accumulation of soil pathogens that impair plant establishment (Van der Putten and Peters, 1997; Bever, 2003). Overall, soil biota has a strong influence on plant performance (Rodríguez et al., 2009; Bever et al., 2010), plant community diversity (Wardle et al., 2004; van der Heijden et al., 2008), succession (Kardol et al., 2006; Lozano et al., 2014), plant invasions (Callaway et al., 2008; Meisner et al., 2013) and ecosystem function (Loreau, 2001; van der Heijden et al., 2008; Wagg et al., 2014). This impact of soil biota on plants at different scales strongly suggests that plant–soil interactions may also have an impact on plant competitive abilities and on the outcome of these plant–plant interactions (Bever, 2003; Callaway, 2007; Bever et al., 2010; Van der Putten, 2009; Martínez-García and Pugnaire, 2011; Montesinos-Navarro et al., 2012). Actually, it is well recognized that neighboring plants commonly tend to share mycorrhizal networks (van der Heijden and Horton, 2009). These mycorrhizal networks may have either positive or negative effects on individual plants, and, for example, can play an important role in nurse–seedling facilitation processes, although there are also negative impacts documented (Selosse, 2006; Booth and Hoeksema, 2010; and see review by van der Heijden and Horton, 2009). Moreover, recent studies have shown that soil microbial communities may have an effect on the outcome of plant–plant competitive interactions (Selosse, 2006; Kardol et al., 2007; Pendergast et al., 2013) and may even have an effect on plant–plant facilitation processes (Rodríguez-Echeverría et al., 2013). Overall, evidence of the impacts of soil microorganisms on plant–plant interactions is growing but is still low, particularly under field conditions in alpine environments.

Plants provide a unique ecological niche for diverse communities of belowground mutualistic microorganisms which in turn often provide multiple benefits to plants such as enhanced photosynthesis, higher resource use efficiency, and tolerance to abiotic and biotic stress (Harley and Smith, 1983; Smith and Read, 2010). These symbiotic relationships are common and fundamental for plants in nature (Sapp, 2004). Particularly, the symbiosis between a plant host and the fungal endophytes residing in plant internal tissues is one of the most documented in harsh environments (Harley and Smith, 1983; Rodríguez et al., 2009, 2010; Redman

et al., 2011). Root-colonizing endophytic fungi are a highly diverse group (Vandenkoornhuysen et al., 2002) that can exert a wide range of plant host effects, i.e., they have different degrees of virulence or even positive effects (Schulz and Boyle, 2005; Rillig et al., 2014). The endophyte–host relationship is thus described as a continuum ranging from mutualism through commensalism to parasitism (Lewis, 1985; Aly et al., 2011; Rillig et al., 2014). The presence of endophytes in plants can alter food webs, community composition, ecosystem processes, and are important to the structure, function, and health of plant communities (Clay and Holah, 1999; Read, 1999; Aschehoug et al., 2014). Endophytic fungi have also been reported to have direct positive effects on a number of plant species (e.g., Read, 1987; Rodríguez et al., 2009; Vos et al., 2012; Johnson et al., 2012; Aschehoug et al., 2014). They can reduce water stress of the host plant (Ortega et al., 2004; Smith and Read, 2010; Harris-Valle et al., 2009; Atala et al., 2012), contribute to the overall plant nutrition (Kipfer et al., 2011), and have been suggested to help some plants to deal with environmental stresses (Clay and Holah, 1999; Redman et al., 2002). Nevertheless, some carbon is exported from the plant to the fungi (Read, 1987; Harris-Valle et al., 2009), reducing the resources available for plant growth and reproduction. By enhancing or impairing their host plant fitness, endophytic plant symbionts may have profound effects on the ability of host plants to interact with other organisms like pathogens, other plant species and consumers and on its competitive ability (Aschehoug et al., 2014), but analysis of the possible impact of root fungal endophytes on facilitative plant–plant interactions is lacking.

In an alpine ecosystem, Casanova-Katny et al. (2011) showed previously that the abundance of root colonizing fungi was higher in plants growing within cushion species than in open areas. These findings were evidence of a particular spatial distribution of alpine microorganisms, however, they did not disentangle the role of these endophytes in the ecophysiological performance and fitness of alpine species facilitated by cushion species. Overall, there is a paucity of studies analyzing the role of soil biota in explaining the observed positive effects of alpine cushions on other species (but see, Casanova-Katny et al., 2011; Gibert et al., 2012). Thus, our aim in this study was to analyze the effect of local soil endophytical fungi associated with the roots of a cushion nurse species, *Laretia acaulis*, on the establishment and performance of three species known to be facilitated by *L. acaulis*: two native (*Phacelia secunda* and *Hordeum comosum*) and one exotic (*Taraxacum officinale*) species. We hypothesized that the presence of the endophytes found preferentially in cushion plant patches of *L. acaulis* would play a fundamental role in the establishment, performance and survival of both native and exotic seedlings. With this purpose we combined a series of greenhouse and field experiments in an alpine community where we analyzed the survival and biomass of beneficiary species planted within artificial cushions with different soil inocula. We also measured the effect of endophytes associated with roots of the cushion species on the physiological and reproductive response of the exotic beneficiary species.

