

Drought increases the freezing resistance of high-elevation plants of the Central Chilean Andes

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Abstract Freezing temperatures and summer droughts shape plant life in Mediterranean high-elevation habitats. Thus, the impacts of climate change on plant survival for these species could be quite different to those from mesic mountains. We exposed 12 alpine species to experimental irrigation and warming in the Central Chilean Andes to assess whether irrigation decreases freezing resistance, irrigation influences freezing resistance when plants are exposed to warming, and to assess the relative importance of irrigation and temperature in controlling plant freezing resistance. Freezing resistance was determined as the freezing temperature that produced 50 % photoinactivation [lethal temperature (LT_{50})] and the freezing point (FP). In seven out of 12 high-Andean species, LT_{50} of drought-exposed plants was on average 3.5 K lower than that of irrigated plants. In contrast, most species did not show differences in FP. Warming changed the effect of irrigation on LT_{50} . Depending on species, warming was found to have (1) no effect, (2) to increase, or (3) to decrease the irrigation effect on LT_{50} . However, the effect size of irrigation on LT_{50} was greater than that of warming

for almost all species. The effect of irrigation on FP was slightly changed by warming and was sometimes in disagreement with LT_{50} responses. Our data show that drought increases the freezing resistance of high-Andean plant species as a general plant response. Although freezing resistance increases depended on species-specific traits, our results show that warmer and moister growing seasons due to climate change will seriously threaten plant survival and persistence of these and other alpine species in dry mountains.

Keywords Alpine · Climate change · Moisture · Freezing temperature · Warming

Introduction

The ability to resist freezing temperatures is an important determinant of plant species' distributions (Woodward 1987), and is one of the first environmental filters that species have to overcome to inhabit high-elevation (i.e., alpine) habitats (Körner 2003). Studies conducted on alpine species from temperate zones show that plants are able to resist temperatures between -7 and -10 °C during the growing season (Sakai and Otsuka 1970; Taschler and Neuner 2004; Bannister et al. 2005). However, studies conducted on alpine plants from high-elevation habitats that experience dry summers have shown that these plants can resist freezing temperatures as low as -20 °C during the growing season (Squeo et al. 1996; Sierra-Almeida et al. 2009, 2010; Venn et al. 2013), which suggests an enormous safety margin for thermal injury, since freezing events of that magnitude are infrequent during this period (Sierra-Almeida and Cavieres 2010; Sierra-Almeida et al. 2010; Venn et al. 2013).

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The greater freezing resistance of alpine species from mountains with summer droughts has been attributed to the high desiccation tolerance of these plants, which is considered a pre- or co-adaptation in high-elevation zones with a dry season (Sakai and Larcher 1987). As freezing is ultimately a cell dehydration phenomenon, plants exposed to freezing temperatures may show similar physiological responses to those exposed to drought (Chaves et al. 2003; Beck et al. 2007). For example, plants favor osmotic adjustments to deal with stress produced by water deficit and also freezing temperatures (Larcher 2003). This close relationship between plant responses to freezing and drought has been used for agricultural purposes (e.g., Cloutier and Siminovitch 1982; Sevillano et al. 2009; Sales et al. 2013) and in forestry (e.g., Blödner et al. 2005; Kreyling et al. 2012a; Navarrete-Campos et al. 2013) to improve either plant survival, productivity, or both. However, the extent of these two factors in co-determining freezing resistance under natural conditions in native plant species has been poorly studied (Blake and Hill 1996; Lambrecht et al. 2007a; Kreyling et al. 2012b). A greenhouse study carried out with plants of *Larrea tridentata* obtained from the high-latitude edge of the Chihuahuan Desert found that drought increased the freezing tolerance of their leaves and xylem. This drought-freezing interaction was suggested as a possible mechanism to explain potential changes in abundance and distribution of this shrub species in the face of changing temperature and precipitation regimes (Medeiros and Pockman 2011).

Mediterranean high-elevation habitats are characterized by growing seasons with low precipitation as well as low temperatures (Valladares et al. 2004; Lara et al. 2005). Thus, plants have to deal with both freezing temperatures and drought during the favorable season for growth (Cavieres et al. 2007; Giménez-Benavides et al. 2007; Sanfuentes et al. 2012; Olano et al. 2013). Several studies have dealt with the freezing resistance of alpine plants in Mediterranean-type mountains (e.g., Loik and Redar 2003; Loik et al. 2004; Sierra-Almeida et al. 2009, 2010; Sierra-Almeida and Cavieres 2012). However, most of these studies evaluated the relevance of temperature on plant freezing resistance. None of them have addressed whether plant freezing resistance in alpine habitats is also influenced by drought. In the Central Chilean Andes, alpine species have shown temporal and spatial variation of freezing resistance that seems to be influenced by summer drought. For example, the freezing point (FP) and lethal temperature (LT_{50}) of several species showed intra-seasonal variation, where lower FP and LT_{50} occur when soil water potential (Ψ_{H_2O}) was at its lowest (Sierra-Almeida et al. 2009). FP may vary according to the cell sap concentration and/or to the accumulation of water-binding substances inside the cell. Thus, its variation is an indicator of osmotic adjustment (Sakai

and Larcher 1987), which could improve plant freezing survival (Loik and Nobel 1993). Likewise, altitudinal variation in FP and LT_{50} also show some correlation with soil moisture availability, although it is not possible to disentangle the effects of temperature and drought (Sierra-Almeida et al. 2009). Thus, an experimental field approach, where temperature and soil moisture effects on plant freezing resistance can be teased apart, could help to elucidate the relative importance of both stressors in explaining temporal and spatial patterns of freezing resistance of high-Andean species and other alpine species from Mediterranean-type mountains.

In this study, we conducted a field experiment in a high-elevation site in the Andes of Central Chile where we varied the temperatures and soil moisture levels experienced by different plant species during two growing seasons to assess their effects on plant freezing resistance. In particular we assessed whether: (1) irrigated plants show less freezing resistance than non-irrigated plants (leaf FP and LT_{50} of irrigated plants should occur at higher temperatures than those of non-irrigated plants); (2) irrigation creates changes in LT_{50} and FP when plants are exposed to increases in environmental temperature (i.e., warming; given that warming and drought have antagonistic effects on the freezing resistance we expected that the LT_{50} increment produced by irrigation would be greater for warmed plants than for unwarmed plants). Our final goal was to assess the relative importance of drought and temperature in controlling the freezing resistance of high-Andean plants.

