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# Annual and perennial high-Andes species have a contrasting freezing-resistance mechanism to cope with summer frosts

Loreto V. Morales<sup>1</sup> · Carla Alvear<sup>1</sup> · Camila Sanfuentes<sup>2,3</sup> · Alfredo Saldaña<sup>1</sup> · Ángela Sierra-Almeida<sup>1</sup>

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## Abstract

In high-mountain habitats, summer frosts have negative consequences for plant fitness, therefore high-mountain plants have developed mechanisms of avoidance and tolerance to cope with freezing temperatures. Various hypotheses have been proposed to explain the prevalence of one freezing-resistance mechanism over another, focusing on thermal conditions without a consensus. We hypothesize that the prevalence of a freezing-resistance mechanism depends on the life-history strategy of the species, and is probably the consequence of a trade-off between growth/reproduction and the cost of the mechanism. Specifically, short-lived annual species should be freezing avoidant, whereas perennial long-lived species should be freezing tolerant. We used thermal analysis to determine the mechanism of freezing resistance of leaves and flowers for 10 annual and 14 perennial herb species from an alpine ecosystem in the Central Chilean Andes. We found that 70% of the annual species, their flowers and leaves were freezing avoidant, indicating that avoidance was their predominant freezing-resistance mechanism. In the case of perennial species, both mechanisms were almost equally represented in flowers and leaves. Overall, our results showed that a species freezing-resistance mechanism depends on its life-history strategy, and that leaves and flowers of single species exhibit the same freezing resistance mechanism, suggesting a common whole plant strategy. Further, freezing resistance strategies were not found to be mutually exclusive. In some cases, a specific combination of phenological, structural, and functional strategies may determine how freezing resistant vegetative and reproductive organs are to freezing during the growing season.

**Keywords** Alpine · Freezing avoidance · Freezing tolerance · Growing season · Reproduction

## Introduction

In high mountain habitats, one of the main abiotic factors that determine a plant's distribution is low temperatures (Körner 2003). In these cold habitats, frosts events and the accumulation of snow during the winter restrict the plant's growth and reproduction to the spring and summer months, where the temperatures are more suitable for plant development. However, unpredictable and stochastic frost events during the growing period occur, occasionally exceeding the frost resistance of alpine plants (Taschler and Neuner 2004), causing significant damage and destruction of vegetative and reproductive structures (Inouye 2000; Taschler and Neuner 2004; Augspurger 2009; Ladinig et al. 2013; Lenz et al. 2013; Vitasse et al. 2014; Caradonna and Bain 2016).

Studies have shown that high-mountain plants have developed physiological mechanisms that allow them to cope with frost events. These physiological mechanisms are categorized into freezing avoidance and freezing tolerance

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✉ Ángela Sierra-Almeida  
angelasierra@udec.cl

Loreto V. Morales  
loretomorales@udec.cl

<sup>1</sup> Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Casilla 160-C, Concepción, Chile

<sup>2</sup> Laboratorio de Ecología Geográfica, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

<sup>3</sup> Instituto de Ecología y Biodiversidad (IEB), Casilla 653, Santiago, Chile

(Levitt 1980). Freezing avoidance is the plant's ability to avoid or delay ice formation in their tissues (e.g., by supercooling, morphological ice barriers, extra-organ freezing). This mechanism has been typically associated with structural traits that determine if, when, and where ice forms and propagates (e.g., small cell size, little intercellular space, thick cuticles; Gusta and Wisniewski 2013). Likewise, freezing tolerance is the plant's ability to survive extracellular ice formation without irreversible damage, and includes biochemical responses regulated by a specific set of genes that allows plants to tolerate the dehydration stress associated with the presence of ice in their tissues (e.g., changes in membrane composition, production of compatible solutes, protein induction, cryoprotective substances; Uemura and Steponkus 1994; Hughes and Dunn 1996; Strand et al. 2003; Hekneby et al. 2006; Gusta and Wisniewski 2013). Due to avoidance mechanisms are commonly associated with anatomical features, they are considered less expensive in energy terms compared to induced responses. In turn, since avoidance mechanisms prevent ice formation, vital functions and cellular processes are not disrupted, and therefore are favorable for plants during periods of metabolic and developmental activity (Sakai and Larcher 1987). Conversely, there is a compromise between normal cell function and the ability to tolerate extracellular ice. Despite freezing tolerance mechanisms are expensive in terms of resource allocation, they are adequate in coping with lower and long-lasting freezing temperatures compared with freezing avoidance mechanisms which are adequate when slight frosts occurred only for a few hours (Goldstein et al. 1985; Rada et al. 1987).

Some authors have proposed that thermal conditions could explain the prevalence of one freezing resistance mechanism over another at the species level, without finding a consensus. Namely, the freezing resistance mechanism presented by a species could be related to the frequency and intensity of frost events that plants experience (Sakai and Larcher 1987; Larcher 2003). On one hand, freezing avoidance should be more frequent in species inhabiting sites where the intensity of the freezing events is moderate (a few degrees below 0 °C) and of short duration. On the other hand, freezing tolerance should be more frequent in locations where freezing events are severe and of long duration (Sakai and Larcher 1987; Larcher 2003). Based on this hypothesis, several authors have proposed that the evolution of freezing avoidance and/or tolerance mechanisms will depend on the microclimatic temperature regimes determined by the plant's height (Azócar et al. 1988; Squeo et al. 1991). Therefore, on a thermal gradient in the air–soil profile, where night-time temperatures near the ground can be much colder than air temperature due to radiative cooling (Körner 2016), selection could favor freezing tolerance in short plants, as rosettes, cushion plants, and grasses (Beck et al. 1982; Azócar et al. 1988; Squeo et al. 1991; Márquez