Methods

Study site

Our study site is located at 3200 m above sea level (a.s.l.) in the Andes mountains in Central Chile (33° S) and close to the Valle Nevado ski complex (33° 20' S 70° 16' W). Central Chile region possesses a Mediterranean-type climate (di Castri and Hajek, 1976), which is characterized by cool rainy winters and long dry summers. During the growing season (November to March) the mean

monthly air temperature ranges from 7.6 °C in February to 4.3 °C in November, with minimum and maximum absolutes of 3.0 and –6.5, respectively (Cavieres and Arroyo, 1999). The mean annual precipitation has been estimated by Santibáñez and Uribe (1990) in ca. 1000 mm at 3200 m a.s.l. The vegetation at the study site is dominated by the native cushion plant *L. acaulis* (Apiaceae). Native perennial species such as *P. secunda* (Hydrophyllaceae) and *H. comosum* (Poaceae), and the invasive species *T. officinale* (Asteraceae) also grow at the site (both within and outside cushions of *L. acaulis*) and all are known to be facilitated by *L. acaulis* (Cavieres et al., 2007, 2008). It has been previously shown that *L. acaulis* cushions facilitate the establishment, survival and ecophysiological performance of *T. officinale* and *H. comosum* in the Andes of central Chile (Cavieres et al., 2007, 2008). Conversely, it has been shown that *P. secunda* tends to grow mainly outside of cushions (Cavieres et al., 2006). Although, some studies have provided evidence related with microclimatic mitigations induced by cushion plants as well as competition among nurse plants, still there is a lack of information to understand the spatial patterns found in field. In this context, the role of fungal endophytes could be a complementary and underlying mechanisms, but seldom been assessed in this ecosystem.

Isolation and identification of endophytes associated with the nurse plant

Samples of *L. acaulis* roots with a cluster of soil (250 g) and from rhizospheric-soil outside of cushions were collected from 50 plants and other 50 points outside them at the site during the 2007 growing season. Roots were then cut into pieces and stored in plastic bags for no more than 24 h at 10 °C before the isolation of endophytic fungi. Roots were superficially sterilized by successive immersion in ethanol 70% (1 min) and 2% sodium hypochlorite (3 min), and then washed with sterile distilled water (2 min) (Collado et al., 1996). Sterilized roots and field soil (5 kg approx.) were placed on 20 Petri dishes containing potato dextrose agar (PDA, Difco, USA) plus chloramphenicol at 100 g/ml/1. The Petri dishes were incubated for up to 60 days at 18 °C. The individual colonies that formed were transferred to PDA medium and stored at 4 °C. Long-term preservation of mycelial fractions was carried out in cryotubes with 30% sterile glycerol at –80 °C, while other mycelia fractions were maintained in PDA at 4 °C to be used in field and greenhouse experiments.

Morphological identification, and analysis of diversity and abundance of isolated endophytes was done under a dissecting microscope, and the emerging fungi were transferred onto cornmeal agar and potato dextrose agar (CMA; PDA; Becton Dickinson & Co, Maryland). Colony morphology was recorded and sporulation studied on CMA and PDA. Different hyphae growing in the same root fragment that showed similar colony morphology were clustered. The isolates were maintained by routine sub-culturing with single-spore isolations. Finally, diversity, abundance and identification of fungal isolates sporulating fungi were identified based on colony morphology, conidiospore and conidiophore characteristics.

In order to validate the early morphological identification of isolated endophytes infecting roots of the nurse *L. acaulis*, we amplified the ITS and the nuclear ribosomal large subunit rRNA (LSU) regions using ITS-5/Lr6 as previously described (Tedersoo et al., 2009). DNA was extracted from fruit bodies using the E.Z.N.A. fungal DNA MiniKit (Omega-Biotek, Doraville, Georgia, USA). PCR reactions were performed using ITS5 (5'-ggaagtaaaagtctgaacaagg-3') as forward primer and ITS4 (5'-tcctccgcttattgatgc-3') as reverse primer and with lrOR (5'-gtaccgctgaacttaagc-3') as forward primer and LR06 (5'-cgccagtctgcttacc-3') as reverse primer for ITS and LSU regions, respectively. Each reaction was conducted in a 15 µl vol-

ume containing 30–50 ng of DNA, PCR buffer, 2 mM MgCl₂, 0.1 µM of each dNTP, 0.5 µM of forward and reverse primers, and 1 U of Taq DNA polymerase. PCR amplification was carried out with an initial denaturation of 4 min at 94 °C, then 35 cycles of 30 s at 94 °C, 60 s at 50 °C and 60 s at 72 °C followed by a final step of 5 min at 72 °C. After this, the PCR product was purified and both strands sequenced with Macrogen sequence service (Seoul, Korea). Once sequenced, forward and reverse sequences were edited using Geneious v 5.4 software (Drummond et al., 2011). The sequence of each isolated endophyte was analyzed with BLAST (Basic Local Alignment Search Tool) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the percentage of maximal identity with the sequences of that global database. Finally, the sequences obtained in the present study were deposited in Gene-Bank.

All experiments were performed with endophyte-free seedlings. To obtain them, we used the fungicide Benlate (containing benomyl (methyl [1-butylamino carbonyl]-1H benzimidazol-2-yl) carbamate, Wilmington, DE, USA). Benomyl was chosen because its application has no phytotoxic effects on perennial plants (Dernoeden and McIntosh, 1991). Leaves and roots were completely submerged in tap water containing 2 gl-1 of Benlate and maintained for 1 h at room temperature. Before the beginning of the experiment, and 10 days after fungicide application, five random plants per treatment were sacrificed to check for endophytes with a microscope (Motic BA310, Motic Chinese Group, CO., Ltda.).

