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Research article

Assessing the importance of cold-stratification for seed germination in alpine plant species of the High-Andes of central Chile

Lohengrin A. Cavieres^{a,b,*}, Angela Sierra-Almeida^{a,b}^a ECOBIOSIS, Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Casilla 160-C, Concepción, Chile^b Instituto de Ecología y Biodiversidad (IEB), Casilla 653, Santiago, Chile

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ABSTRACT

Snow cover duration, which depends on winter precipitation and temperature, is decreasing along several alpine areas around the world as a consequence of the global warming and further increases are expected in the future. Thus, alpine species that requires cold stratification to initiate seed germination may be threatened in the future. Here we report the results of an exploratory experimental survey to assess the importance of cold stratification for the seed germination in alpine plant species from the High-Andes of central Chile. In addition, we assessed how this requirement varies with elevation at the community level. Seeds of a total of fifty plant species were collected along an altitudinal range from 2200 to 3600 m a.s.l. Eighteen species corresponded to the Low-Andean vegetation belt, while thirteen and nineteen species corresponded to the Mid- and High-Andean vegetation belts, respectively. For each species, seeds that experienced cold-stratification at 4 °C for 3 months and control seeds (stored at room temperature and dry conditions for 3 months) were placed on moist paper in 4 Petri dishes containing 50 seeds each, and arranged in a growth chamber set at 20 °C/10 °C and a photoperiod of 14/10 h. While 36% of the studied species were able to germinate without cold-stratification, after this pre-treatment 74% of these species germinated, suggesting that in general cold-stratification promotes seed germination of these alpine plant species. This positive effect was particularly evident on the species from the lower elevations. At highest elevation both the number of species that showed seed germination and the percentage and velocity of seed germination were low. Nonetheless, non-germinated seeds remained viable, suggesting a deeper dormancy. Our results suggest that cold stratification could be an important requirement for successful seed germination in species from lower elevations, while species from higher elevations could require other factors than cold-stratification to break seed dormancy. Nonetheless, as we did not use temperatures as those experienced in the field, further studies are needed to gain insights into the importance of the duration of cold stratification and the underlying mechanisms involved in the seed germination in the field of high-Andean plant species.

1. Introduction

The germination of seeds, that is the transition from seed to seedling, is a high-risk period in the life cycle of many plant species (Baskin and Baskin, 2014); hence, the mechanisms regulating the timing of this transition are expected to be under strong selective pressure (Angevine and Chabot, 1979; Willis et al., 2014). If seeds germination timing is an adaptive process, then natural selection should favor seed germination requirements that reduce the probability of facing environmental conditions that are not appropriate for seedlings establishment (Vleeshouwers et al., 1995; Willis et al., 2014; Baskin and Baskin, 2014). Thus, seed germination requirements should vary depending on the environmental risks associated to seedlings mortality (Meyer and

Monsen, 1991; Meyer et al., 1989, 1990, 1995).

Alpine environments are characterized by low temperatures, strong winds, unstable substrates, and short growing seasons (Körner, 2003). The short growing season of alpine habitats is a major barrier for plants recruitment as it constrains seedlings growth and the period favorable for their establishment (Chambers et al., 1990; Forbis, 2003) because seedlings have to attain a critical biomass by the end of growing season to withstand the harsh and long-lasting winter conditions (Schütz, 2002).

It has been suggested that seeds from plant populations that normally experience long snow cover periods and adverse winter conditions, as occur in alpine habitats, would require an amount of time experiencing the moderately low and constant temperatures that occur

* Corresponding author at: ECOBIOSIS, Facultad de Ciencias Naturales y Oceanográficas, Departamento de Botánica, Universidad de Concepción, Casilla 160-C, Concepción, Chile.
E-mail address: lcaviere@udec.cl (L.A. Cavieres).