et al. 2006; Sklenář et al. 2010). Conversely, freezing avoidance could be selected in taller plants (e.g., shrubs) experiencing a milder thermal microclimate (Squeo et al. 1991, 1996; Márquez et al. 2006). Evidence that supports this hypothesis is limited and inconclusive: some supporting evidence has been found by Goldstein et al. (1985) and Rada et al. (1985, 1987) who reported that in *Polylepis sericea* and several species of *Espeletia*, which have vegetative buds at more than 1 m above ground, the main mechanism against freezing injury in this tropical environment is freezing avoidance by supercooling. However, other studies have found the opposite pattern. For example, Afroalpine (Beck et al. 1982, 1994) and Hawaiian giant rosettes (Melcher et al. 1994) are freezing tolerant despite their plant heights. Finally, others found no relationship between the freezing resistance mechanism and the plant height for Chilean (Sierra-Almeida et al. 2010) and Equatorial alpine species (Sklenář et al. 2010).

The above findings suggest that the prevalence of one freezing resistance mechanism over another is determined by a combination of frost regimes and an integrative group of plant traits associated with life-history strategy (LHS). This is because the energetic investment in functions like growth and reproduction, determined by the LHS, could cause plants to have less resources to allocate to other functions like freezing resistance (a survival mechanism; Agrawal et al. 2004; Neuner and Beikircher 2010; Wingler 2015; Bucher et al. 2019). Supporting the above, Morales et al. (2020) found that the degree of freezing resistance in leaves and flowers of herbaceous species of Chilean central Andes depends on the LHS. For these studied species, 58% and 83% of perennial species resist temperatures  $\leq -10$  °C in their flowers and leaves, respectively, compared with only 30% and 40% of annual species. However, whether the freezing resistance is consequence of the underlying mechanism is still uncertain. Based on above, it is to be expected that plants with contrasting LHS should select different freezing-resistance mechanisms according to the energetic cost and budget. For example, in annual species which have a high growth rate in the pre-reproductive phase and invest most of their energetic and mineral resources in reproduction (Bazzaz et al. 1987; Hancock and Pritts 1987; Garnier 1992), it should be expected that freezing avoidance mechanisms are commonly utilized in their organs. Since an avoidance mechanism implies a lower energy cost, annual plants would avoid or diminished the trade-off with the resource intensive demands of growth and reproduction. Conversely, in perennial plants which have a more conservative use of energy due to a longer growth period and a lower relative growth rate than annual plants (Bazzaz et al. 1987; Bazzaz and Morse 1991; Garnier 1992), it should be expected that freezing tolerance mechanisms are commonly utilized in their organs to increase plants long-term survival.

In this study, we present an alternative explanation that could help to explain the determinants of the freezing resistance mechanism in alpine plant species. We hypothesize that the occurrence of the freezing-resistance mechanism in the high-Andean plants of central Chile depends on their LHS, where short-lived annual herbs should be freezing-avoidant, whereas long-lived perennial herbs should be freezing-tolerant. For this, we determined the freezing-resistance mechanism at the whole-plant level (including reproductive and vegetative organs) of fourteen perennial and ten annual species that live in the Central Chilean Andes. Specifically, we ask if there are differences in the freezing-resistance mechanism between annual and perennial herbs?

## Materials and methods

### Study site and species

This study was carried out near Farellones (33°17'S 70°25'W), in the central Chilean Andes, 50 km east of Santiago. The climate in this area is alpine with a strong Mediterranean influence, which predominates at lower elevations, generating a dry growing season (Santibañez and Uribe 1990). This area is characterized by the dominance of shrubs and dwarf shrubs such as *Anarthrophyllum cumingii*, *Chuquiraga oppositifolia* and *Tetraglochin alatum*, perennial herbs such as *Alstroemeria exerens* and *Stachys philippiana*, and annual species such as *Chaetanthera spp*, *Collomia biflora* and *Microsteris gracilis* (lower Andean vegetation belt, Teillier et al. 2011).

The growing season begins with the snowmelt in October and ends in April with the first snowfall (Sierra-Almeida and Cavieres 2012). During this period, the daily mean air temperature is 15.4 °C, with daily maximum and minimum temperatures of 32 and 5 °C, respectively (average temperatures between 2200 and 2800 m above sea level; Table S1). Frost events are distributed throughout the growing season, though they are more frequent at the beginning and end of the season (October–November and February–March). There is high inter-annual variation in the number and intensity of frost events, ranging from 2 to 59 frost events per growing season. The duration of these frost events is also highly variable, ranging from 1.5 h to 13 h. Minimum temperature can drop below –10 °C in spring (Morales et al. 2020).

A total of 24 species was studied, including 10 annual and 14 perennial herbaceous species (Table 1). Plant material for each studied species was collected between 2200 and 2800 m above sea level. The studied species were primarily selected based on their observed abundance in the plant herbaceous community. Then, we considered the morphological characteristics of reproductive (flowers/inflorescences) and vegetative (leaves) organs, which should be present at

the same time, to simultaneously determine their freezing resistance. Plant material was collected between 17-Oct-2017 and 01-Jan-2018, according to the phenology of each studied species.