Field experiment

Seedlings of *T. officinale* (invasive species) and *P. secunda* (native) approx. 4–5 cm tall were collected in a site at 3100 m a.s.l., adjacent to the study site and were kept in well watered plastic boxes with native soil until the beginning of the experiment. Seedlings of the native species *H. comosum* were obtained from seeds collected at the study site. After germination, seedlings were planted in small plastic bags (100 cc with native soil) and were maintained in a growth chamber (Forma Scientific Inc. Marietta, Ohio) at 10/5 °C (day/night) for 50 days.

We performed a manipulative experiment at the study site to assess the effect of endophytes on the facilitation of cushions on other species by planting seedlings of the above-mentioned species in artificial cushions filled with different soil treatments. Artificial cushions were circular in shape (50 cm diameter) and were constructed using green plastic mesh containing 15 l of soil. Our artificial cushions mimicked the shape, texture and consistency of natural cushions. To prepare soil treatments, soil beneath different cushions (10 individuals) of *L. acaulis* was collected at a depth of approx. 3–5 cm and mixed all together as described in other studies (Callaway et al., 2011; Meisner et al., 2013; Pendergast et al., 2013). Pooling soil samples reduces variability but allows for the testing of differences between soil treatments considering experimental replicates as technical replicates (Rodríguez-Echeverría et al., 2013). One-third of the soil volume was maintained intact and the other two-thirds were sterilized in a thermal-autoclave during 3 h at 130 °C to kill all soil biota. There were three cushion-soil treatments: **AC**, artificial cushions filled with non-sterilized soil; **ACS**, artificial cushions filled with sterilized soil; **ACI**, artificial cushions filled with sterilized soil inoculated with a mixture of endophytes inocula. Inoculation with endophytes was done with a concentrated mix of spores (1000 spores g⁻¹) from the two most abundant endophytes isolated from the cushion soil. Thermal soil sterilization can change resource availability (De Deyn et al., 2004) and thus we analyzed if there were any differences in soil nitrogen, phosphorus and potassium availability between sterilized and non-sterilized soils from *L. acaulis* ($n = 7/\text{treatment}$). There were no differences in either soil nitrogen ($p = 0.45$;

$t=0.78$), phosphorus ($p=0.11$; $t=1.70$) or potassium ($p=0.44$; $t=0.79$) contents between sterilized and non-sterilized soils ($n=7$).

Within each artificial cushion we planted a group of 10 seedlings of *T. officinale* or *P. secunda*, or seven seedlings for *H. comosum* ($n=10$ cushion-soil per soil treatment; n total = 30 artificial cushions). Each group of seedlings was randomly assigned to the different cushion-soil treatment. Seedlings were planted no less than 5 cm from each other and survival was evaluated after 70 days and alive seedlings were then harvested and total dry mass (72 h at 60 °C) of each plant was measured with an electronic balance (Boeco BBL-52; precision 0.01 g).

Greenhouse experiment

Before starting the greenhouse experiment, we assessed the presence of fungal endophytes in roots of *T. officinale*, the invasive species, growing within and outside cushions. We used a clearing and staining procedure to quantify endophyte colonization, abundance, and richness. The abundance and richness of endophyte in roots was determined by a histological staining method (Hiatt et al., 1999). Endophyte concentration in host tissues was quantitatively determined by counting aniline blue-stained endophyte hyphae in leaf cross-sections. This method is a reliable and direct measure of the amount of viable endophyte mycelium (Spiering et al., 2005). Root colonization by endophytes fungi was quantified using a dissection microscope (Motic BA410, Chinese Group, CO., Ltda.) after cleaning the roots in 10% KOH (w/v) and staining with trypan blue in an acid glycerol solution. A variation of the gridline intersection method, developed by Giovannetti and Mosse (1980), was used to determine the percentage of root colonization and occurrence of different morphotypes. All root samples were photographed with a digital camera (Motic 3000 Cooled) and each picture was analyzed with specific software for analysis of digital images (Motic Images advance 3.2, Motic Chinese Group, CO., Ltda.).

Following this assessment seeds were collected nearby the study site at the 3300 m a.s.l., in Los Andes. Around four to five seeds per plant were collected from a relatively large number of maternal plants (>150) growing both within and outside *L. acaulis* cushions. Seeds were germinated on Petri dishes with wet filter paper, located inside a room at 24 ± 2 °C. After germination, we planted them in 300-ml plastic pots filled with soil from the study site and maintained them in a growth chamber (Forma Scientific Inc.) with a photosynthetic photon flux density (PPFD) of $170 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 16/8 h light/dark photoperiod. Two weeks after the appearance of the first true leaf, seedlings were transferred to the greenhouse and assigned to two treatments: (i) seedlings growing in pots filled with sterilized field soil and (ii) seedlings growing in pots filled with sterilized field soil that was inoculated with a mixture of endophytes.