Alpine habitats have been suggested to be one of the most sensitive to global climate change (Guisan and Theurillat 2000; Körner 2000; Thuiller et al. 2008) because they are strongly controlled by environmental conditions and most of the plants in them function near their physiological limits (Körner 2003). Recent studies indicate that warming will increase alpine plants vulnerability to freezing damage (Martin et al. 2009; Sierra-Almeida and Cavieres 2010; Wheeler et al. 2014). However, we do not know how summer rainfall will vary in the future, and how this might influence alpine plant responses to the predicted increases in temperature.

Materials and methods

Study area

This research was carried out near Farellones, an alpine village 50 km east of Santiago (Chile). The study area was located at 2900 m a.s.l., and covers approximately 3200 m² on a northwest-facing slope, near La Parva ski

Table 1 Microclimate of plants exposed to experimental irrigation and warming in the Central Chilean Andes during two growing seasons

	−W		+W	
	−H ₂ O	+H ₂ O	−H ₂ O	+H ₂ O
Growing season 2011–2012				
Length of the growing season (days)	114			
Daily mean air temperature (°C)	11.2 ± 0.3 b	11.1 ± 0.3 b	13.6 ± 1.1 a	14.1 ± 0.1 a
Maximum air temperature (°C)	21.8 ± 0.3 c	21.5 ± 0.6 c	29.3 ± 0.9 b	30.8 ± 0.3 a
Minimum air temperature (°C)	3.9 ± 0.2 a	3.8 ± 0.2 a	3.9 ± 0.7 a	3.9 ± 0.2 a
Frequency of freezing events (%)	4.4 ± 2 a	4.4 ± 2.6 a	4.8 ± 4.4 a	5 ± 2.1 a
Intensity of freezing events (°C)	−1 ± 0.1 a	−1 ± 0.1 a	−1 ± 0.2 a	−1.1 ± 0.2 a
Growing season 2012–2013				
Length of the growing season (days)	134			
Mean air temperature (°C)	9.7 ± 0.04 b	–	12.2 ± 0.3 a	–
Maximum air temperature (°C)	19.2 ± 0.4 b	–	26.7 ± 0.5 a	–
Minimum air temperature (°C)	2.6 ± 0.1 a	–	2.6 ± 0.1 a	–
Frequency of freezing events (%)	19.4 ± 3.1 a	–	19.4 ± 3.9 a	–
Intensity of freezing events (°C)	−1.5 ± 0.1 a	–	−1.6 ± 0.04 a	–

Values correspond to mean ± SE ($n = 3–4$)

−W Unwarmed, +W warmed, −H₂O non-irrigated, +H₂O irrigated

Different lowercase letters indicate significant differences ($P < 0.05$)

resort (33°21'S, 70°19'W). This area has a plant coverage of <50 % and is characterized by dominance of the cushion plant *Laretia acaulis* Gillies and Hook. (Apiaceae), and the presence of shrubs such as *Berberis empetrifolia* Lam. (Berberidaceae) and *Nassauvia looseri* Cabrera (Asteraceae), annuals and several perennial herbs (Cavieres et al. 2000; Teillier et al. 2011). The climate in the study area is alpine, strongly influenced by the Mediterranean-type climate that predominates at lower elevations, which generates a dry growing season (Santibañez and Uribe 1990). The growing season usually starts with the snowmelt in October and finishes in April with the first snowfall. In this period, the mean air temperature is 9.8 °C, with a maximum and minimum of 19 and 3 °C, respectively (Table 1), and freezing events below −5 °C may occur unpredictably (Sierra-Almeida et al. 2010). Water shortage here becomes important for plants towards the second half of the growing season (January–March), when soil Ψ_{H_2O} may decrease below −3 MPa at 20-cm soil depth (Sierra-Almeida et al. 2009; Sierra-Almeida and Cavieres 2010).

Plant species

We studied a total of 12 plant species selected from the most abundant species within the study area (Table 2). The selected species included: five native shrubs, *Berberis empetrifolia*, *Chuquiraga oppositifolia* D. Don

Table 2 List of plant species considered for freezing-resistance determinations

Species	Family	Growth form	Vegetation belt
<i>Acaena pinnatifida</i>	Rosaceae	PH	SA–A _{inf}
<i>Berberis empetrifolia</i>	Berberidaceae	SHR	A _{sup}
<i>Chuquiraga oppositifolia</i>	Asteraceae	SHR	SA–A _{inf}
<i>Euphorbia collina</i>	Euphorbiaceae	PH	SA–A _{inf}
<i>Haplopappus anthyloides</i>	Asteraceae	SHR	A _{sup} –HA
<i>Laretia acaulis</i>	Apiaceae	C	A _{sup}
<i>Nassauvia looseri</i>	Asteraceae	SHR	A _{sup}
<i>Perezia carthamoides</i>	Asteraceae	PH	A _{inf} –A _{sup}
<i>Phacelia secunda</i>	Boraginaceae	PH	SA–A _{inf} –A _{sup} –HA
<i>Poa mendocina</i>	Poaceae	PH	HA
<i>Senecio polygaloides</i>	Asteraceae	SHR	A _{inf} –A _{sup}
<i>Taraxacum officinale</i>	Asteraceae	PH	SA–A _{inf} –A _{sup} –HA

Altitudinal distribution of target species along vegetation belts: sub-Andean (SA; 1800–2100 m), inferior Andean (A_{inf}; 2100–2800 m), superior Andean (A_{sup}; 2800–3300 m), HA high Andean (HA; 3300–3800 m). Nomenclature and altitudinal distribution follow Teillier et al. (2011)

PH Perennial herb, SHR shrub, C cushion plant

(Asteraceae), *Haplopappus anthylloides* Meyen and Walp (Asteraceae), *Nassauvia looseri*, and *Senecio polygaloides* Phil. (Asteraceae); the cushion plant *Laretia acaulis*; and six perennial herbs, of which five were natives, *Acaena pinnatifida* Ruiz et Pav. (Rosaceae), *Euphorbia collina* Phil. (Euphorbiaceae), *Perezia carthamoides* Hook and Arn. (Asteraceae), *Phacelia secunda* J.F. Gmel (Boraginaceae), *Poa mendocina* Nicora and F.A. Roig (Poaceae), and one was non-native, *Taraxacum officinale* (L.) Weber.

