Germination responses of *Lycium humile*, an extreme halophytic Solanaceae: understanding its distribution in saline mudflats of the southern Puna

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ABSTRACT

Although knowledge about halophytic Solanaceae is scarce, it is known that several species within genus *Lycium* tolerate salinity. *Lycium humile* grows in highly saline soils in mudflats near saline Andean lakes. This study evaluated the germination responses of *L. humile* under different scarification methods, photoperiods, temperatures and saline conditions and, simultaneously, tested seedling survival under different iso-osmotic conditions. Dormancy and germination were found to be regulated by interactions with different factors, with the highest germination percentages being obtained by immersion in sulfuric acid, with a temperature of 25 °C and a temperature regime of 5/25 °C, under which seeds were neutrally photoblastic. As osmotic potential of saline solutions decreased, germination also decreased drastically but the seedling survival percentage was higher than 30 % at 600 mM NaCl. No seeds germinated in any of the polyethylene glycol (PEG) solutions and no seedling survival was observed from -1.2 MPa PEG solutions. More than 90 % of seeds incubated in NaCl were able to recover germination after being transferred to distilled water, independently of NaCl treatments. We concluded that the effects of extreme environmental conditions on germination responses and seed tolerance to salinity may determine the occurrence and restricted distribution of *L. humile*.

Keywords: Andes, halophyte, *Lycium humile*, Puna, salinity, salt tolerance, Solanaceae

Introduction

Salinity is one of the major abiotic stresses that adversely affect worldwide crop productivity, as well as the growth and development of plants (Llanes *et al.* 2016), hence the importance of finding new sources to improve crop salt tolerance through biotechnological strategies (Reginato *et al.* 2012), especially in families with great productive value. Specifically, seed viability, germination and seedling growth are negatively affected by salinity. Low germination and decreased seedling growth result in poor plant establishment and, occasionally, in crop failure (Bajehbaj 2010). Salinity can inhibit the germination and growth of plants because it induces low external water potential (preventing water uptake) and ion toxicity; it also interferes with nutrient uptake (Munns & Tester 2008; Guma *et al.* 2010; Nasri *et al.* 2015; Silva *et al.* 2015). Plants adaptation to salinity during germination and early seedling stages is essential for a successful establishment and depends on the tolerance to

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both osmotic and ionic effects. Ionic effects also depend on the chemical nature of the ions involved, which may interact in a synergistic or antagonistic way (Llanes *et al.* 2005).

Caryophyllales and Alismatales are the orders with the greatest number of halophytic species; on the other hand, Solanales has been reported on a smaller scale, and little is known about Solanaceae in particular (Flowers & Colmer 2008; Flowers *et al.* 2010), a family of fundamental economic importance with worldwide distribution (Solanaceae Source 2020). Only few species of Solanaceae genera have been reported to grow in saline environments. However, *Lycium* is the genus with the greatest number of recorded species growing in saline soils (Flowers *et al.* 2018) and some of them have salt tolerance, *e.g. L. barbarum* (Wei *et al.* 2006; Wu *et al.* 2015; Dimitrova *et al.* 2017a; b), *L. carolinianum* (Butzler & Davis III 2006), *L. chinense* (Dimitrova *et al.* 2017a; b), *L. nodosum* (Tezara *et al.* 2003) and *L. ruthenicum* (Wang *et al.* 2015). Recently, Cantero *et al.* (2016) asserted that Solanaceae is one of the five families with the highest number of taxa in the halophytic flora of Argentina, *Lycium* being the most species-rich genus inhabiting saline environments. Nonetheless, there are no studies about the physiological responses of *Lycium* species growing in saline soils.

Lycium humile (tribe Lycieae) is a dwarf shrub endemic to northeastern Chile and northwestern Argentina, inhabiting salars (salt flats) in the Puna region, between 3000 and 4000 m a.s.l. (Cabrera 1957; Bernardello 1986; 2013). The Puna is a high-altitude cold desert, with an average annual temperature of 8 °C and large diurnal temperature fluctuations. Precipitations are below 200 mm per year, being even lower (<50 mm) on the western edge. The Puna plateau comprises broad, internally drained depocenters; these sedimentary basins contain continental evaporites and clastic deposits, and have high salt concentrations, halite and gypsum being the dominant minerals (Alonso *et al.* 1991; 2006; Strecker *et al.* 2007). In this region, *L. humile* plants grow in the clayey mudflats near to the saline lakes (Fig. 1), where highly saline soils can be found. Additionally, this species plays a relevant ecological role in the ecosystem dynamics of the salars, showing high plant cover and being, on several occasions, one of the few growing in these sites (Cabrera 1957; Luebert & Gajardo 2000; Carilla *et al.* 2018).