Two inoculation treatments (with and without endophytes) with seedlings from two origins (seeds of maternal plants growing within and outside cushions) resulted in four experimental groups ($n=20$ seedlings per group). Each seedling received 50 cc of tap water every two days. Pot position within the greenhouse was changed every four days to avoid block effects. Experimental treatments lasted for 90 days, at which time we measured the maximum quantum efficiency of PSII (Fv/Fm), total dry biomass, and seed production. The Fv/Fm was measured at room temperature using a pulse-amplitude modulated fluorometer (FMS 2, Hansatech, Instruments Ltd., Norfolk, UK). One fully-developed leaf from each individual ($n=15$ seedlings per group) was dark-adapted for 30 min to obtain open PSII centers, carefully avoiding leaf detachment from the plant (for more details see Maxwell and Johnson, 2000). We then harvested the plants and recorded plant

oven-dry biomass as described earlier. All plants fruited, and we bagged each capitulum with a transparent nylon mesh to prevent seed loss. Seed production per capitulum was determined and seed output was calculated as the ratio between the number of filled seeds and the total number of seeds (i.e., including aborted and predated seeds) produced per capitula (Molina-Montenegro et al., 2008).

Statistical analyses

To compare the effect of fungal endophytes on the dry biomass of seedlings growing within the artificial cushions was conducted one-way ANOVAs with cushion-soil treatments (ACS, AC or ACI) as a fixed-factor. Survival curves of seedling growing within the artificial cushions were compared by means of the Kaplan–Meier method, and differences among species and treatments were assessed with the Cox–Mantel test (Fox, 1993). For field experiments, all analyses were conducted independently for each species. We also compared the differences in the abundance and richness of different endophytes recorded in adults of the invasive *T. officinale* growing within and outside of *L. acaulis* cushions using one-way ANOVA. Differences among treatments in greenhouse measurements (Fv/Fm, total dry biomass, and seed production) recorded on the invasive *T. officinale* were also compared using one-way ANOVA with inoculation treatment (with or without endophytes) as a fixed-factor we did not include seed origin as a fixed factor as responses of *T. officinale* seedlings originated from seeds collected from maternal plants growing within and outside cushions were not statistically different, and thus we merged the results from both origins. For all one-way ANOVAs, assumptions of normality and homogeneity of variances were tested using the Shapiro–Wilks and Bartlett tests, respectively (SAS, 2003). Tukey's post hoc test was used after significant ANOVAs. Values reported throughout the text are means \pm 1SE.

Results

Isolation and identification of fungal endophytes

Abundance, isolation and identification measurements were conducted in five to seven secondary roots from 50 *L. acaulis* individuals (between 150 and 210 total samples) and 50 points outside of them. The abundance of morphotypes as well as the frequency of occurrence were significantly higher in the rhizospheric-soil beneath cushions compared with outside of cushions (Table 1). Ten different endophytes were isolated from all samples, and two of them represented more than 97% of total recorded abundance. Based on the genetic analyses we identified two dominant species: *Geomyces pannorum* (65% of abundance of all records), and *Lecanicillium lecanii* (32%) (Fig. 1).

Field experiment

We found a strong positive effect of endophytes on plant growth and survival in the field. Growth rates and survival of seedlings of the three studied species were always greater in cushion-soils where endophytes were present (intact native soil [AC] and sterilized soil with endophytes inoculated [ACI]) than when endophytes were removed (Fig. 2). Survival percentage of native *H. comosum* and *P. secunda* seedlings was between 20% and 60% lower when endophytes were removed from soil (ACS treatment) than when present (value of Cox–Mantel test = 12.6, $p=0.007$, and C–Mt = 10.3, $p=0.021$ for *H. comosum* and *P. secunda*, respectively). The invasive *T. officinale* also suffered a sharp and significant decrease in seedling survival (ca. 60% lower) when growing in ACS cushion-soils compared to treatments with endophytes present

Table 1
Rhizospheric-soil samples (50 g) collected from beneath the cushions of *L. acaulis* and outside the cushions (total $n=100$). It shows the diversity of fungal endophytes (morphological identification) and number of samples with endophytes. Isolation and identification of endophytes within each sample was performed as described in the main text for endophytes infecting *L. acaulis* roots. We found a total of 17 different endophytes morphotypes in rhizospheric-soil beneath the cushions, whereas only eight morphotypes were found in rhizospheric-soil outside the cushions. Moreover, 46 of the rhizospheric-soil samples from beneath the cushions had at least one type of endophyte fungi, whereas endophytes were only present in 20 of the samples of rhizospheric-soil from outside the cushions.

	Within cushions	Outside cushions	t-Student test	Randomization test
Abundance of morphotypes	17.3 (± 3.6)	8.2 (± 2.9)	$p=0.015$	
Frequency of occurrence	46	20		$p=0.024$

(Cox–Mantel test = 10.4, $p=0.0134$). *H. comosum* and *T. officinale* seedlings growing in the presence of endophytes (AC and ACI treatments) had similar survival rates, whereas *P. secunda* seedlings survived more in cushions filled with sterilized soil plus endophytes inoculation compared to those transplanted on artificial cushions filled with native soil (Fig. 2).

Seedling biomass showed a similar trend as survival for all species and it was always greater in treatments with the presence of endophytes than when they were absent (Fig. 3; $F_{2, 27} = 202.25$, $p < 0.001$; $F_{2, 27} = 113.51$, $p = 0.021$; and $F_{2, 27} = 79.74$, $p = 0.032$ for *H. comosum*; *P. secunda* and *T. officinale*, respectively). The difference in biomass for *H. comosum*, *P. secunda* and *T. officinale* when growth in the artificial cushions with endophytes (native soil) and without them (sterilized soil) was 30%, 10% and 5%, respectively (Fig. 3). Similarly, *H. comosum*, *P. secunda* and *T. officinale* showed an increase in 31%, 18% and 9% of biomass when growth in the artificial cushions with re-inoculated soils compared with sterilized soil, respectively (Fig. 3). In addition, biomass of seedlings growing in the presence of soil endophytes (AC and ACI) was again similar in *H. comosum* and *T. officinale*, whereas *P. secunda* seedlings growing in sterilized soils inoculated with endophytes (ACI) had greater biomass than those transplanted on the artificial cushions filled with intact native soil (AC) (Fig. 3).