### Plant sample collection

Plant samples were collected from randomly selected plant individuals ( $n=7$  individuals per species), and at the same developmental stage. For vegetative structures, only healthy, mature and fully-expanded leaves were collected. For reproductive structures, only open flowers and inflorescence were collected. Seven leaves and seven flowers were collected from each individual plant; each was covered with a moist paper towel and immediately placed inside a plastic sealed bag. These plant samples were stored inside a cooler at approx. 5 °C to avoid changes in the tissue water status. Within 2 h of collection, all plant samples were transferred to a refrigerator at 4 °C until freezing resistance determinations were carried out. To avoid senescence and dehydration of the plant samples, freezing resistance determinations were conducted in a field laboratory within 24 h of the samples' collection.

### Thermal analysis

For each species, an expanded mature leaf and an open flower was removed from seven different plant samples taken in the field ( $n=7$  flowers and 7 leaves per species). Each leaf and flower was attached to a thermocouple (Type T, gauge 30 copper-constantan thermocouples, 2 mm diameter of junction; Cole Parmer Instruments, Vernon Hills, IL). For solitary and large flowers, the thermocouple was placed among petals (e.g., *Alstroemeria exerens*, *Scyphanthus elegans*). In small flowers and capitulum type inflorescences (e.g., *Chaetanthera sp*, *Perezia carthamoides*), the thermocouple was placed inside the flower's head to make contact with the fertile flowers. Once the thermocouple was secured to the tissue with a padded clamp, the samples were immediately enclosed in a hermetically re-sealable plastic bag. The plastic bags were placed in a cryostat (F34-ME, Julabo Labortechnik GmbH, Germany), and the temperature was decreased from 0 to –20 °C, at a cooling rate of 5 °C h<sup>-1</sup>. The cooling rate was selected according to the maximum cooling rate during natural summer freezing events in the Central Chilean Andes obtained from weather station recordings (HOBO, Onset Computer Corporation) located 1.5 m above the ground (See Fig. S1 for examples of extreme frost events when the cooling rate reach values close to –5 °C h<sup>-1</sup>). The temperature of individual leaves and flowers was monitored every second with a DAQ/32 multi-channel thermocouple USB data acquisition module (USB-2416 connected to an AI-EXP32 expansion module;

**Table 1** Freezing resistance of flowers and leaves of 24 species from the central Chilean Andes measured during the growing season

Species	Family	Flowers			Leaves		
		INT (°C)	LT <sub>50</sub> (°C)	Mechanism	INT (°C)	LT <sub>50</sub> (°C)	Mechanism
<b>Annual species</b>							
<i>Chaetanthera flabellata</i>	Asteraceae	-9.4±0.7	-12.9±0.2	FT	-15.5±0.7	-20.0±0.0	FT
<i>Chaetanthera linearis</i>	Asteraceae	-9.0±0.9	-20.0±0.0	FT	-9.5±0.9	-20.0±0.0	FT
<i>Collomia biflora</i>	Polemoniaceae	-11.6±0.9	-9.1±0.9	FA	-9.8±1.1	-6.6±0.5	FA
<i>Lupinus microcarpus</i>	Fabaceae	-10.7±1.1	-9.9±0.9	FA	-8.5±1.2	-20.0±0.0	FT
<i>Madia sativa</i>	Asteraceae	-7.8±0.2	-6.4±0.4	FA	-8.4±0.2	-9.3±1.1	FA
<i>Microsteris gracilis</i>	Polemoniaceae	-12.2±1.4	-17.9±0.3	FT	-11.9±1.1	-8.2±0.6	FA
<i>Phacelia brachyantha</i>	Boraginaceae	-15.0±0.9	-5.5±0.4	FA	-7.0±0.3	-8.1±0.4	FA
<i>Quinchamalium parviflorum</i>	Schoepfiaceae	-10.5±0.6	-7.4±0.6	FA	-10.7±1.0	-11.8±0.4	FA
<i>Schizanthus hookeri</i>	Solanaceae	-8.1±0.5	-7.5±0.6	FA	-8.6±0.9	-7.5±0.8	FA
<i>Scyphanthus elegans</i>	Loasaceae	-6.8±0.5	-6.5±0.2	FA	-8.2±0.2	-7.0±0.8	FA
<b>Total mean</b>		<b>-10.1±0.4</b>	<b>-10.2±0.6</b>		<b>-9.8±0.4</b>	<b>-11.7±0.8</b>	
<b>Perennial species</b>							
<i>Acaena splendens</i>	Rosaceae	-14.6±1.1	-14.4±1.1	FA	-5.9±0.12	-20.0±0.0	FT
<i>Alstroemeria exerens</i>	Alstroemeriaceae	-6.6±0.2	-6.2±0.4	FA	-8.5±0.7	-10.9±1.0	FA
<i>Barneoudia chilensis</i>	Ranunculaceae	-9.9±1.1	-11.0±2.1	FA	-7.2±0.5	-7.8±0.4	FA
<i>Calceolaria segethii</i>	Calceolariaceae	-7.6±1.12	-11.3±1.9	FA	-6.3±0.8	-6.3±0.4	FA
<i>Draba gilliesii</i>	Brassicaceae	-11.9±0.4	-20.0±0.0	FT	-10.1±0.4	-12.7±0.8	FT
<i>Hordeum comosum</i>	Poaceae	-11.5±0.5	-14.3±0.1	FT	-12.1±1.11	-11.5±0.8	FA
<i>Ipeion sessile</i>	Liliaceae	-5.3±0.1	-10.4±1.1	FT	-8.4±0.6	-13.4±1.0	FT
<i>Olsynium junceum</i>	Iridaceae	-12.3±0.3	-19.9±0.1	FT	-10.8±0.9	-15.5±0.9	FT
<i>Oxalis cinerea</i>	Oxalidaceae	-8.0±0.5	-9.0±0.8	FA	-13.1±0.9	-16.2±1.6	FA
<i>Perezia carthamoides</i>	Asteraceae	-9.3±0.5	-17.7±1.3	FT	-10.4±0.5	-12.5±0.8	FT
<i>Poa holciformis</i>	Poaceae	-11.8±2.1	-8.9±0.8	FA	-8.4±0.7	-7.3±0.3	FA
<i>Sanicula graveolens</i>	Apiaceae	-9.7±1.0	-6.8±0.3	FA	-12.2±1.0	-16.3±1.2	FT
<i>Senecio polygaloides</i>	Asteraceae	-6.9±0.3	-20.0±0.0	FT	-6.2±0.1	-20.0±0.0	FT
<i>Stachys philippiana</i>	Lamiaceae	-16.8±0.5	-8.5±0.8	FA	-14.1±1.1	-10.5±0.4	FA
<b>Total mean</b>		<b>-9.5±0.4</b>	<b>-13.2±0.5</b>		<b>-10.3±0.4</b>	<b>-12.9±0.6</b>	