Lycium humile is an interesting case of study because it grows exclusively in the Puna salt flats which present multi-stressed environmental conditions such as extreme temperatures, water deficit, and salinity (Malatesta *et al.* 2016), but particularly because it lives in highly saline soils. Moreover, no studies have yet been reported on the germination responses to salinity in American *Lycium* species. Thus, *L. humile* may be considered a new model for studying salt tolerance mechanisms. Based on this background, we hypothesized that seed germination and seedling growth are regulated by different environmental factors (*i.e.* temperature, photoperiods, salts concentration,

water availability). We expect that specific seed responses to extreme factors will determine the occurrence and restricted distribution of *L. humile* plants in saline mudflats of the southern Puna. Therefore, the aims of the present study were: (1) to evaluate the germination responses of *L. humile* under different scarification methods, temperatures and photoperiods; (2) to establish the effect of increasing salinity with monosaline and bisaline iso-osmotic solutions of NaCl and KCl (salts present in the soils of the study site) on germination and seedling survival, and at the same time, differentiating the osmotic effects from the ion toxicity effects; (3) to assess the ability of *L. humile* seeds to recover their germination in distilled water after being exposed to NaCl solutions; and (4) to discuss the possible causes that explain the occurrence and restricted distribution of *L. humile*. To our knowledge, this is the first study focused on the germination responses of an extreme halophytic Solanaceae.

Figure 1. *Lycium humile* growing in the saline mudflat surrounding the saline lake.

Materials and methods

Plant materials and site conditions

Mature fruits of *Lycium humile* Phil. were collected during summer 2017 from "Salar del Diablo" (24º37'52'' S, 67º15'46'' W, 3841 m a.s.l.) in the Puna region, province of Salta, Argentina. The voucher specimen was identified by G.E. Barboza and was deposited in the herbarium of the Botanical Museum of Cordoba (CORD), National University of Cordoba (Argentina): voucher number CORD 00053244. Berries were randomly collected from at least 50 plants, with

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a minimum distance of three meters between each plant; seeds were separated from the fruits and stored at 4 °C until used two–eight months later in the experiments. Seeds were selected visually for uniform size and healthy aspect.

Fifteen random soil sub-samples were taken from the collection site and pooled as a composite sample for saturated paste extract analysis. The clay-textured soil has high salinity and its saturated paste extract shows 290 dS.m^{-1} electrical conductivity, 29 g.l^{-1} Na$^+$, 1.6 g. l^{-1} K$^+$, 47 g.l^{-1} Cl$^-$ and pH 8.04 at the surface.

Climate data were obtained from WorldClim 2 (Fick & Hijmans 2017; https://www.worldclim.org/) at a spatial resolution of 30 seconds (~1 km^2) (Tab. S1 in supplementary materials).

Scarification methods

Seeds were submitted to four scarification methods which included: 1) Mechanical scarification by: A) manual abrasion with sandpaper; B) incision at the rounded end of the seed with a scalpel (Baes *et al.* 2002; Patanè & Gresta 2006; Can *et al.* 2009). 2) Heat scarification by soaking the seeds in a hot-water bath at 100 °C for four minutes and then gradually placing them into cold water overnight (Uzun & Aydin 2004; Can *et al.* 2009). 3) Acid scarification by soaking in sulfuric acid (96 %) for four, eight, 10, 15, 20 and 30 minutes, then soaking overnight in tap water (Martin & Cuadra 2004; Kim *et al.* 2008; Alderete-Chavez *et al.* 2010). 4) Soaking the seeds in tap water for 24 hours. After scarification, the seeds were placed in Petri dishes with two filter-paper discs saturated with distilled water and incubated in darkness at 30 °C. Seeds without scarification were used as the control treatment. Four replicates of 25 seeds each were used for each treatment. Germinated seeds were counted at 2-day intervals for 14 days.