Greenhouse experiment

The abundance and richness of different endophytes morphotypes found in root samples of the invasive *T. officinale* was higher in adults growing in the field within cushions of *L. acaulis* than outside of them (Fig. 4; $F_{1, 48} = 231.12$, $p < 0.001$ for abundance and $F_{1, 48} = 183.61$, $p = 0.0043$ for endophytes richness).

Inoculation of endophytes positively affected the ecophysiological performance and fitness-related traits of *T. officinale* individuals grown in greenhouse conditions (Fig. 5). Maximum efficiency of PSII (Fv/Fm), dry biomass and seed production at the end of the experiment were higher in those *T. officinale* plants growing in sterilized soils inoculated with endophytes compared to those growing

in sterilized soil (control) ($F_{1, 58} = 84.22$, $p = 0.041$ for Fv/Fm; $F_{1, 58} = 133.42$, $p = 0.023$ for plant mass; and $F_{1, 58} = 267.56$, $p < 0.001$ for seed production).

Discussion

Our results show a strong positive effect of root fungal endophytes associated with the nurse cushion species *L. acaulis* on the performance of native and exotic beneficiary species. These results suggest that facilitation by this alpine cushion species can be mediated by their associated endophytic fungi. This positive effect was fundamental for plant survival and enhanced plant biomass and fitness, suggesting that belowground interactions between soil biota and beneficiary plants growing in nurse cushion species could be important drivers of plant–plant facilitation in alpine ecosystems.

The relevance of positive interactions in the structuring of plant communities is already well documented globally (Wright et al., 2014). These interactions are particularly important in alpine ecosystems (Callaway et al., 2002; Butterfield et al., 2013; Cavieres et al., 2013). In such environs, cushion plants like *L. acaulis* facilitate other species by providing more favorable abiotic conditions (Cavieres et al., 2008; Reid et al., 2010; Molenda et al., 2012). Direct effects of benefactor species on beneficiaries are nowadays well documented (Callaway, 2007), but the effects of plant–soil biota interactions mediating facilitation are not (Callaway and Pugnaire, 2007; Rodríguez-Echeverría et al., 2013). We found that fungal endophytes in our system were more abundant under the cushion *L. acaulis* than in open areas, as Casanova-Katny et al. (2011) already showed for other soil fungi–mycorrhizae and alpine cushion species. Our screening also showed that the abundance and richness of endophytes in roots of adult plants of one of the beneficiary species, *T. officinale*, were higher in individuals growing within cushions of *L. acaulis* than outside of them, suggesting a greater probability of cushion–beneficiary plant species to be infected by endophyte fungi when growing within the cushion than surrounding open areas. Moreover, our field experiment with

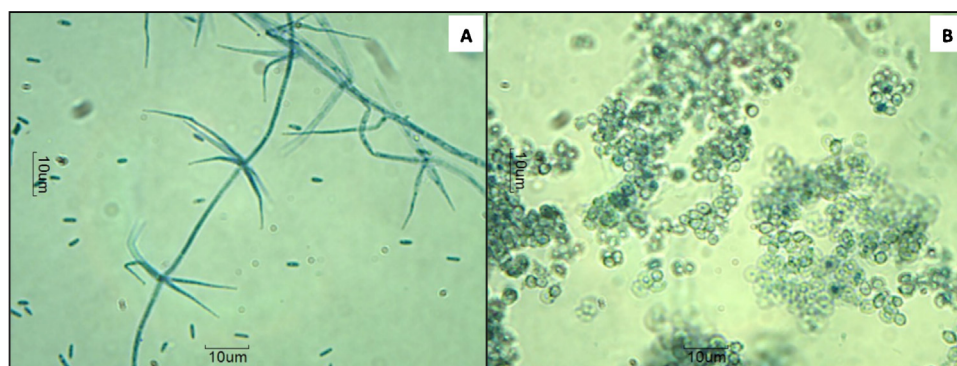


Fig. 1. Phialides and conidia of *Lecanicillium lecanii* mycelium (A) and conidia of *Geomyces pannorum* (B) growing on potato dextrose agar plates (PDA) after 15 days of incubation.