Values are means ± standard error. LT<sub>50</sub> were obtained from Morales et al. (2020)

INT ice nucleation temperature, LT<sub>50</sub> temperature producing 50% damage and Mechanisms, FA freezing avoidance, FT freezing tolerance

Measurement Computing Corporation, Norton, MA). The sudden rise in leaf and flower temperature (exotherm) produced by the heat released during the freezing process was used to determine the ice nucleation temperature (INT). INT is the lowest temperature before the start of the exotherm and indicates the beginning of the formation of ice crystals in the apoplast (Larcher 2003).

### Assessment of freezing damage

The temperature that caused 50% damage (LT<sub>50</sub>) in flowers and leaves was estimated by using the membrane electrolyte leakage method (Wilner 1960; Lipp et al. 1994). Single flowers or capitula and leaves from different individuals per species were exposed to six independent freezing treatments: -5, -7.5, -10, -12.5, -15, and -20 °C. Once each temperature treatment finished, the tubes were

removed from the cryostat, and 10 ml of deionized water was then added to each tube. The electrical conductivity (EC) of the solution and the maximum ion leakage by freezing-induced damage were measured with a conductance/resistance meter (Hanna HI 8733, Hanna Instruments, Woonsocket, RI, USA) and used to estimate the relative electrical conductivity (REC). REC was corrected with the control treatment to obtain a corrected relative EC (REC of the freezing treatment—REC of the control treatment). LT<sub>50</sub> was determined by linear interpolation using the temperature that caused the highest corrected REC below 50% and the temperature that caused the lowest corrected REC above 50%. All the LT<sub>50</sub> data were obtained from Morales et al. (2020), due to LT<sub>50</sub> determinations were made simultaneously with the thermal analysis, using the same plant individuals (same elevation and date). For full experimental details see Morales et al. (2020).

## Freezing resistance mechanism

For each species, the freezing resistance mechanism was determined by comparing the temperature that produced 50% damage ( $LT_{50}$ ) and INT obtained in the thermal analyses. When  $LT_{50}$  occurred at a lower temperature than INT, the plant was classified as tolerant to extracellular ice formation (freezing tolerant, FT). Conversely, when  $LT_{50}$  was minor or not significantly different from INT, the resistance mechanism was classified as freezing avoidance, FA (Squeo et al. 1991; Bravo et al. 2001; Sierra-Almeida et al. 2009, 2010). In the case of FT species, the safety margin was obtained by calculating the difference between  $LT_{50}$  and INT (Sierra-Almeida et al. 2009; Lazarus et al. 2019) and was used as an indicator to quantify how tolerant to freezing each species is. When this margin is narrow, the plant can tolerate little additional ice related stress within its tissues, and any additional freezing poses a hazard of tissue damage (Lazarus et al. 2019).

## Statistical analyses

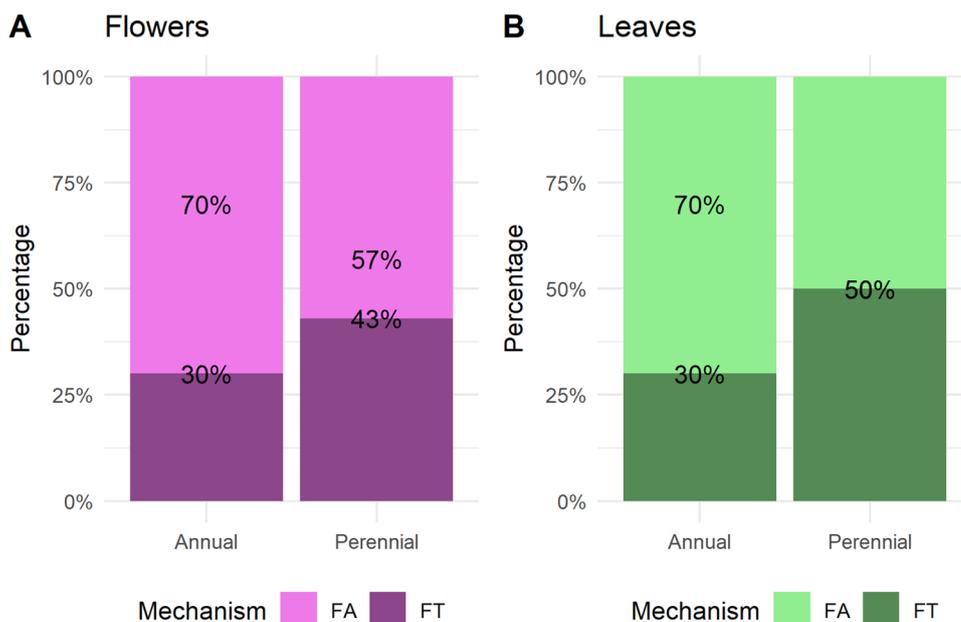
To estimate INT and  $LT_{50}$  differences and determine the freezing resistance mechanism on each organ per species an unpaired two-samples *t* test was used. Additionally, the non-parametric Wilcoxon test was used when assumptions for parametric analyses were not met. A linear model (LM) was used to determine the extent of the relationship between  $\Delta INT-LT_{50}$ , and the independent variables: flower and leaf type, growth form, elevation, and date of measurement, per all annual and perennial species independently. We assumed a Gaussian distribution and checked the normal distribution