Temperature treatments

Sulfuric acid-scarified seeds were germinated in darkness at different temperatures: 10, 25, 27, 30 and 38 ºC. The number of germinated seeds was recorded at 2-day intervals for 10 days. Then, in order to examine the effects of light and temperature requirements during germination, seeds were incubated daily (12 h/12 h) at four temperature regimes (10/20, 5/25, 10/25 and 15/25 °C) in a 12 h dark/12 h light photoperiod (where the higher temperature coincided with the light period (1300 lux) and in continuous darkness. These temperatures regimes were selected because germination was higher at 25 °C (previously tested) and due to the high daily temperature fluctuation in the Puna region, of even 20 °C (Alonso 2013). Darkness was attained by wrapping the Petri dishes with aluminum foil. Germinated seeds were counted at 2-day intervals for 30 days. Four replicates of 25 seeds each were used for each treatment.

Salt and osmotic treatments

To differentiate the osmotic effects from the ion toxicity effects, comparisons between salt solutions and the osmotic agent polyethylene glycol (PEG) were carried out. Each treatment had 100 seeds divided among four replicates of 25 seeds.

Sulfuric acid-scarified seeds were placed in Petri dishes with two discs of filter paper saturated with distilled water for the control treatments [osmotic potential (Ψo) = -0.011 MPa] and the corresponding solutions of NaCl, KCl, their iso-osmotic mixture (NaCl + KCl) and PEG 6000 in concentrations calculated to obtain the following Ψo: -0.4, -0.8, -1.2, -1.5, -1.9 and -2.2 MPa (Sosa *et al.* 2005) (Tab. S2 in supplementary material). The osmotic potentials of salt and PEG solutions were measured with a vapor pressure osmometer (Model 5500, Wescor Inc. Logan, UT, USA).

Seeds were incubated for 14 days in darkness at 25 °C. Seedling survival under different concentrations of salts and PEG was tested. Scarified seeds were placed in Petri dishes with two discs of filter paper saturated with distilled water. Germinated seedlings with a radicle of 1 cm (approx. seven days) were transferred to Petri dishes saturated with salt or PEG solutions at concentrations previously mentioned (Tab. S2 in supplementary material), distilled water was used as the control treatment. Seedlings were incubated in a 12 h light (1300 lux)/12 h dark photoperiod at 25 °C and on the seventh day the percentage of living plants was recorded (living plants: seedlings with no visible signs of plant senescence; Ougham *et al.* 2005).

Germination recovery

Four replicates of 25 sulfuric acid-scarified seeds each were sown under six different salinity concentrations (0, 50, 100, 200, 400 and 600 mM NaCl). Seeds were germinated in Petri dishes with two discs of filter paper saturated with the test solution. Petri dishes were incubated at 25 °C (12 h light (1300 lux)/12 h dark). Germinated seeds were counted at 2-day intervals for 30 days. After 30 days all seeds that failed to germinate in the different NaCl solutions were transferred to distilled water. Germinated seeds in distilled water were counted at 2-day intervals for 14 days.

Data analyses

The experiments followed a completely randomized design. All treatments were carried out with 100 seeds (four replicates of 25 seeds each), and the experimental unit was one Petri dish.

Germination responses were evaluated using the final germination percentage and / or germination rate, the

latter was estimated using a modified Timson's velocity index (Timson 1965): ΣG/t, where G is the percentage of seeds germinated after 2-day intervals and t the total time of germination (Khan & Ungar 1984). A higher value of Timson's index indicates more rapid germination.

Data were arcsine transformed and analyzed using a one-way ANOVA. Least Significant Difference (LSD) Fisher test (p < 0.05) was used a posteriori. Normality and analysis of variance assumptions were corroborated, and for cases without homogeneity, a non-parametric test (Kruskal Wallis) was performed followed by nonparametric multiple comparisons (Conover 1999).

InfoStat v. 2016 (Di Rienzo *et al.* 2016) software was used to perform all statistical data analysis.

Results

Scarification methods

The highest germination percentages were obtained with acid scarifications for eight, 15 and 20 min (Fig. 2A). Remarkably, although acid scarification for 15 min was not the treatment with the highest germination percentage, it was the fastest since ≈50 % germination was obtained by the fifth day of incubation (Fig. 2B). On the other hand, germination was null for heat scarification, seed incision and acid scarification of 30 min treatments (Fig. 2).