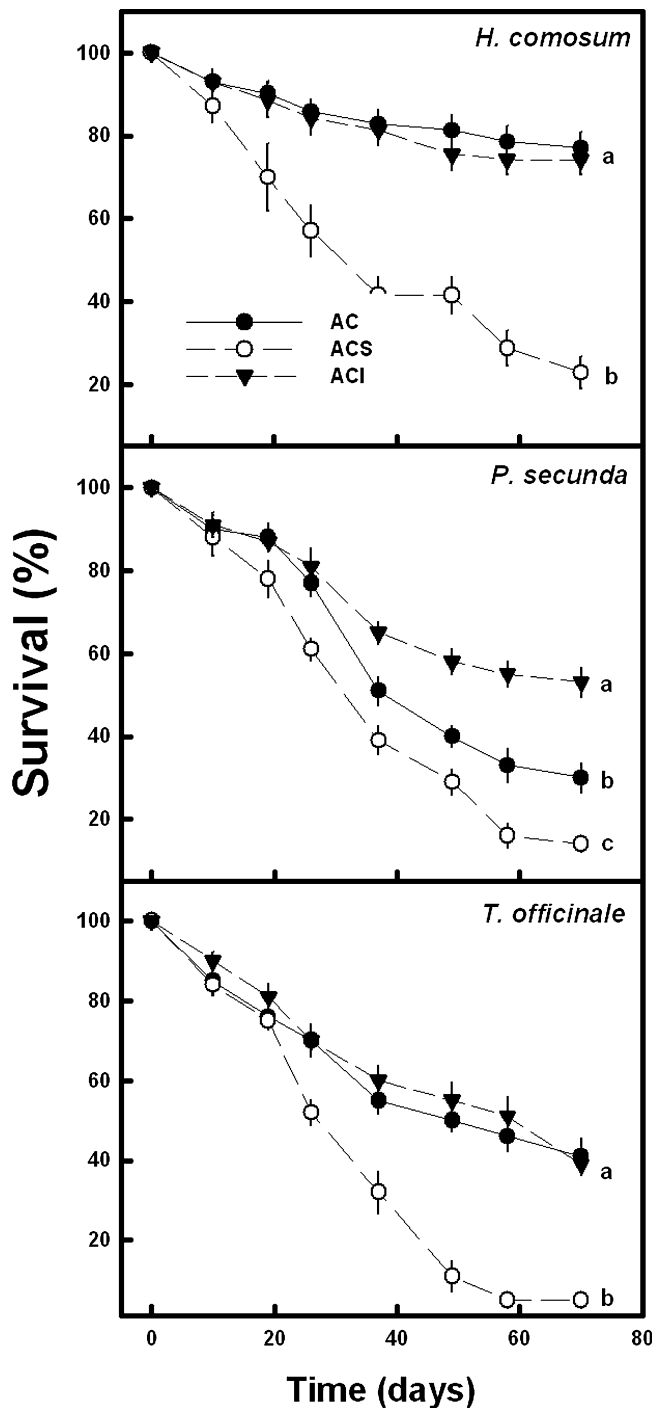


Fig. 2. Survival percentage (%) of individuals of *Hordeum comosum*, *Phacelia secunda*, and *Taraxacum officinale* transplanted in artificial cushions at 3200 m a.s.l., in the central Chilean Andes. Treatments used for survival experiment were: AC, artificial cushions filled with soil from *L. acaulis*, non-sterilized; ACS, artificial cushions filled with soil from *L. acaulis*, sterilized; ACI, artificial cushions filled with soil from *L. acaulis* sterilized and inoculated with a mixture of endophytes inoculums. Symbols are mean values \pm 1SE; initial $n = 100$ for *T. officinale* and *P. secunda*, and $n = 70$ for *H. comosum*. Different letters indicate significant differences ($p < 0.001$, Cox–Mantel test).

artificial cushions filled with soils with different microbiota communities (none, only two fungi species, and natural/native/field communities) showed a strong positive effect of endophytes inhabiting cushion roots on the survival and performance of the three cushion-beneficiary plant species. Native soil included all soil microbiota such as fungi and bacteria which

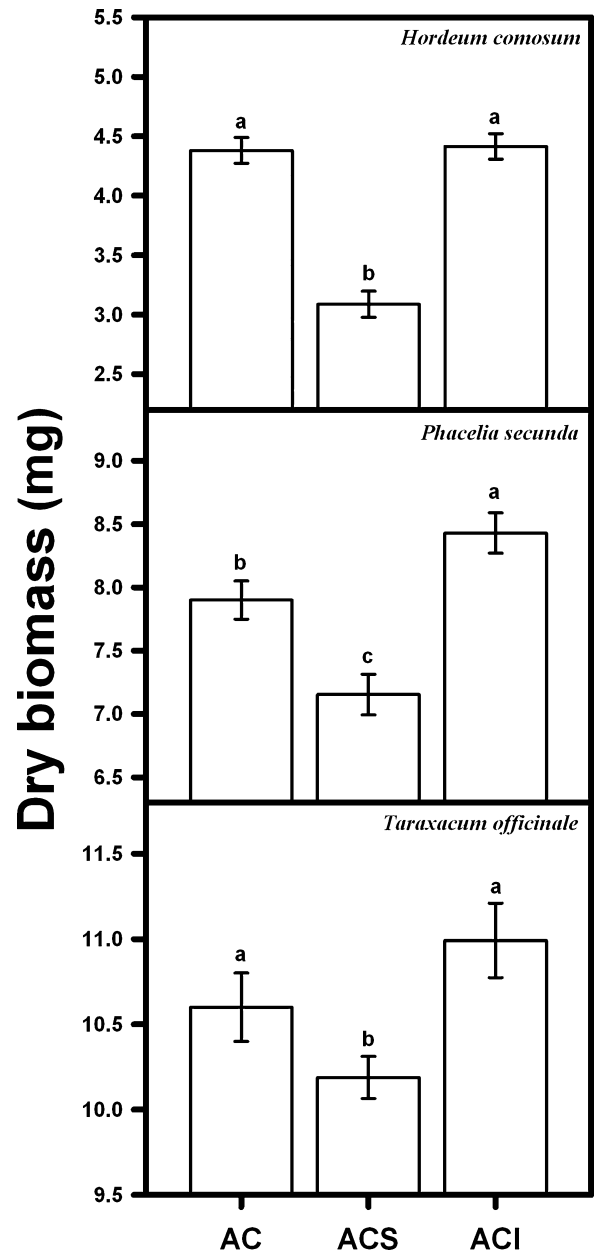


Fig. 3. Dry biomass (mg) of individuals of *Hordeum comosum*, *Phacelia secunda*, and *Taraxacum officinale* transplanted in artificial treatment cushions in the field site. Treatments and legend as in Fig. 2. Bars are means \pm 1SE; initial $n = 100$ for *T. officinale* and *P. secunda*, and $n = 70$ for *H. comosum*. Different letters indicate significant differences ($p < 0.001$, Tukey's test).