Experimental design

Soon after snowmelt, we selected 20 mature individuals per target species across the study area. For each species, we randomly assigned ten of these individuals to a drought-alleviation or irrigation treatment. For this, we installed a sprinkler on each of these individuals, which was connected to a valve controller programmed to give 7 L h⁻¹ twice a day during the entire growing season. Water was taken from a nearby stream. The remaining ten individuals per species were exposed to the natural increase in water shortage throughout the growing season. Thus, the irrigation treatment had two levels: irrigated plants (+H₂O) and non-irrigated plants (−H₂O).

To assess the interaction of drought and temperature (i.e., warming) on LT₅₀ and FP we randomly selected five irrigated and five non-irrigated plants per species. On each of these selected individuals we placed a hexagonal open top chamber (OTC), made of transparent Plexiglass®, with walls of 40-cm height and 115-cm diameter (warmed; +W). The OTCs walls were punched with 25 holes of 1.5-cm diameter each to allow some wind to pass through and hence avoid an excessive increase in air temperature. The OTCs are passive warming systems that have been widely used in warming experiments in alpine and arctic tundras (Bokhorst et al. 2013). The other five individuals were kept under natural temperatures conditions (unwarmed; −W). Hence, we obtained four experimental conditions with five replicates each: irrigated and unwarmed plants (+H₂O −W), non-irrigated and warmed plants (−H₂O +W), irrigated and warmed plants (+H₂O +W) and the natural control of non-irrigated and unwarmed plants (−H₂O −W). Each experimental condition was located 2–6 m from the nearest replicate to reduce potential topographic differences within the study area and also to prevent microclimatic manipulations in one experimental patch influencing plant responses in adjacent patches. Plants were exposed to experimental conditions during an entire growing season, and the study lasted 2 years. In the first growing season (1 December 2011 to 31 March 2012) we studied *B. empetrifolia*, *C. oppositifolia*, *H. anthylloides*, *N. looseri*, *Phacelia*

secunda, *S. polygaloides*, and *Poa mendocina*. In the second growing season (17 November 2012 to 6 April 2013) the studied plant species were: *A. pinnatifida*, *E. collina*, *L. acaulis*, *P. carthamoides* and *T. officinale*.

Microclimatic measurements

We characterized microclimatic changes produced by the experimental irrigation and warming, by measuring soil $\Psi_{\text{H}_2\text{O}}$ and air temperature (Table 1). For soil moisture monitoring we used psychrometers (PST-55; Wescor, Utah, US), which were buried at a depth of 20 cm at the center of each treatment ($n = 3$). Soil $\Psi_{\text{H}_2\text{O}}$ (MPa) was measured two times during each growing season: early (December) and late (March), by connecting psychrometers to a data-logger (Psypro; Wescor). We also recorded air temperature with mini-loggers (HOBO U23 Pro V2; Onset, Bourne, MA) that were placed 15 cm above the soil surface, and protected with plastic shelters from direct exposure to sunlight ($n = 3$ –4). Air temperature sensors were placed thusly because no differences in thermal conditions around the plants have been found at distances below 15 cm (Sierra-Almeida et al. 2010). The mini-loggers were programmed to record the temperature every hour during each growing season. Unfortunately, temperature sensors placed on irrigated plots stopped working during the second growing season. Thus, no data are shown in Table 1 for those experimental conditions and period.

Plant material collection

We collected five plant samples for each experimental condition. Plant samples corresponded to small branches with at least six leaves for dwarf shrubs, small rosettes or modules for cushion plants and some herbaceous plants, and complete individuals for other herbaceous plants. We collected all plant material between 9:00 a.m. and 12:00 p.m. Plant samples were wrapped in wet paper and aluminum foil and placed into a cooler to prevent changes in tissue water content and mechanical damage. Samples were then transported to a field laboratory less than 10 min away. We kept plant samples in a refrigerator (i.e., 4 °C) until freezing resistance determinations were performed, within 24 h of collection.

Low-temperature damage

For each species and experimental condition, six detached leaves from different plant samples were separated into six subsamples to estimate the freezing temperature producing 50 % damage [lethal temperature (LT)₅₀]. We chose the youngest fully expanded leaf

from each plant sample to reduce ontogenetic influences in plant responses, and also to ensure that leaves were formed under experimental conditions. Each subsample was separately incubated at five different target temperatures: -6 , -10 , -14 , -18 and -22 °C, in a previously cooled cryostat (F34-ME; Julabo Labortechnik, Germany). As a control we used a sixth subsample kept in darkness and stored in plastic bags at 4 °C for 24 h. All samples were transferred from the refrigerator to the cryostat and incubated for 2 h to reach homogeneous leaf temperatures. After this freezing treatment, samples were removed from the cryostat and placed back into the refrigerator, under darkness and at 4 °C during 24 h for thawing. We used this method of direct rather than gradual cooling and thawing because it measures the current freezing resistance rather than the hardening capacity of plant material (Larcher et al. 2010). At fast cooling rates, water cannot diffuse normally to extracellular spaces producing anomalous ice-crystal formation, and as consequence, more plant tissue damage (Wisniewski et al. 2014). Therefore, this method could underestimate the freezing resistance of the studied species. However, we have recorded sudden drops in night temperatures that occurred at a cooling rate above 8 °C h⁻¹ in early spring (Cavieres et al., unpublished data) which could be even greater during autumn and winter in this area where snow cover melts very early. Thus, we considered that although this method could be improved, it provides adequate and reliable information relevant to the study site.

Leaf damage was assessed as the percentage of photoinactivation (PhI) as described by Larcher (2000). For this, we measured the ratio of variable to maximum fluorescence of a dark-adapted leaf by using a chlorophyll fluorometer (MINI-PAM; Walz, Germany). LT_{50} corresponds to the temperature at which PhI reaches a 50 % value in subsamples, and was determined by linear interpolation using the temperature of the highest PhI of <50 % and the temperature of the lowest PhI of >50 % (Bannister et al. 2005). PhI was chosen because it measures changes in photosynthetic performance that correlate very well with direct measurements of tissue damage [i.e., visual freezing injuries and vital stain (Boorse et al. 1998; Neuner and Buchner 1999)].