Temperature treatments

The optimal germination temperature was determined to be 25 °C (Fig. 3A-B), since seeds reached about 90 % of germination and showed the highest germination rate index. On the other hand, germination was scarce at 38 °C and null at 10 °C. Regarding the temperature regimes for germination, 5/25 °C was the treatment that showed the highest germination rate index and percentage; moreover, seeds were neutrally photoblastic at this regime (Fig. 4A-B).

Salt and osmotic treatments

No seeds germinated at the different PEG concentrations used in the experiment. Similarly, seeds placed in salt solutions at potentials lower than Ψo: -0.4 MPa did not germinate. In addition, the percentage of germination at 100 mM (=Ψo: -0.4 MPa) was significantly lower compared to the seeds germinated in distilled water (Fig. 5).

Furthermore, 7-day seedlings showed no significant differences in survival between the control and salts treatments at -0.4 MPa. However, PEG treatments showed a decrease in the percentage of survival being null at osmotic potentials lower than -0.8 MPa. Seedlings showed a higher percentage of survival in the NaCl treatments than under the other salt treatments (Fig. 6).

Germination recovery

The final germination percentage and germination rate index were severely affected by NaCl treatments (Fig. 7A-B). Thus, a significant decrease in the final germination and germination rate index was observed following an increase in NaCl concentration. However, the ability of seeds to recover their germination in distilled water after being exposed to NaCl solutions was not significantly affected. The final germination percentage during the recovery phase
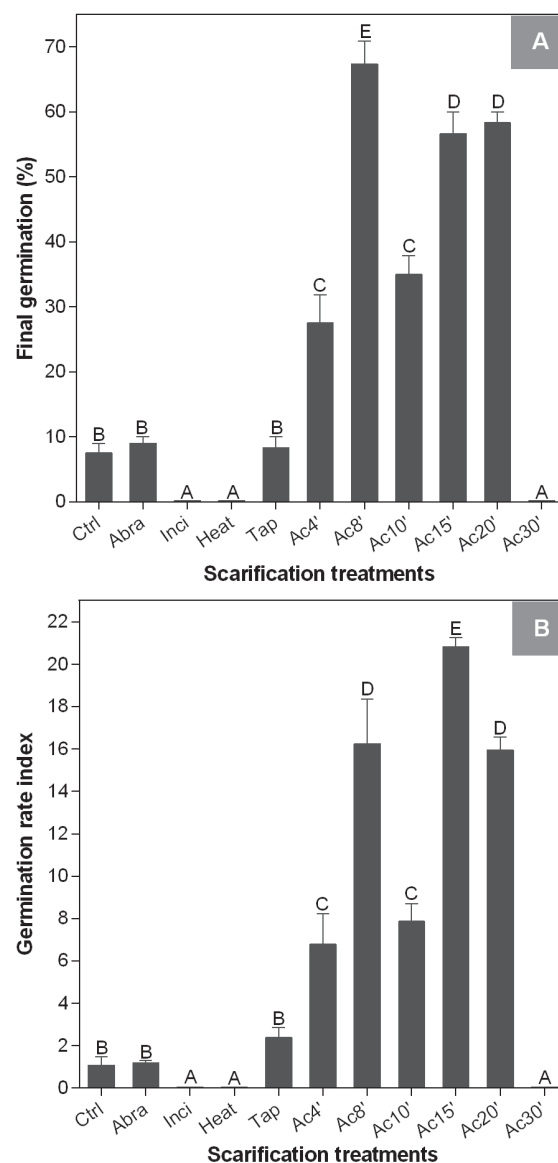
Figure 2. Germination of *Lycium humile* seeds after different scarification methods during 14 days (mean ± SE). **A)** Accumulated germination percentage; **B)** Germination rate. Treatments: distilled water (Ctrl), sandpaper abrasion (Abra), incision (Inci), hot-water bath (Heat), washing for 24 h (Tap), sulfuric acid (96 %) (Ac) and different time periods (4 to 30 min). Bars with different letters are significantly different (p < 0.05).

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(seeds germinated after being transferred to distilled water) did not differ significantly between treatments; however, the germination rate index was lower in seeds exposed to higher NaCl concentrations.

Discussion

This is the first study performed to evaluate and analyze the germination responses of *Lycium humile* under different scarification methods, temperatures, photoperiods, and salt and osmotic treatments.