effects on plants can range from being pathogenic which effects on plants can range from being to beneficial (Kardol et al., 2006; van der Heijden and Horton, 2009; Rodríguez et al., 2009; van der Putten et al., 2013). Plants growing in this native soil performed much better than those in sterile soil (without soil biota), but, interestingly, plants growing in soil only inoculated with some particular endophyte species performed equally well (the case of *H. comosum* and *T. officinale*) or even better (*P. secunda*) than under native soils. These results suggest that: (i) the positive effects of soil biota on plant performance could be mainly attributable to the inoculated endophyte fungi species and (ii) in this system, soil pathogens seemed to have a less important effect – for *H. comosum* and *T. officinale* – than soil fungi since plants in sterile soil

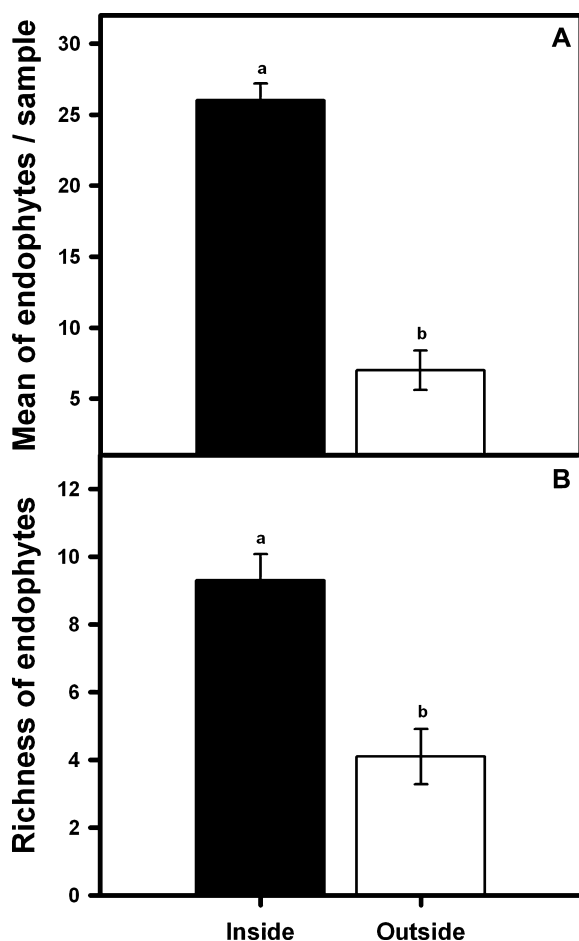


Fig. 4. Abundance (A) and richness (B) of endophytes present in samples from the rhizosphere of *Taraxacum officinale* individuals growing inside and outside of *Laretia acaulis* cushions in the field site. Bars are means \pm 1SE. Different letters indicate significant differences ($p < 0.001$, ANOVA).

(without pathogens and fungi) performed poorly, whereas plants growing in native soils performed better, probably due to increased pathogen resistance in plants associated with endophyte fungi (Sikes et al., 2009; Vos et al., 2012). Nevertheless, we found an opposite trend for *P. secunda* because its survival percentage was lower in the native soils (AC) compared to re-inoculated soils (ACI) treatment.

Root endophyte networks are composed of different fungal species and can connect different plant species (Newman, 1988). It is known that symbiotic association with endophytes can provide drought tolerance to plants (Auge, 2001; Atala et al., 2012), and may improve plant performance in environments with low nutrients (Smith and Read, 2010) such as alpine environments (Cavieres et al., 2006). Thus, in our system in the Mediterranean Andes of central Chile, where summer drought is common (Cavieres et al., 2005; Molina-Montenegro et al., 2011), the positive effects of endophytes on the three cushion-beneficiary plant species might be due to increased nutrient uptake and increased resistance to drought stress. We used the two most common endophytes isolated from the cushion plant roots, *G. pannorum* and *L. lecanii*. These fungi species have been suggested to provide environmental tolerance and biotic protection to infected plants, respectively (Hayes, 2012; Jackson et al., 2012). For instance, *G. pannorum* have been found to improve the cold tolerance and water stress in the associated plants growing in an Antarctic environment (Rosa et al., 2010). On the other hand, *L. lecanii* has been described as an effective