of model residuals to confirm the goodness of fit. We also checked the plotted residuals of the model to ensure homoscedasticity. A  $\chi^2$  test was used to determine if the observed proportion of the freezing resistance mechanisms (FA and FT) differ with the hypothesized proportion in each LHS. Fisher's Exact Test was used to determine whether the proportions of FA and FT recorded in the annual and perennial species, per flowers and leaves independently, were statistically different. Rstudio (Version 1.1.463—© 2009–2018 RStudio, Inc.) was used to perform the statistical analyses.

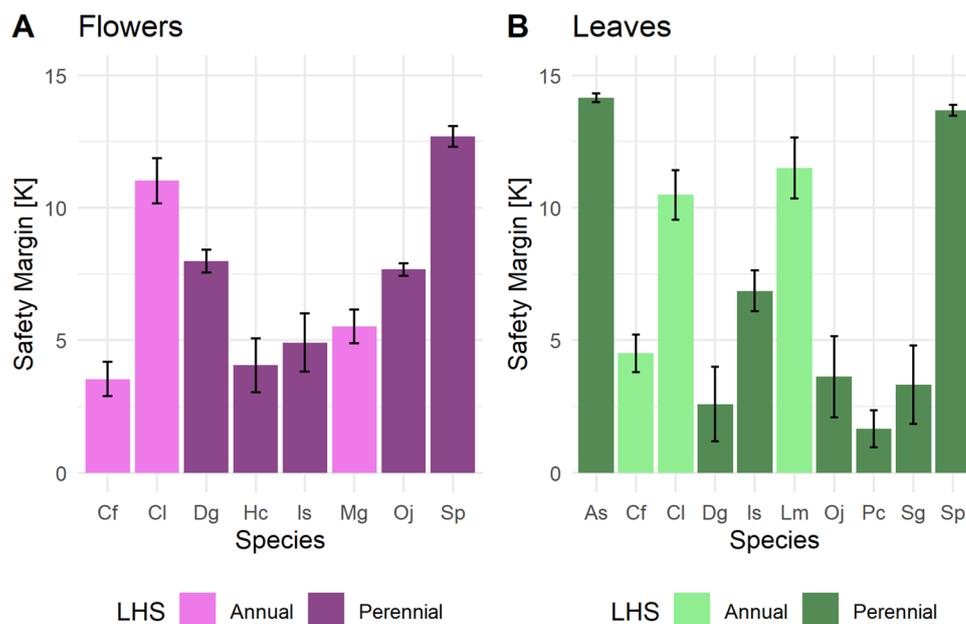
## Results

In annual species, ice nucleation temperature (INT) occurred at  $-10\text{ }^{\circ}\text{C} \pm 0.4\text{ SE}$  in flowers and leaves (Table 1). These values are similar to the mean freezing resistance temperature ( $LT_{50}$ ) reported previously for the same species ( $-10.2\text{ }^{\circ}\text{C} \pm 0.6$  and  $-12\text{ }^{\circ}\text{C} \pm 0.8\text{ SE}$  for flowers and leaves, respectively; Table 1). Accordingly, seventy percent ( $n=7$ ) of annual species were classified as freezing-avoidant (FA) and thirty percent ( $n=3$ ) as freezing-tolerant (FT), in their flowers and leaves, respectively (Fig. 1;  $\chi^2 = 3.53$ ,  $df=1$ ,  $p>0.05$ ). In FT annual species, the mean safety margin was  $6.4\text{ K} \pm 0.9$  in flowers, meanwhile in leaves it was  $8.3\text{ K} \pm 0.9$  (Fig. 2). Linear model indicated that for annual species,  $\Delta INT-LT_{50}$  of flowers was determined by flower type (40%;  $p<0.001$ ), the date of measurement (22%;  $p<0.001$ ), and elevation (5%;  $p<0.01$ ). In contrast, the  $\Delta INT-LT_{50}$  of leaves was mainly determined by the leaf type (71%;  $p<0.001$ ) (Table S2).