under the snow cover to allow or promote their germination (Meyer and Monsen, 1991; Meyer et al., 1989; Meyer et al., 1990; Schütz and Rave, 1999). In the seed germination literature the process of seeds experiencing low and constant temperatures to simulate natural winter conditions as if they were under snow is called cold stratification (Bewley et al., 2013; Baskin and Baskin, 2014). The requirement of a cold stratification period would allow seeds to sense the presence of snow, thereby timing the germination to a period appropriate for seedling survival and establishment. This way, cold stratification requirements would prevent precocious germination under autumn conditions where appropriate soil moisture and temperatures to complete seed germination and seedling establishment are not likely to persist for more than a few days (Meyer and Monsen, 1991). In addition, cold stratification requirements allow germination to occur only in spring after the snow melt, preventing young seedlings from being damaged by freezing temperatures (Billings and Mooney, 1968). Thus, it may be expected that a cold stratification period would be a common requirement for seed germination in alpine plant species. In an early review, Amen (1966) concluded that cold stratification is not a common requirement for the seed germination of several alpine plant species from different mountains in the USA (see also Sayers and Ward, 1966; Marchand and Roach, 1980; Kaye 1997). In contrast, Söyrinki (1938, cited in Körner, 2003) experimenting with 91 alpine species from the Alps, found that storage at winter temperatures (cold stratification) increased seed germination in the great majority of species. More recently, Sommerville et al. (2013) and Hoyle et al. (2015) showed that in 19 and 54 Australian alpine plant species, respectively, a cold stratification period significantly increased seed germination only in half of the species tested. Shimono and Kudo (2005) reported that cold stratification increased the seed germination over a range of temperatures in most of the 27 plant species studied in the alpine zone of Japan. Baskin and Baskin (2014) showed that seeds of most tundra species are dormant, and only those with physiological dormancy require cold stratification to release dormancy. Therefore, how important is cold stratification for seed germination in alpine plant species remains elusive. Further, although studies conducted in species with broad altitudinal distribution (e.g., Cavieres and Arroyo, 2000; García-Fernández et al., 2015) and in species living in habitats with contrasting snow cover duration (Meyer et al., 1989, 1995), have found a positive relationship between the duration of snow cover and duration of cold stratification for maximum seed germination (but see Schütz and Milberg, 1997; Schütz, 2002), few studies have analyzed seed germination patterns in high elevation species at the community level, and as far as we are aware, none has evaluated the altitudinal variation of cold stratification requirements for seed germination at this scale.

Snow cover duration, which depends on winter precipitation and temperature, is decreasing on several alpine areas around the world as a consequence of the recent climate change (Beniston, 1997, 2012), and further decreases are expected to occur in the future due to global warming (op. cit.). Thus, alpine species that require to experience a time of low and constant temperatures as occur under snow (a cold stratification) to initiate their seed germination may be threatened in the future, whilst species with no such requirements may be indirectly favored, generating important changes in the composition and dynamics of alpine plant communities (Briceño et al., 2015; Walder and Erschbamer, 2015). In this study we evaluated the importance of a cold stratification period for seed germination in 50 high elevation plant species from the Andes of central Chile, and whether the requirement of cold stratification varies with elevation at the community level. We expected that a cold stratification period is an important factor for seed germination in these species, and that this importance increases with the altitude (i.e., higher duration of snow cover) at which a community develops.

2. Materials and methods

2.1. Seeds sources

Seeds of the studied alpine plant species were collected in the central Chilean Andes, in the surroundings of La Parva (33°21'S, 70°19'W) and Valle Nevado (33°19'S, 70°15'W) Ski resorts, distanced at 50 and 80 km east of Santiago, respectively. This area is characterized by an alpine climate with strong influence of the Mediterranean-type climate that prevails in the lowlands (di Castri and Hajek, 1976). Precipitation mainly occurs as snow during winter, with summer months usually receiving very few or no rain (Santibáñez and Uribe, 1990). Snow cover remains for 3–5 months depending on the altitude and slope aspect (Rozzi et al., 1989).