Thermal analyses

For each species and experimental condition, an expanded leaf was removed from each of five plant samples taken in the field. Each leaf was attached to a thermocouple (gauge 30 copper-constantan thermocouples; Cole Palmer Instruments, Vernon Hills, IL), and immediately enclosed in a small, tightly closed cryotube. The cryotubes were placed in a cryostat (Haake Phoenix II-C25P; Thermo, Karlsruhe, Germany), and the temperature was decreased from 0 to

-18 °C, at a cooling rate of 2 °C h⁻¹. The temperature of individual leaves was monitored every second with a Personal Daq/56 multi-channel thermocouple USB data acquisition module (IOtech, Cleveland, OH). The sudden rise in leaf temperature (exotherm) produced by the heat released during the freezing process was used to determine the FP, which corresponds to the highest point of the exotherm, indicating the freezing of water in the apoplast, including symplastic water driven outwards by the Ψ_{H_2O} difference caused by the apoplastic ice formation (Larcher 2003).

Statistical analyses

Differences in air temperature (i.e., mean, maximum, minimum, intensity and frequency of freezing events) among experimental conditions at the end of the growing season were compared using factorial ANOVAs with irrigation and warming as predictors. Soil Ψ_{H_2O} differences among experimental conditions were assessed with repeated-measure ANOVAs, where the inside/outside OTC and with/without irrigation were considered fixed factors and time (early vs. late in the growing season) as the within-subject factor. Differences in the effect of irrigation and warming on LT_{50} and FP were assessed by using factorial ANOVAs as well. Data were checked for normality before analyses. The effect size of irrigation and warming on LT_{50} and FP was calculated as ω^2 following Olejnik and Algina (2003).

Results

Microclimatic conditions

Soil Ψ_{H_2O} in non-irrigated patches significantly decreased from -0.7 to -0.1 MPa at the beginning of the growing season to -2.8 ($F_{1,8} = 29.5$, $P < 0.001$) and to -2.6 MPa ($F_{1,8} = 13.2$, $P = 0.007$) towards the end of the first and the second growing season, respectively (Fig. 1). Irrigation treatment increased the soil Ψ_{H_2O} , with average values ranging around -0.9 MPa throughout the entire growing season (Fig. 1). Soil Ψ_{H_2O} was not affected by warming during the first ($F_{1,8} = 1.3$, $P = 0.283$) or the second growing season ($F_{1,8} = 0.1$, $P = 0.731$; Fig. 1).

OTCs increased daily mean air temperatures by 2.6 °C in both growing seasons (Table 1). Mean maximum air temperature was on average 8.1 °C greater inside OTCs regardless of irrigation treatment and growing season. Mean minimum air temperature was similar among experimental conditions and fluctuated between 2.6 and 3.9 °C in both growing seasons (Table 1). Intensity of freezing events ranged from -1 to -1.6 °C, and the frequency of these events was 4.6 and 19.4 % of the days for the first and the second growing season, respectively. Neither intensity nor

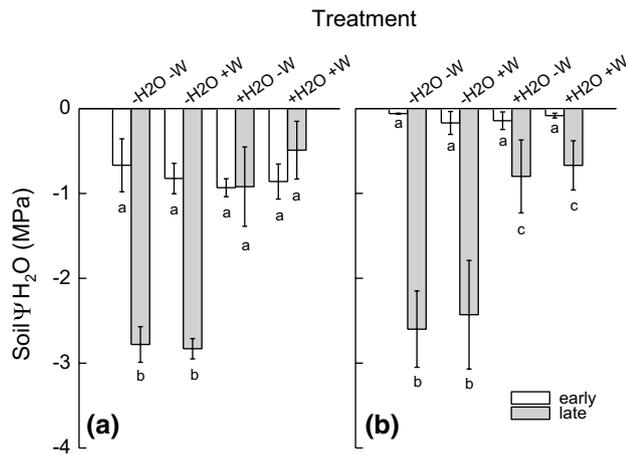


Fig. 1 Water potential ($\Psi_{\text{H}_2\text{O}}$; MPa) measured in the soil under four experimental conditions in the Central Chilean Andes. Data correspond to mean $\Psi_{\text{H}_2\text{O}} \pm \text{SE}$ ($n = 3$) measured early and late in the first (a) and the second growing season (b). Different letters indicate significant differences ($P < 0.05$). $-H_2O -W$ Non-irrigated and unwarmed, $-H_2O +W$ non-irrigated and warmed, $+H_2O -W$ irrigated and unwarmed, $+H_2O +W$ irrigated and warmed

frequency of freezing events was affected by irrigation or warming (Table 1).

Irrigations effect on plant freezing resistance

Seven out of the 12 studied species increased their LT_{50} (i.e., less negative values) when they were irrigated (Fig. 2; Table ESM1). These species were *C. oppositifolia*, *E. collina*, *H. anthylloides*, *N. looseri*, *P. carthamoides*, *S. polygaloides* and *T. officinale*. LT_{50} of irrigated plants was on average 3.5 K higher than that of non-irrigated plants, where increases of LT_{50} with irrigation ranged from 1.4 °C ($F_{1,16} = 62.2$, $P < 0.0001$) in leaves of *T. officinale* to 7 °C in leaves of *E. collina* ($F_{1,16} = 56.6$, $P < 0.0001$; Fig. 2). Among these species, only three showed that FP was affected by irrigation (Table 3). For *N. looseri*, the FP of irrigated plants was on average 2.5 K ($F_{1,36} = 13.5$, $P < 0.001$) higher than that of non-irrigated plants. In contrast, irrigation decreased FP by 1.3 K ($F_{1,15} = 21.2$, $P < 0.001$) and 2.7 K ($F_{1,14} = 13.6$, $P = 0.002$) for *C. oppositifolia* and *T. officinale*, respectively (Table 3).

Irrigation effect on freezing resistance changes with warming

The combined effects of irrigation and warming on LT_{50} and FP were not consistent between species (Fig. 2; Tables 3; ESM1). Firstly, warming reduced the LT_{50} increment with irrigation in three studied species. For *N. looseri*, the LT_{50} increment was reduced from 5.3 to