Different scarification methods are useful for softening hard seeds and improving germination and seedling establishment. However, the most effective scarification method varies between species since prolonged time treatments can cause seed injuries (Taia 2004; Kimura & Islam 2012). Our results show that the germination

percentage of *L. humile* seeds without scarification was very low and the sulfuric acid treatment for 15 minutes was the most effective method for *L. humile*; suggesting that in natural conditions, *L. humile* seeds are dormant, *i.e.* seeds that do not have the ability to germinate under otherwise favorable environmental conditions (Baskin & Baskin 2004), and may require passage through animal digestive tracts, such as birds or mammals, where gastric acids are found. The passage of seeds through the digestive tract of herbivores could result in seed dormancy release. This is attributed to the gastric acid that scarifies the seed coats, making them permeable and leading to higher germination percentages after defecation (Jaganathan *et al.* 2016). In fact, during our visits to the Puna salt flats, we observed birds from the
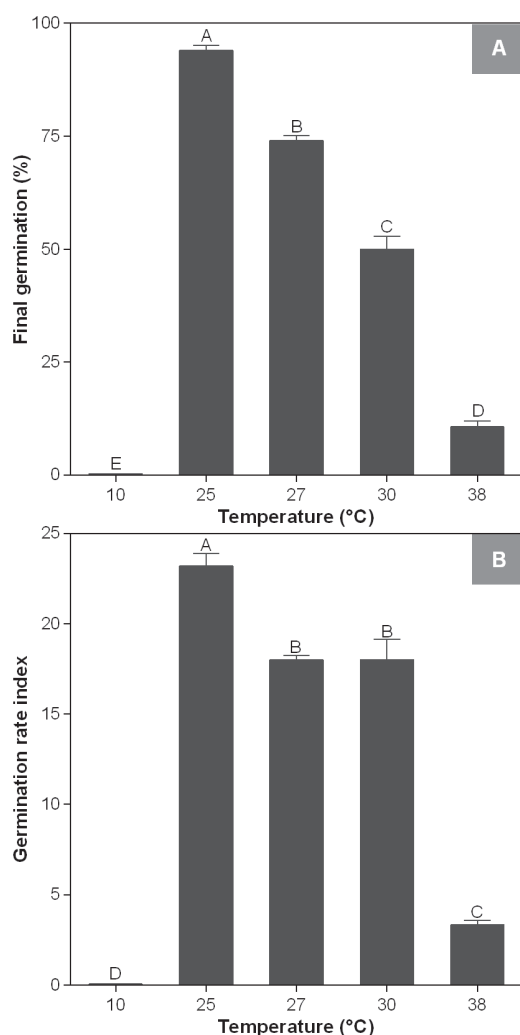
Figure 3. Effects of incubation temperature on germination of *Lycium humile* seeds during 10 days (mean ± SE). **A)** Accumulated germination percentage; **B)** Germination rate. Bars with different letters are significantly different (p < 0.05).
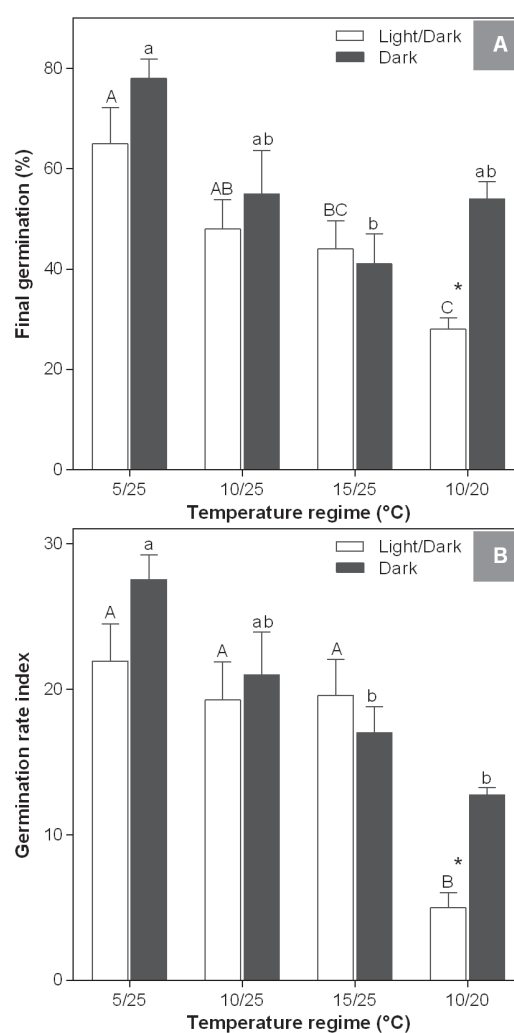
Figure 4. Effect of the temperature regime and photoperiod on germination (mean ± SE) of *Lycium humile* seeds. **A)** Accumulated germination percentage; **B)** Germination rate. Capital letters were used to compare germination between different temperature regimes for light/dark photoperiod, while lowercase letters were used for dark treatment. Bars with different letters are significantly different (p < 0.05). *: p < 0.05 was used for comparing germination between different photoperiods at the same temperature.
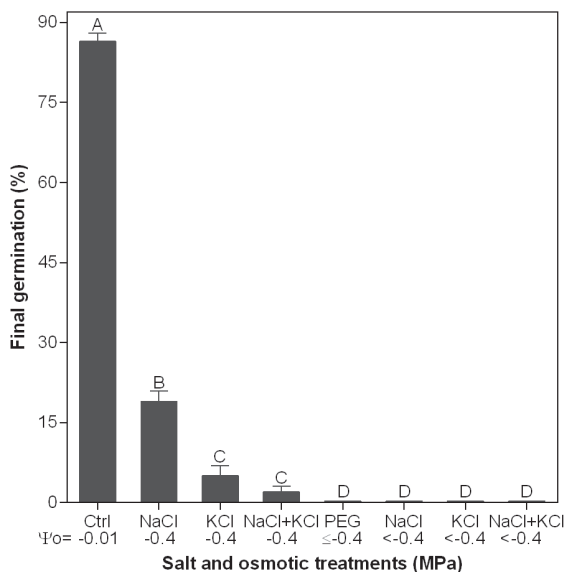
**Figure 5.** Effect of salinity on germination of *Lycium humile* seeds (mean ± SE) during 14 days. Bars with different letters are significantly different (p < 0.05).