**Fig. 1** Percentage of total annual and perennial herbs species that showed freezing avoidance or tolerance mechanism in flowers (a) and leaves (b). **a** Light purple: freezing avoidant (FA); purple: freezing tolerant (FT). **b** Light green: freezing avoidant (FA); green: freezing tolerant



**Fig. 2** Freezing safety margin ( $LT_{50} - INT$ ) for freezing tolerant species determined in (a) flowers of annual (light purple) and perennial (purple) species; and (b) leaves of annual (light green) and perennial (green) species. Abbreviations species: **Cf** *Chaetanthera flabellate*, **Cl** *Chaetanthera linearis*, **Lm** *Lupinus microcarpus*, and **Mg** *Microsteris gracilis*. For tolerant perennial herbs species: **As** *Acaena splendens*, **Dg** *Draba gilliesii*, **Hc** *Hordeum comosum*, **Is** *Ipheion sessile*, **Oj** *Olsynium junceum*, **Pc** *Perezia carthamoides*, **Sg** *Sanicula graveolens*, **Sp** *Senecio polygaloides*. Values are means  $\pm$  SE



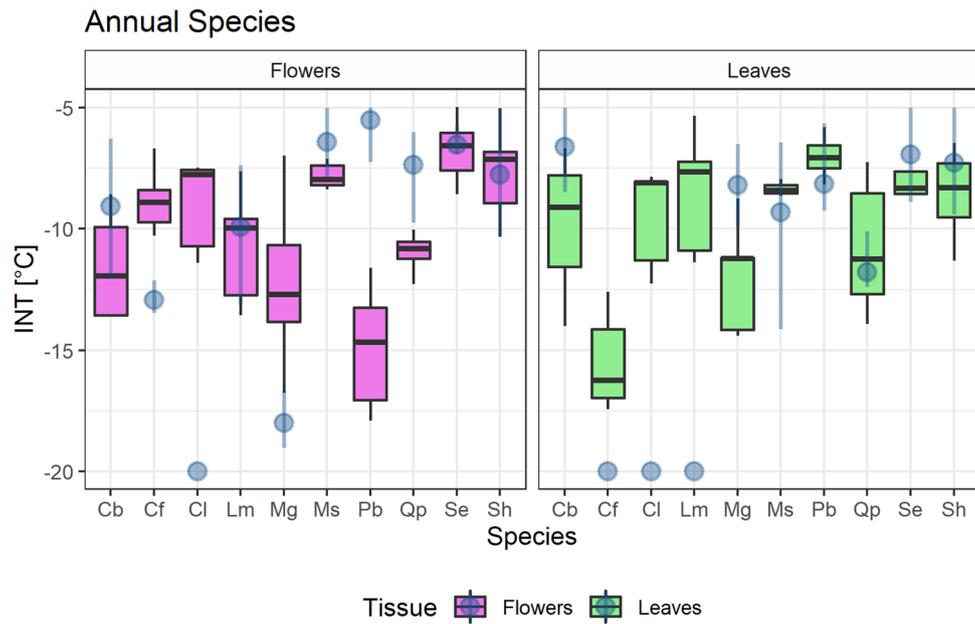
For perennial species, INT occurred at  $-10.3\text{ }^{\circ}\text{C} \pm 0.4$  and  $-9.5\text{ }^{\circ}\text{C} \pm 0.4$  SE, in flowers and leaves, respectively (Table 1), meanwhile the mean  $LT_{50}$  was reported at  $-12.9\text{ }^{\circ}\text{C} \pm 0.6$  SE and  $-13.2\text{ }^{\circ}\text{C} \pm 0.5$  SE in flowers and leaves, respectively (Table 1). Accordingly, 57 of perennial species ( $n=8$ ) were classified as FA and forty seven percent ( $n=6$ ) as FT (Fig. 1;  $\chi^2=11.2$ ,  $df=1$ ,  $p<0.05$ ) in their flowers. In their leaves, 50 percent of species were classified as FA and 50 percent as FT (Fig. 1;  $\chi^2=9.33$ ,  $df=1$ ,  $p<0.05$ ; Table S3). In FT perennial species, the mean safety margin was  $6.9\text{ K} \pm 0.7$  in flowers and  $7.2\text{ K} \pm 0.9$  in leaves (Fig. 2). Linear model indicated that for perennial species, the  $\Delta INT-LT_{50}$  in flowers was determined by flower type (22%;  $p<0.001$ ), date of measurement (16%;  $p<0.01$ ), and growth form (13%;  $p<0.01$ ). Meanwhile in leaves,  $\Delta INT-LT_{50}$  was determined by leaf type (44%;  $p<0.001$ ), growth form (14%;  $p<0.001$ ), and elevation (6.5%;  $p<0.001$ ) (Table S3). Fisher's Exact Test showed no statistically significant differences in the proportions of FA and FT recorded in the annual and perennial species, neither for flowers ( $p>0.05$ ) nor for leaves ( $p>0.05$ ).

Most of the studied species showed the same mechanism of freezing resistance in flowers and leaves (Figs. 3 and 4). Nevertheless, there were some exceptions. For example, in annual species leaves of *L. macrocarpus* were FT whilst flowers were FA; for *M. gracilis* their leaves were FA but their flowers were FT (Table 1; Fig. 3). Similarly, the leaves of perennial species *A. splendens* and *S. graveolens* were FT but their flowers were FA, while *H. comosum* showed freezing avoidant leaves but freezing tolerant flowers (Table 1; Fig. 4).