3 K, which was accompanied by a reduction in the FP increment from 2.5 to 1.3 K under warmer temperatures. For *E. collina* and *H. anthylloides* the LT_{50} increment was reduced from 7.4 to 2.9 K, and from 4.1 to 3.2 K, respectively. In addition, irrigation was associated with FP increments of 2 K for *E. collina*, and of 2.6 K for *H. anthylloides* that were not observed among unwarmed plants (Table 3). Secondly, LT_{50} differences between irrigated and non-irrigated plants disappeared with warming (Fig. 2). This pattern was observed in *C. oppositifolia*, *P. carthamoides*, *S. polygaloides* and *T. officinale*, where warmer temperatures produced LT_{50} values similar to those observed in irrigated plants, except for *S. polygaloides*, for which LT_{50} of warmed plants was similar to that of control plants. Interestingly, the irrigation effect on FP did not disappear with warming in two of these species (Table 3). For instance, in *C. oppositifolia* following irrigation treatment the FP increased from 1.3 to 2.2 K. Meanwhile, in *T. officinale*, FP decreased from 2.7 to 1.9 K following irrigation treatment and warming. In contrast, irrigation did not affect the FP of *P. carthamoides* or *S. polygaloides* either under natural or warmer temperatures (Table 3). Thirdly, the effect of irrigation on LT_{50} only appeared in combination with warm temperatures (Fig. 2; Table ESM1). For example, LT_{50} increased by 3 K ($F_{1,15} = 3.2$, $P < 0.001$) and 2.3 K ($F_{1,16} = 54$, $P < 0.0001$) in leaves of irrigated and warmed plants of *Phacelia secunda* and *Poa mendocina*, respectively. In both species, warming also reduced the FP increment produced by irrigation, from 2.8 to 0.7 K in *Phacelia secunda*, and from 2.6 to 1.2 K in *Poa mendocina* (Table 3). LT_{50} and FP of *A. pinnatifida*, *B. empetrifolia* and *L. acaulis* were not affected by irrigation under either natural or warmer temperatures (Fig. 2; Table 3; ESM1).

The effect size of irrigation and warming on freezing resistance

Irrigation and warming increased the LT_{50} in most of the studied alpine plant species, but their impact varied considerably (Table 4). Effect size estimations for irrigation was 90.2 % greater than that produced by warming in leaves of *E. collina* ($\omega^2 = 0.61$ vs. 0.06). This pattern was also observed in *S. polygaloides* (60 %), *H. anthylloides* and *Poa mendocina* (30.8 %), and *Perezia carthamoides* (28.6 %) (Table 4). Only in *C. oppositifolia* was the effect size of warming on LT_{50} greater than that of irrigation ($\omega^2 = 0.42$ vs. 0.14).

Contrary to LT_{50} , FP measured in most of studied species was separately affected by irrigation or warming. Only in *P. mendocina* and *T. officinale* was the FP affected by both experimental conditions. Thus, effect size estimations

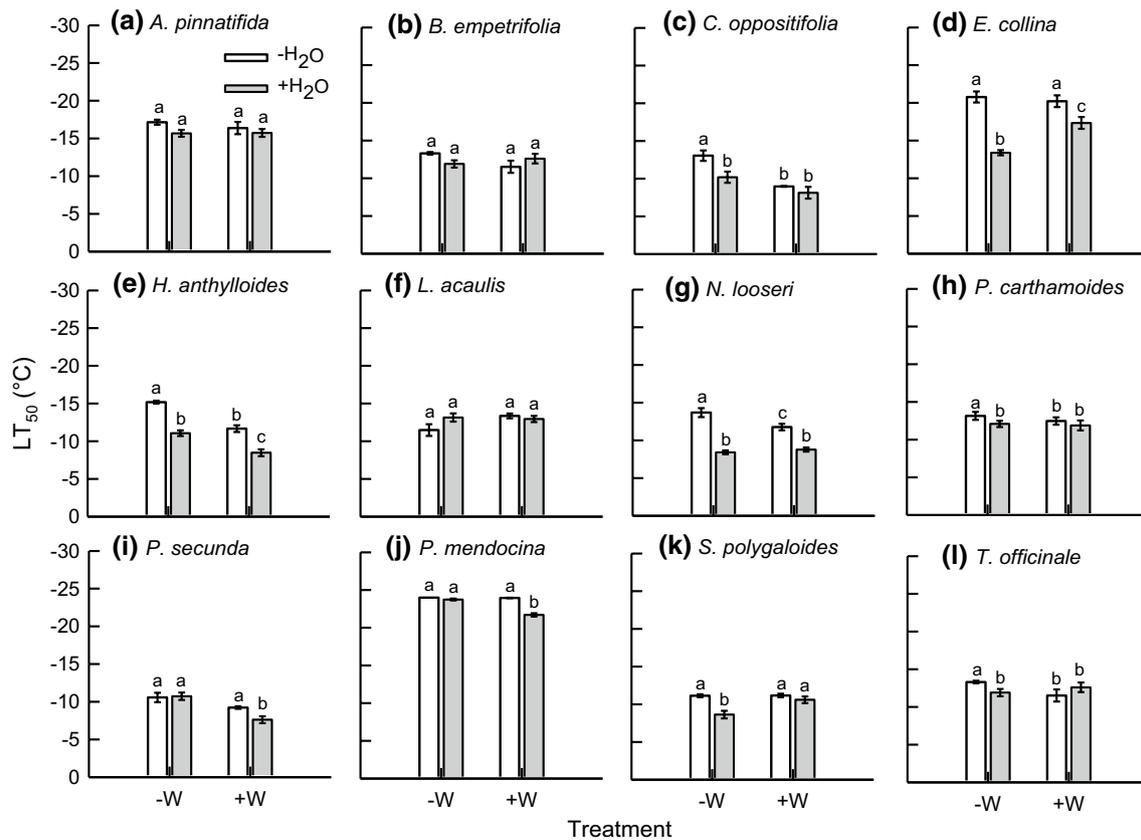


Fig. 2 Freezing resistance of 12 high-elevation species growing in the Central Chilean Andes after exposure to irrigation and warming conditions in the field. **a** *Acaena pinnatifida*, **b** *Berberis empetrifolia*, **c** *Chuquiraga oppositifolia*, **d** *Euphorbia collina*, **e** *Haplopappus anthylloides*, **f** *Laretia acaulis*, **g** *Nassauvia looseri*, **h** *Perezia*

carthamoides, **i** *Phacelia secunda*, **j** *Poa mendocina*, **k** *Senecio polygaloides*, **l** *Taraxacum officinale*. Lethal temperature (LT) causing 50 % damage (LT_{50}) is shown as mean \pm SE ($n = 5-10$). Different letters indicate significant differences ($P < 0.05$). For other abbreviations, see Fig. 1

of irrigation and warming on FP were conducted only in these species. In the case of *P. mendocina*, both irrigation and warming had an effect size of $\omega^2 = 0.08$. For *T. officinale*, the effect size of irrigation was slightly greater than that of warming ($\omega^2 = 0.32$ vs. 0.24).