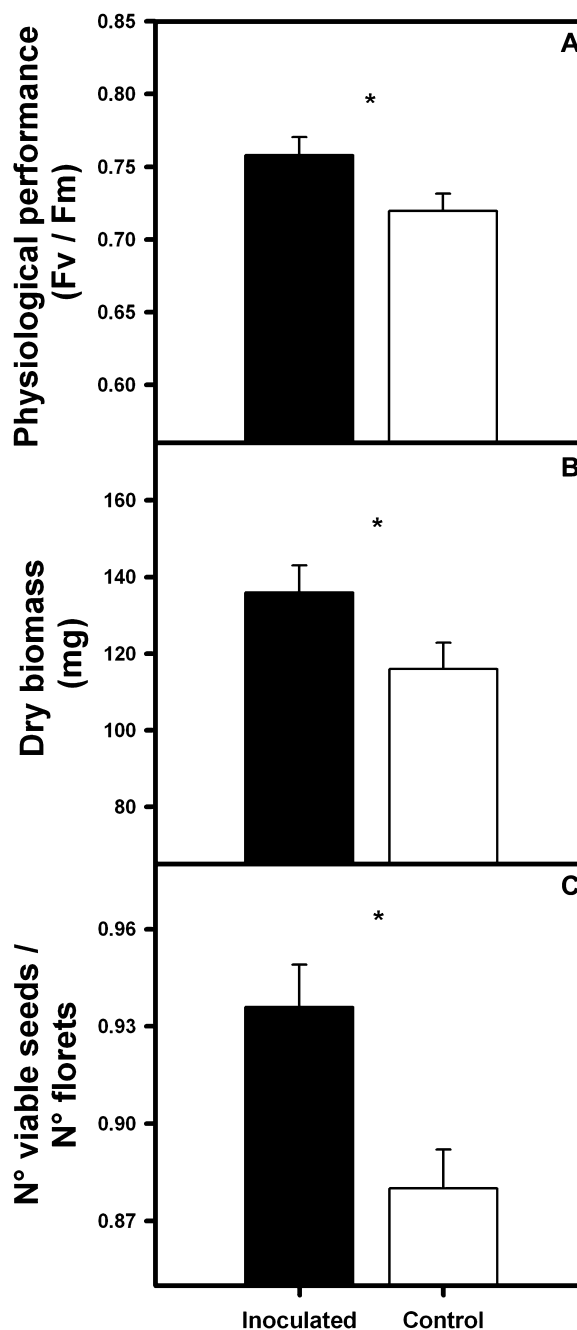


Fig. 5. Maximum quantum yield (Fv/Fm), dry biomass (mg) and seed production of *Taraxacum officinale* individuals inoculated and non-inoculated with a mix of endophytes. Bars are means \pm 1SE; total $n = 80$ individuals. Asterisks indicate significant differences ($p < 0.001$, ANOVA).

entomopathogenic fungi that prevents or reduces the attack of insects on many plant species in field conditions (Jackson et al., 2012). Thus, the strong positive effects induced by fungal endophytes on native and invasive plants displayed in this study, could be explaining, in one hand, the increasing abiotic tolerance to harsh conditions (including water and nutrient deficits) and, in other hand, mediating biotic defense from herbivores or other detrimental organisms.

In the alpine ecosystems in the Mediterranean Andes, the invasive species *T. officinale* is known to be facilitated by native cushion species (e.g., Cavieres et al., 2005, 2008) enhancing alien invasion. Our results show not only that *T. officinale* can be infected

by fungal endophytes associated with the native cushion species *L. acaulis* but also displaying compatibility and profitability. Soil biota could be acting as a facilitating agent for exotic and invasive species (Reinhart et al., 2003; Callaway et al., 2004; Reinhart and Callaway, 2006; Mangla et al., 2008; Aschehoug et al., 2014), particularly endophytic fungi (Núñez et al., 2009b), although it can depend on the particular community and environmental conditions (Shah et al., 2009). Many of the studies that propose plant–soil and plant–plant interactions as significant drivers for biological invasions identify interference (e.g., allelopathy) and negative interactions (i.e., competition) as the most relevant biotic interactions among native and exotic species (Vilà and Weiner, 2004; Callaway and Aschehoug, 2000; Aschehoug et al., 2014). However, some studies have highlighted that positive interactions among plant species may help the invasion process as well (Simberloff and Von Holle, 1999; Richardson et al., 2000; Cavieres et al., 2008; Molina-Montenegro et al., 2008). Examples of direct positive interactions among plants, either between invasive species or between native and invasive species, are scarce (Richardson et al., 2000; Molina-Montenegro et al., 2008). In our study, the three species (two natives and one invasive) performed equally well when infected with fungal endophytes. However, we have not tried yet whether this better performance has relation with differences in the competitive abilities of the species. Therefore, we cannot make a cause-effect relationship among the positive effects of fungal endophytes and the invasion process or enhanced invasiveness of *T. officinale* observed in the field. However, the higher survival, improved fitness-related traits such as plant biomass and seed production, and the higher ecophysiological performance showed by *T. officinale* plants when growing with endophytes suggest that the interaction of root endophytes in soils under *L. acaulis* and this nurse-cushion species could facilitate the establishment and colonization of this invasive species in the field.

In conclusion, our study shows that fungal endophytes associated with a nurse species exert a strong positive effect on native and invasive alpine plants and improves plant survival and establishment in this harsh environment. Facilitation by nurse species in this harsh environment may likely be the result of several factors acting in concert. On one hand, beneficiary species are directly facilitated by cushion plants through the amelioration of abiotic conditions within the benefactor canopy compared to open areas. On the other hand, facilitation of cushion-beneficiary species could be also explained by the positive effect of microbial endophytes (and, probably, other soil biota as well) inhabiting cushion roots and its rhizosphere. Future surveys should consider these positive effects of soil biota from nurse plant species as a possible pivotal driver in the structuring of plant communities inhabiting alpine ecosystems.

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