## Discussion

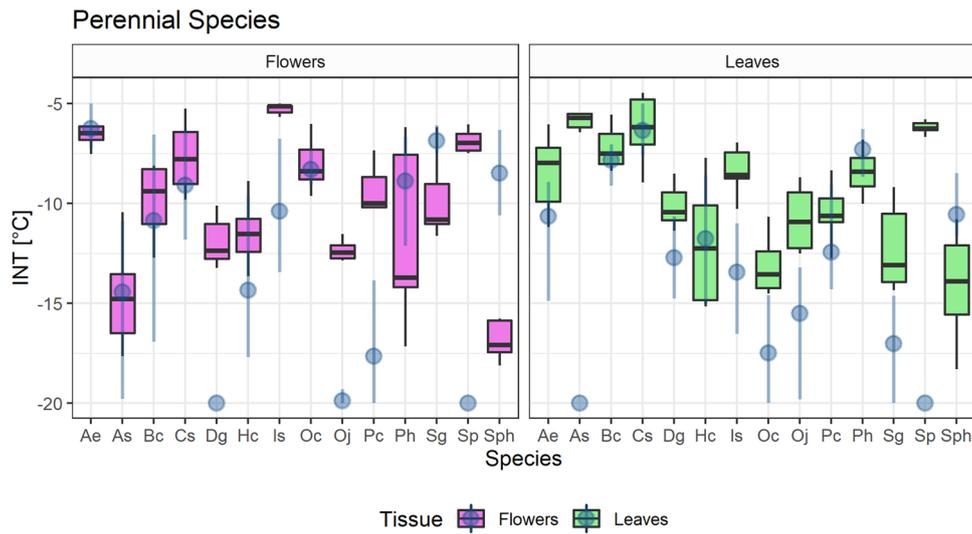
In this study, we evaluated the importance of life-history strategy (LHS) on the type of freezing-resistance mechanism of flowers and leaves in a subalpine community of the central Chilean Andes. In particular, our results partially supported our hypothesis. As we expected, in annual species avoidance is the principal freezing-resistance mechanism. Nevertheless, tolerance and avoidance mechanisms were almost equally represented among perennial species. Most of the studied species exhibited the same freezing-resistance mechanism in flowers and leaves, indicating that the freezing resistance mechanism is conserved at the whole-plant level. To the best of our knowledge, this study represents the first report that simultaneously shows the freezing resistance mechanism of both flowers and leaves for both perennial and annual species, including the highest number of annual-alpine species reported to date.

As we hypothesized, FA was the main mechanism of freezing resistance among studied annual species and could be explained by the trade-off theory (Stearns 1989; Reich et al. 2003). Contrary to the majority of perennial plant species, which have 'slow' life cycles, annual-plants have 'fast' life cycles (e.g., short lifespan, early maturity and high reproductive rates) (Bazzaz and Morse 1991). To assure the success of their only reproductive event during their lifespan, annual plants have a high growth rate and invest most of their energy and mineral resources into reproduction (Primack 1979; Bazzaz et al. 1987; Hancock and Pritts 1987; Garnier 1992). Resource availability is finite; consequently, the high- resource demands of fast



**Fig. 3** Ice nucleation temperatures (INT, °C) in flowers (purple bars) and leaves (green bars) of annual species are shown in comparison to mean values (blue circles) and the minimum and maximum values (range) of freezing resistance of flowers and leaves (blue lines). The boxes indicate the median (second quartile; line inside the box) and extend from the first to the third quartile. The whiskers show at

maximum the 1.5-fold interquartile range. Abbreviations species: **Cb** *Colomia biflora*, **Cf** *Chaetanthera flabellate*, **Cl** *Chaetanthera linearis*, **Lm** *Lupinus microcarpus*, **Mg** *Microsteris gracilis*, **Ms** *Madia sativa*, **Pb** *Phacelia brachyantha*, **Qp** *Quinchamalium parviflorum*, **Se** *Scyphanthus elegans*, **Sh** *Schizanthus hookeri*



**Fig. 4** Ice nucleation temperatures (INT, °C) in flowers (purple bars) and leaves (green bars) of perennial species are shown in comparison to mean values (blue circles) and the minimum and maximum values (range) of freezing resistance of flowers and leaves (blue lines). The boxes indicate the median (second quartile; line inside the box) and extend from the first to the third quartile. The whiskers show at

maximum the 1.5-fold interquartile range. Abbreviations species: **Ae** *Alstroemeria exerens*, **As** *Acaena splendens*, **Bc** *Barneoudia chilensis*, **Cs** *Calceolaria segethii*, **Dg** *Draba gilliesii*, **Hc** *Hordeum comosum*, **Is** *Ipheion sessile*, **Oj** *Olsynium junceum*, **Oc** *Oxalis cinerea*, **Pc** *Perezia carthamoides*, **Ph** *Poa holciformis*, **Sg** *Sanicula graveolens*, **Sp** *Senecio polygaloides*, and **Sph** *Stachys philippiana*

growth and reproduction could produce a trade-off with the freezing-resistance mechanism.

In this sense, annual plants likely avoid or diminished this trade-off through the use of FA mechanisms. Since many avoidance mechanisms have been associated with anatomical features (e.g., supercooling; ice barriers; Kuprian et al. 2014, extraorgan freezing; Ishikawa et al. 2015) they are highly convenient in terms of energy and response time compared with non-constitutive responses. Through these mechanisms, annual plants by-pass the damaging effects of freezing and hence the vital functions and cellular processes are not interrupted, ensuring high growth rates, reproduction, and seed output during its single reproductive cycle. Supporting the above, the results of the linear model showed that  $\Delta\text{INT-LT}_{50}$  in annual species is mainly determined by the flower and leaf type, highlighting the importance of structural traits as part of the mechanism of freezing resistance in annual plants. In addition, seed germination of annual plants is particularly regulated by environmental signals, which synchronize seedling emergence towards periods of low mortality risk (Bazzaz and Morse 1991). Thus, even for annuals that emerge early in the growing season, when minimum temperatures are near zero in the central Chilean Andes (Sierra-Almeida et al. 2009; Sierra-Almeida and Cavieres 2012), investing resources in FT mechanisms during their vegetative growth stage, may be disadvantageous due to the uncertainty of the occurrence of frost events (Sklenář 2017), and may reduce plant fitness when frost damage does not occur (Agrawal et al. 2004).