Discussion

Drought alleviation through our irrigation treatment increased LT_{50} in most of the studied species, but this reduction of freezing resistance was not mediated by changes in FP as we expected. We had expected irrigation and warming to have additive effects, reducing plants' freezing resistance. However, the additive effect of irrigation and warming was poorly represented among the studied species. Irrigation produced greater changes in freezing resistance of high-Andean plants than warming according to the effect size estimations, and as far as we are aware, this is the first multi-species approach that directly assessed, in a field experiment, the

extent to which drought affects the summer freezing resistance of alpine plants species. Below we discuss our results in more detail addressing the implications of these findings in the context of climate change.

Irrigation effect on plant freezing resistance

Irrigation significantly affected LT_{50} for several herbaceous and shrubby high-Andean plant species, indicating that summer drought leads to a significant increase in LT_{50} (i.e., less negative values), and suggesting a generalized plant freezing response to soil moisture regimes. Although studies examining the freezing vulnerability of plants native to areas where drought and freezing co-occur naturally are scarce, our results agree with previous findings. For example, Kreyling et al. (2012b) found that drought exposure in the preceding growing season increased the resistance to late-spring freezing events of four native grass species in central Europe. Likewise, drought improved whole-plant functioning for the shrub species *Larrea tridentata*

Table 3 Freezing point (°C) measured in leaves of plants exposed to experimental irrigation and warming in the Central Chilean Andes during two growing seasons

Species	–W		+W	
	–H ₂ O	+H ₂ O	–H ₂ O	+H ₂ O
<i>A. pinnatifida</i>	–2.4 ± 0.4 a	–1.6 ± 0.2 a	–0.7 ± 0.5 b	–0.9 ± 0.2 b
<i>B. empetrifolia</i>	–4.8 ± 0.8 a	–5.0 ± 0.8 a	–3.8 ± 0.4 a	–4.5 ± 0.7 a
<i>C. oppositifolia</i>	–1.9 ± 0.2 a	–3.2 ± 0.4 b	–2.1 ± 0.4 a	–4.3 ± 0.4 b
<i>E. collina</i>	–4.2 ± 0.5 a	–4.8 ± 0.2 a,b	–5.5 ± 0.4 b	–3.5 ± 0.3 a
<i>H. anthylloides</i>	–2.2 ± 0.1 a	–2.9 ± 0.5 a,c	–6.4 ± 0.8 b	–3.8 ± 0.3 c
<i>L. acaulis</i>	–4.2 ± 0.5 a	–4.8 ± 0.3 a	–5.6 ± 0.2 b	–5.4 ± 0.02 b
<i>N. looseri</i>	–5.1 ± 0.6 a	–2.6 ± 0.5 b	–4.1 ± 0.3 a	–2.8 ± 0.7 b
<i>Perezia carthamoides</i>	–5.0 ± 0.7 a	–4.1 ± 1.0 a	–4.1 ± 0.9 a	–3.5 ± 0.8 a
<i>Phacelia secunda</i>	–3.7 ± 1.2 a	–0.9 ± 0.4 b	–1.9 ± 0.2 c	–1.2 ± 0.4 b
<i>Poa mendocina</i>	–7.8 ± 0.2 a	–5.2 ± 0.2 b	–5.2 ± 0.2 b	–6.4 ± 0.2 c
<i>S. polygaloides</i>	–3.0 ± 0.7 a	–2.6 ± 0.4 a	–2.9 ± 0.3 a	–2.5 ± 0.4 a
<i>T. officinale</i>	–2.6 ± 0.6 a	–5.3 ± 0.7 b	–1 ± 0.3 c	–2.9 ± 0.5 a

Values correspond to mean ± SE ($n = 5–10$). For other abbreviations, see Table 1

Different lowercase letters indicate significant differences ($P < 0.05$)

following exposure to –15 and –19 °C in a greenhouse study with plants from the Chihuahuan Desert (Medeiros and Pockman 2011). In contrast, plants of the shrub species *Calluna vulgaris* that were previously exposed to drought decreased their photosystem II performance after freezing events (Albert et al. 2013). But this response was likely caused by an earlier senescence of aboveground biomass, triggered by freezing injuries experienced by plants during preceding winter months rather than a freezing response to drought per se (Albert et al. 2013).

Evidence from high-elevation habitats where plant freezing resistance is crucial for plant survival is scarce. Subalpine populations of *Banksia marginata* (Proteaceae) showed lower LT₅₀ during summer compared with winter on Mt Wellington (Tasmania) (Blake and Hill 1996), suggesting that drought favors physiological responses that improve freezing resistance during the dry season. Lambrecht et al. (2007a) compared the seedling responses to freezing of two subspecies of *Artemisia tridentata* from the Western USA: *A. tridentata* ssp. *vaseyana*, which inhabits higher elevation sites with moist soils, and *A. tridentata* ssp. *tridentata* that is found on the xeric soils of lower elevation valleys. They found that drought-exposed seedlings of *A. tridentata* ssp. *vaseyana* reduced their photosynthetic capacity compared to well-irrigated seedlings following exposure to –5 °C. In contrast, the drought-exposure treatment did not increase the susceptibility of *A. tridentata* ssp. *tridentata* seedlings to freezing (Lambrecht et al. 2007a), suggesting that drought tolerance is an important trait that favors better plant performance under freezing stress.

Physiological mechanisms underlying freezing-drought responses of high-Andean plants are still unknown. Osmotic adjustment triggered by drought exposure is described as one of the most common mechanisms among vascular

plants (Chaves et al. 2003; Beck et al. 2007), and is thought to have a direct effect on the FP depression as has been reported for plants from tropical alpine zones (Goldstein et al. 1985; Rada et al. 1985). Thus, we expected that those species that increased their LT₅₀ with irrigation would also show an increase in their FP. However, this phenomenon was observed only in the shrub *N. looseri*. In this species, non-irrigated plants showed a FP depression of 2.5 K, which was accompanied by a decrease of 16 % in the relative water content of leaves compared to irrigated plants (Table ESM2). These results suggest that the FP depression through osmotic adjustment might be a physiological mechanism underlying freezing-drought response in *N. looseri*, but clearly it is not the most common mechanism used to cope with freezing temperatures among the studied species. Interestingly, we found that in *C. oppositifolia* and *T. officinale* irrigation increased their LT₅₀ (i.e., less negative) but it decreased their FP (i.e., more negative). We do not have a concrete explanation for this response, but we propose a possible explanation. It is likely that drought-alleviation favors carbon gain, producing plants that have accumulated a great amount of non-structural carbohydrates (NSC) in their leaves. A high concentration of NSC contributes to a delay in ice crystal formation in plant tissues (e.g., Zúñiga et al. 1996; Kasuga et al. 2007), which could explain the lower FP of irrigated plants compared to non-irrigated plants. At the same time, Sakai and Larcher (1987) recognized a trade-off between resistance to freezing stress and growth intensity. Thus, better access to water supply might be favoring plant growth to the detriment of freezing resistance, which could explain the less negative values of LT₅₀ observed in *C. oppositifolia* and *T. officinale*.