genus *Muscisaxicola* and "vicuñas" (*Vicugna vicugna*) eating *L. humile* fruits which would favor seed germination.

The highest germination of *L. humile* occurs at 25 °C and in the 5/25 °C temperature regime. This temperature regime corresponds to those observed during summer months (December to February) in the saline areas where it grows; it should be noted that *L. humile* had ripe fruits by the end of January (pers. obs.) coinciding with the rainy season in these areas. Therefore, high summer temperatures and diurnal temperature fluctuation could be important cues for seed dormancy-breaking. In addition, this broad temperature regime for germination of *L. humile* is similar to those temperatures registered for germination of other *Solanum* species (Ahmed *et al.* 2006). In this sense, Jaganathan *et al.* (2017) suggested that dormant seeds might be a dominant trait in dry ecosystems because the low humidity and high temperatures prevalent at these sites, would dry the seeds to lower moisture content; and because soil seed persistence would be important for the successful establishment of plants in dry environments due to the narrow window of opportunity for germination compared to other habitats
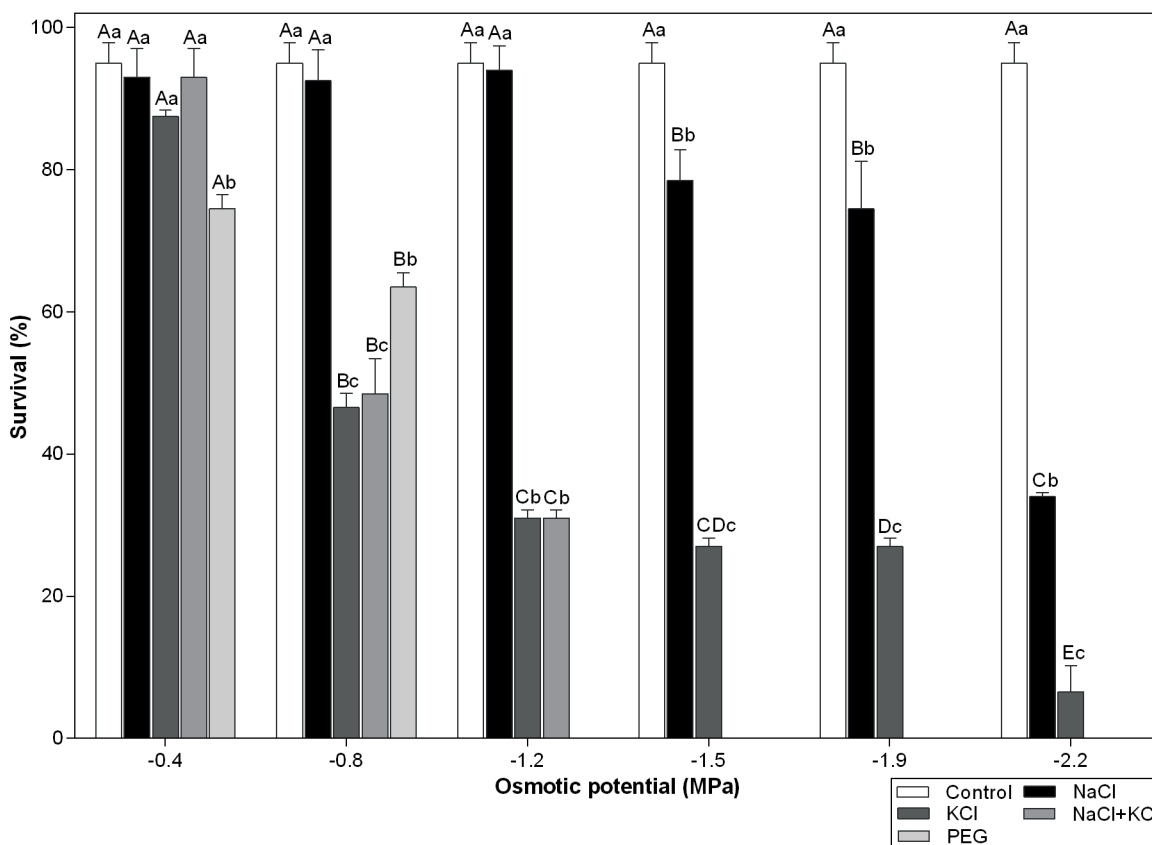
Figure 6. Final survival percentage (mean ± SE) of seven-day-old *Lycium humile* seedlings transferred to different iso-osmotic solutions. The final percentage of live seedlings was measured on the seventh day; distilled water was used as control (Ctrl). Bars with different letters are significantly different (p < 0.05). Capital letters were used to compare the survival percentage between different concentrations (osmotic potential) of the same salt or PEG, while lowercase letters were used to compare the survival percentage between iso-osmotic treatments.
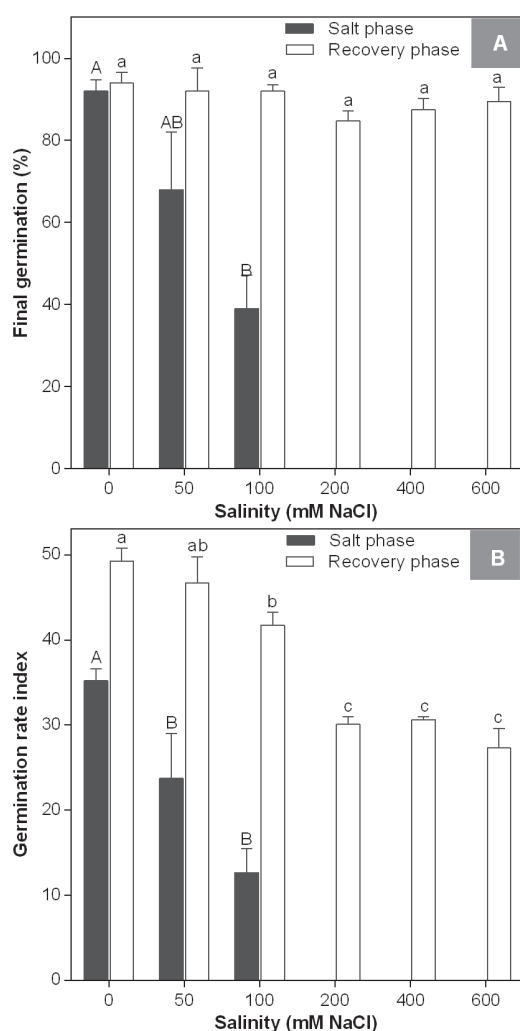
where water and appropriate temperature required for germination are available throughout the year.