Conversely, the use of resources in a more conservative way in perennial plants, as a result of slower growth rates and longer lifespan compared to annual plants, gives them a greater availability of resources to invest in other functions as tolerance mechanisms (Bazzaz and Morse 1991). Likewise, the presence of reserve organs, where non-structural carbohydrates are stored, gives perennial plants the capacity of subsidizing the imbalance produced by the simultaneous demand for resources for both growth and protection and may give perennial plants, over their lifetime, the ability to cope with environmental stresses (Bazzaz and Morse 1991). According to the above, the linear model indicated that  $\Delta\text{INT-LT}_{50}$  in perennial species is determined, in addition to structural traits, by traits associated with environmental conditions as the date of measurement, altitude, and the growth form. These results demonstrated that  $\Delta\text{INT-LT}_{50}$  and, in consequence, the freezing resistance mechanism is more flexible in perennial plants and can vary according to demands for protection provoke by environmental stresses, which would explain the almost equal representation of both avoidance and tolerance mechanisms in perennial plants. These results are in line with other authors who reported that freezing resistance mechanisms in perennial plants could change throughout the growing season and

elevation in response to environmental variables as temperature and water availability (Sierra-Almeida et al. 2009; Sklenář 2017). For perennial species, maintaining freezing resistance strategies, either through tolerance or avoidance mechanisms, could be particularly important to ensure their survival during their long lifespan (Bouزيد et al. 2019), as living for multiple seasons implies a high probability of exposure to summer frosts.

Our results showing that FA was the principal mechanism of freezing resistance in annual plants, meanwhile, in perennial species FA and FT mechanisms were almost equally represented, are supported by the analysis of the data reported by Sklenář (2017). Specifically, three of five annual species studied were FA. In perennial species, both mechanisms were almost equally represented. Even though there were no statistical differences in the proportions of FA and FT recorded in the annual and perennial species, to obtain a similar trend in alpine species from temperate mountain regions validate the biological significance of our findings, and support our hypothesis concerning the effect of LHS on the freezing resistance mechanism. In addition to the above, Bouزيد et al. (2019) reported that the mechanism of drought resistance (another dehydrative stress, Xin and Browse 2000) differed between *Arabidopsis* species according to LHS. The only annual species studied, *Arabidopsis thaliana*, employs a drought avoidance strategy. Meanwhile, the perennial species *A. lyrata* employs a drought tolerance strategy. Another perennial species *A. halleri*, combined avoidance, and tolerance strategies. According to the authors, these strategic differences response to the distinct ecological priorities of the stress-tolerant *A. lyrata*, the competitive *A. halleri*, and the ruderal *A. thaliana*, and are agree with the discussion about the ability of perennial species to change their freezing resistance mechanism in response to abiotic conditions and, even, in the case *A. halleri* in response to biotic interactions.

It is important to note that species of the genus *Chaetanthera* (*C. linearis* and *C. flabellata*) were the only freezing tolerant species among studied annuals (for both organs). Apparently, these species use mixed strategies to withstand temperatures down to  $-20\text{ }^{\circ}\text{C}$  in leaves and flowers. In the first instance, they prevent the ice formation by presenting INT close to  $-10\text{ }^{\circ}\text{C}$  in both tissues. Subsequently, they can tolerate the presence of ice in their tissues, reaching a safety margin in leaves and flowers of 4 K in *C. flabellata* and 10 K in *C. linearis*. In the case of reproductive structures, this delay in the ice formation may be the result of the arrangement of the flowers in a floral capitulum (Fig. S2 and Fig. S3). Their numerous and small flowers have a spatially compressed arrangement that can maintain steady temperatures inside the inflorescence (LV Morales, UDEC, Concepción, Chile, unpubl. Res). In the same way, the rosette-like arrangement of the foliaceous implicated bracts surrounded the capitulum can serve as

cold isolation morphological strategies (Davies 2013). The above represents a large safety margin for both species, especially considering the characteristics of frost events described for the study area, and reflects a long evolutionary history of the genus to this type of environment (Hershkovitz et al. 2006) since they have achieved high freezing tolerance despite having “fast” life cycles.

In summary, we found a close relationship between the life-history strategy and the freezing-resistance mechanism of herbaceous species in the Central Chilean Andes. Similar relationships have been previously reported for mechanisms to resist drought and herbivory (Kooyers 2015; Bouzid et al. 2019; Agrawal et al. 2004). These findings suggest that regardless of the stressor, annual species tend to be stress-avoidant, while perennial species could exhibit both types of mechanisms according to the environmental condition. Hence, our results constitute a remarkable contribution to the ecological theory about the connection between life history and plant stress response. Although leaves and flowers tend to present the same type of freezing-resistance mechanism reflecting a whole-plant response, some exceptions exist (e.g., *A. splendens*, *H. comosum*, *L. macrocarpus*, *M. glacialis*, and *S. graveolens*). Thus, further studies dealing with freezing resistance mechanisms have to include different plant organs/structures to develop a whole plant view of the stress response and consider that freezing avoidance and tolerance mechanisms are not necessarily mutually exclusive. Indeed, in some cases, they combine to develop a high degree of freezing resistance (e.g., *Chaetanthera*).

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This study does not involve research on human participants or animals.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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