We believe that plant traits or responses other than osmotic adjustment could be operating as more common

Table 4 Factorial ANOVAs of irrigation and warming effects on the lethal temperature causing 50% photoinactivation (LT_{50}) of studied species and their effect sizes

Species	Factor	SS	df	MS	F	p	ω^{2a}
COPP	Irrigation	16.1	1	16.1	7.3	0.016	0.14
	Warming	44.4	1	44.4	20.1	<0.0001	0.42
	Interaction	4.8	1	4.8	2.2	0.0161	
	Error	33.1					
ECOL	Irrigation	131.9	1	131.9	56.6	<0.00001	0.61
	Warming	14.3	1	14.3	6.1	0.025	0.06
	Interaction	25.4	1	25.4	10.9	0.004	
	Error	37.3	16	2.3			
HANT	Irrigation	66.9	1	66.9	89.5	<0.00001	0.52
	Warming	46.7	1	46.7	62.4	<0.00001	0.36
	Interaction	1.1	1	1.1	1.4	0.247	
	Error	12	16	0.7			
PCAR	Irrigation	0.06	1	0.06	24.6	<0.0001	0.28
	Warming	0.04	1	0.04	17.6	<0.0001	0.20
	Interaction	0.03	1	0.03	13	0.001	
	Error	0.06	27	0.002			
PMEN	Irrigation	0.003	1	0.003	84	<0.0001	0.39
	Warming	0.002	1	0.002	59	<0.0001	0.27
	Interaction	0.002	1	0.002	54	<0.0001	
	Error	0.0005	16	0.00003			
SPOL	Irrigation	0.05	1	0.05	18.5	0.0001	0.25
	Warming	0.02	1	0.02	8	0.007	0.10
	Interaction	0.02	1	0.02	7.7	0.009	
	Error	0.09	36	0.003			

This table includes only those species with significant irrigation and warming effects

COPP *C. oppositifolia*, ECOL *E. collina*, HANT *H. anthylloides*, PCAR *Perezia carthamoides*, PMEN *Poa mendocina*, SPOL *S. polygaloides*

^a ω^2 corresponds to the effect size of irrigation and warming on LT_{50}

mechanisms of freezing resistance for high-Andean species. For example, Blake and Hill (1996) found that the most freezing resistant plants of *B. marginata* also showed high osmotic potential, more elastic cells and a higher apoplastic water content. These traits are common among freezing-tolerant plants, and enable plants to avoid intracellular ice formation through the gradual displacement of symplastic water to the apoplast, protecting cells from sudden loss water during subsequent dehydration (Sakai and Larcher 1987; Anisko and Lindstrom 1996). In our case, all studied high-Andean species that were responsive to drought-alleviation have been previously classified as freezing tolerant (Sierra-Almeida et al. 2009). Thus, regular summer droughts could be favoring mechanisms of freezing tolerance among high-Andean plant species instead of freezing avoidance through osmotic adjustment. Further studies dealing with freezing resistance mechanisms in high-elevation plants from dry mountains have to consider their morpho-anatomical traits (e.g., size of mesophyll cell and intercellular spaces) as well as their biochemical traits (e.g., fatty acids, sugars, antifreeze proteins), in order

to understand the mechanisms and physiological changes underlying the influence of drought on freezing resistance.

Irrigation effect on freezing resistance changed with warming

Several studies have assessed the effect of warming on plant vulnerability to freezing damage during the growing season in cold climates. The findings so far are mixed: warmed plants are more susceptible (Loveys et al. 2006; Marchand et al. 2006; Sierra-Almeida and Cavieres 2010), less susceptible (Loik et al. 2004; Martin et al. 2009), or warming did not affect plant freezing resistance (Ögren 2001; Loik et al. 2004; Martin et al. 2009; Sierra-Almeida and Cavieres 2010; Kimball et al. 2014). Species-specific responses to warming, added to its indirect effects on soil properties, might interfere with the direction and magnitude of plant freezing responses (Arft et al. 1999; Elmen-dorf et al. 2012). Our results showed that the irrigation effect on LT_{50} and FP of high-Andean species changed with warming, and the consequences for their summer

freezing resistance differed among species. We predicted that a greater LT_{50} increment would be produced through irrigation in leaves of warmed plants compared with unwarmed plants, but we did not find this response for any of studied species. Nevertheless, we found that the greatest LT_{50} values (least negative) were produced by the simultaneous effects of irrigation and warming in the shrub *H. anthylloides*, the perennial herb *Phacelia secunda* and the grass *Poa mendocina*. Under such conditions, we recorded an LT_{50} above -8.5 °C for *H. anthylloides* and *P. secunda*, with FP occurring at freezing temperatures above -4 °C. Although growing season freezing events when this study was conducted were relatively mild, their frequency, duration and intensity vary within and among growing seasons in the Central Chilean Andes (Sierra-Almeida et al. 2010; Sierra-Almeida and Cavieres 2012). For example, temperature records from a climatic station installed in the same area since 2006 indicate that during the growing season, freezing events lower than -8 °C occurred in 30 % of the days, exposing plants to even lower temperatures near the ground (Cavieres et al., unpublished data). Thus, warmer and moister conditions during the summer in the Central Chilean Andes would leave *H. anthylloides* and *P. secunda* more vulnerable to freezing damage in their leaves. In contrast, although warming combined with irrigation also increased the LT_{50} of *P. mendocina*, this species kept a wide safety margin between its LT_{50} (i.e., -21.7 °C) and the intensity of growing season freezing events recorded in the area, suggesting that *P. mendocina*, similar to other grass species in high-elevation habitats, is less susceptible to freezing damage due to climate change (Taschler and Neuner 2004; Martin et al. 2009; Pescador et al. 2016).