Seed germination is an important stage of plant development and can be influenced by many abiotic factors, salinity being one of major ones affecting it. Regarding the effect of salinity on *L. humile* germination and seedling survival, our results showed that KCl treatments reduced germination more than NaCl treatments. Similarly, seed germination of *Prosopis strombulifera*, a genus commonly found in halophytic plant communities of America, was more affected by KCl than NaCl demonstrating that a specific ion effect of the salts present in the medium modifies the germination responses (Llanes *et al.* 2005; Sosa *et al.* 2005). Furthermore, PEG solutions demonstrated deleterious

Figure 7. Germination of *Lycium humile* seeds at different saline conditions (salt phase) during 30 days and then transferred to distilled water (recovery phase) for 14 days (mean ± SE). **A)** Final germination percentage; **B)** Germination rate index. Bars with different letters are significantly different (p < 0.05). Capital letters were used to compare germination during the salt phase, while lowercase letters were used to compare germination during the recovery phase.

effects on *L. humile* seed germination. These results indicate that the seeds or seedlings absorb ions that allow them to incorporate water due to lower salt-induced water potential. Therefore, although *Lycium* species are characterized by their drought-tolerance and salt-tolerance (Dimitrova *et al.* 2017b), our results suggest that *L. humile* has a greater tolerance to ionic effects of salt solutions than to osmotic effects; this phenomenon has also been reported in other species from arid environments of South America (*e.g.*, *Prosopis*; Villagra & Cavagnaro 2006; Meloni 2017).

Regardless of the saline conditions, *L. humile* germinates better and faster in distilled water, as has already been reported for most halophytes (Ahmed & Khan 2010; El-Keblawy *et al.* 2017; Bhatt *et al.* 2019a; b; 2020). On the other hand, results of recovery experiments suggest that the viability of *L. humile* seeds is not affected by salt concentrations up to 600 mM NaCl. However, the speed of recovery is lower for those incubated under higher salinity conditions. This result supports the hypothesis that seeds do not germinate until the salinity levels under natural conditions are reduced by dissolution, produced either by rain or the thawing of snow. This response has been reported by several researchers in other halophytes (Poljakoff-Mayber *et al.* 1994; Ungar 1996; Keiffer & Ungar 1997; Bhatt & Santo 2016). In the Puna habitats, water is available after snowfalls, which occur mainly during June and July (Alonso 2013). However, the most appropriate conditions for germination would be during the summer months when precipitation and high temperatures occur (Tab. S1 in supplementary materials). In addition, precipitations in the Puna region show great interannual variations (Maggi *et al.* 2010). Thus, *L. humile* seeds might remain viable and dormant for long periods waiting for optimal germination conditions. Indeed, perennial halophytes do not necessarily have to recruit seedlings every year; they can even use ramets to reproduce clonally. The occasional recruitment via seeds, which occurs during years of good growth conditions, promotes genetic variation among populations (Gul *et al.* 2013).

As we previously stated, *L. humile* distribution is apparently restricted to mudflats of the Andean salt flats (see Fig. 1) were plants form isolated plates as mosaics (Carilla *et al.* 2018). This distribution may be related to water availability. It is known that water availability in the Puna region is a restricting factor; however, there are sites such as salars where plants have access to water from the water table near to the surface; but at the same time, high concentrations of water-soluble salts promote physiologically dry soils. So, halophytes are the only plants able to grow there, since they have mechanisms that allow them to incorporate water by decreasing their water potential. In accordance with this, Grigore *et al.* (2012) suggested that the distribution of halophytes in nature does not only depend on soil salinity *per se*, but also on the availability of water and nutrients in natural saline habitats.

Conclusion

Lycium humile is one of the few halophytic Solanaceae species that can germinate and survive under saline conditions. We found that acid scarification and temperature regime promote dormancy release in *L. humile* seeds. We also found that low osmotic potential solutions have negative effects on the seed germination and seedling survival, which can be attributed to osmotic rather than ionic stress. However, seed viability is not affected by high salt concentrations. Further studies on the effects of extreme environments on germination and seedling growth of endemic halophytes are crucial for the conservation of halophytic plant communities in the Puna region.

Acknowledgements

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