Other trends for freezing resistance were observed among high-Andean plant species. First, the perennial herb *E. collina* and the shrub *N. looseri* showed a decrease in the irrigation effect on LT_{50} when these species were warmed. For *E. collina*, this trend was produced because the LT_{50} of irrigated plants under warming were more negative compared to the LT_{50} of plants growing under natural temperatures. Previous studies have reported that experimental warming can increase plant water deficit by lowering soil Ψ_{H_2O} (Loik et al. 2000; Lambrecht et al. 2007b; Sanfuentes et al. 2012). In our study, OTCs did not affect soil Ψ_{H_2O} . Nevertheless, we cannot completely discount the greater evaporative demand occurring for warmed plants of *E. collina*. An indication of this is the fact that non-irrigated plants under warming decreased their FP to values below those recorded for non-irrigated plants under natural temperatures conditions (i.e., -4.2 vs. -5.5 °C). This result suggests that warming altered leaf water status for *E. collina* and also its freezing processes. In the case of *N. looseri*, the decrease in the irrigation effect was due to warming increasing LT_{50} and FP of non-irrigated plants

compared to the natural temperature conditions, which is in agreement with our expectations. Second, in the shrubs *C. oppositifolia* and *S. polygaloides* and in the perennial herbs *P. carthamoides*, and *T. officinale*, the irrigation effect on LT_{50} disappeared among warmed plants. Regardless of irrigation condition, warming increased the LT_{50} of *C. oppositifolia*, *P. carthamoides* and *T. officinale* to similar values of those obtained for irrigated plants growing at natural temperatures, indicating that warming ruled out the enhancement effects of drought on their freezing resistance. In contrast, warmed plants of *S. polygaloides* showed LT_{50} similar to those of non-irrigated and unwarmed plants. But contrary to the observations for *E. collina*, the FP depression and relative water content in their leaves (Table ESM2) did not vary to support an increase of freezing resistance in warmed plants mediated by a greater evaporative demand. In the case of *C. oppositifolia*, FP decrease produced by irrigation was greater under warmed rather than natural temperatures. However, for *T. officinale*, we observed the opposite response. Likely, the shrub *C. oppositifolia* took advantage of the warmer conditions to increase growth, as has been reported for other shrub species from cold climates (e.g., Tape et al. 2006; Hudson et al. 2011; Anadon-Rosell et al. 2014), favoring high concentrations of NSC in its leaves. In contrast, for the rosette of *T. officinale*, warm conditions inside OTCs could have been hotter and drier than those outside the OTC to favor a similar response.

Finally, freezing resistance of *A. pinnatifida*, *B. empetrifolia*, and *L. acaulis* was not affected by irrigation or warming. Freezing resistance determinations were conducted in a late flowering-fruiting phase for all studied species, and we were especially careful to select leaves at the same phenological stage for comparisons. However, plant phenological behaviors in high-Andean species are variable (Arroyo et al. 1981), and they certainly may determine plant responses, or the lack of them, to freezing temperatures. For example, *A. pinnatifida* is a perennial herb that dies back immediately after fruiting, a behavior that appears to be genetically determined (Arroyo et al. 1981). Thus, it is likely that the lack of freezing responses to irrigation and/or warming were due to dormancy processes that were initiated at the moment of freezing. In contrast, flowering and fruiting in the shrub *B. empetrifolia* are preceded by a long period of shoot and leaf growth, and the plant continues growing after fruiting until the beginning of winter (Arroyo et al. 1981). Its moderate mean LT_{50} of -9.4 °C observed across irrigation and warming conditions suggests two things. Firstly, irrigation did not affect its LT_{50} and FP because *B. empetrifolia* possesses a deep and well-developed tap root system that allows it to take up water from deeper soil layers, ensuring a high water supply throughout the growing season. Further, this species should have a reduced rate of loss of water through transpiration

because it possesses very small, bent and sclerophyllous leaves. Secondly, warming did not affect its LT_{50} and FP because cold hardening processes in leaves of *B. empetrifolia* might be triggered by changes in photoperiod, or in combination with temperature, as occurs in other woody species from cold climates (Beck et al. 2004; Bannister et al. 2005). *L. acaulis* is a cushion plant that possesses an extremely deep and well-developed tap root system that ensures free and continuous access to water during the entire growing season. Moreover, *L. acaulis* exhibits thick and waxed leaves that maximize water conservation in addition to its flat, tight form that reduces wind desiccation (Martínez 1989). With regards to warming, our results contrast with those previously reported by Sierra-Almeida and Cavieres (2010), where warming increased the LT_{50} of *L. acaulis* by >3 °C. These contrasting responses to warming cannot be related to thermal microclimates experienced by plants during the studies, as these were similar across the studies. However, winter precipitation preceding the growing seasons when freezing resistance measurements were conducted was 28.3 % lower in 2012 compared to 2007 (E. Sánchez Ad. Station, Chilean Meteorological Service, 33°45'S 70°55'W, <http://www.meteochile.gob.cl/climatologia.php>). Leaf buds of *L. acaulis* burst immediately after snow melts, and cushion plants continue their vegetative activity throughout the entire growing season, though they are noticeably less active from late summer onwards (Arroyo et al. 1981). Thus, it seems likely that dormancy processes in leaves of *L. acaulis* were initiated earlier in 2013 compared with 2008, because plants experienced a drier growing season as a consequence of less snow accumulation during the preceding winter, affecting LT_{50} determinations with warming.

Implications for climate change scenarios

We found that the alleviation of summer drought produced greater changes of LT_{50} but not of FP for high-Andean plant species compared with warming. These results have important implications for understanding alpine plant community responses to climate change. Whereas recent climate trends and projections predict a consistent increase of mean temperature for the Central Chilean Andes (CONAMA 2006; Quense 2011), current and future trends in precipitation are highly uncertain (Carrasco et al. 2005; CONAMA 2006). Despite increases in the mean temperature of high-elevation habitats in the Central Chilean Andes, the occurrence of episodic strong freezing events during the growing season has remained barely constant during the last 30 years (Quense 2011). Regular summer drought exposure determines that most of the high-Andean plant species exhibit great levels of freezing resistance during the growing season (i.e., mean LT_{50} of non-irrigated plants was -14.3 °C), suggesting that

though warming could reduce the plant freezing survival of several of these species, others have a safety margin between their freezing resistance and the magnitude of the growing season freezing events. But this optimistic scenario could vary dramatically depending on whether warmer temperatures due to climate change are accompanied by summer precipitation increases. In such a case, the survival of high-Andean plant species would be seriously threatened by strong freezing events during the summer. Thus, further studies dealing with this topic should to be conducted on numerous plants species in order to gain a more accurate picture of the likely consequences of climate change for plant survival and persistence in alpine communities.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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