The alpine vegetation of the central Chile Andes can be found from immediately above the *Kageneckia angustifolia*'s tree line at 2200 m up to 3600 m a.s.l. (Cavieres et al., 2000). Based on the altitudinal distribution of plant life-forms, this vegetation has been divided in three altitudinal vegetation belts (Cavieres et al., 2000). The Low-Andean belt span from 2200 to 2500 m a.s.l., and is dominated by prostrate shrubs species such as *Chuquiraga oppositifolia* D. Don (Asteraceae), and *Anarthrophyllum cumingii* Hook. et Arn. J.F. Phil. (Fabaceae), with several annual species growing in open spaces between shrubs (Cavieres et al., 2000). The Mid-Andean belt extends from 2600 to 3200 m a.s.l., and is characterized by the dominance of cushion plants (e.g., *Laretia acaulis* Gillies & Hook. (Apiaceae), *Anarthrophyllum gayanum* (A. Gray) B.D. Jacks (Fabaceae)), and perennials herbs such as *Phacelia secunda* J.F. Gmel (Boraginaceae), *Nassauvia aculeata* (Less.) Poepp. et Endl. (Asteraceae), and *Melosperma andicola* Benth. (Plantaginaceae) (Cavieres et al., 2000). The High-Andean belt can be found from 3200 to 3600 m elevation, and is characterized by a low plant cover, where the dominant species are small rosette-forming herbs such as *Pozoa coriacea* Lag. (Apiaceae), *Chaetanthera* sp. (Asteraceae) and *Montiopsis potentilloides* (Barneoud) Ford (Montiaceae) (Cavieres et al., 2000).

The studied species were selected on basis of their seeds availability, to represent different growth-forms and families, and for to be important component of the plant communities in the study area (Table 1). Seeds of fifty plant species were collected along an altitudinal range from 2200 to 3600 m a.s.l.; eighteen species corresponded to the Low-Andean belt, while thirteen and nineteen species corresponded to the Mid- and High-Andean belts, respectively.

All seeds were collected during the natural period of seed dispersal (January–March), stored in paper bags, and transported to the laboratory at the University of Concepción. To assure high intra-specific representation for each species seeds were collected from at least 50 different individuals sparsely distributed within the corresponding vegetation belt. Depending on the species, some fruits (e.g., caryopses, achenes) were treated as seeds. In the laboratory, seeds were immediately cleaned, removing non-seed material by hand and blow away. Then visually-healthy seeds were selected, discarding those aborted and/or predated. The selected seeds of each species were allocated to treatments of cold stratification and control as follow.

2.2. Cold stratification

A widely used standard protocol for the stratification of seeds was used (Baskin and Baskin 2014). For each species, 200 seeds were placed in plastic boxes (20 × 15 × 6 cm) between two layers of paper previously wet. For this, we added distilled water until the papers were saturated and the excess of water was drain out from the plastic boxes. The boxes were completely wrapped with aluminum foil to avoid the passage of light, and were arranged into a growth chamber (Pitec, Bioref-19L) at 4 °C in darkness during three months. We monthly checked that papers remained moist during the cold stratification treatment, and we added distilled water when it was necessary. This procedure was done in dark conditions to avoid light effects on seed

Table 1

Effect of cold stratification on the seed germination of plant species from the Andes of central Chile. Germination parameters: **FPSG**, Final percentage of seed germination (%); **CVG**, coefficient of velocity of germination. Seeds of studied species were collected along three altitudinal vegetation belts: Low-Andean (2100–2500 m), Mid-Andean (2600–3200 m) and High-Andean belt (3200–3600 m). Growth forms: **S**, Shrub; **C**, Cushion plant; **PH**, Perennial herb; **A**, Annual. Statistical differences are shown as: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (ns) not significant. Plant species nomenclature follows Teillier et al. (2011):.

Species	Family	Growth form	FPSG		CVG	
			Control	Cold-stratified	Control	Cold-stratified
Low-Andean belt						
<i>Acaena alpina</i>	Rosaceae	PH	0	2.2 ^{ns}	–	2.4
<i>Acaena splendens</i>	Rosaceae	PH	0	70 ^{***}	–	14.3
<i>Acaena pinnatifida</i>	Rosaceae	PH	0	5.6 ^{ns}	–	5.5
<i>Adesmia papposa</i>	Fabaceae	S	13.3	100 ^{***}	4.0	33.3*
<i>Anarthrophyllum cumingii</i>	Fabaceae	S	23.3	0 ^{***}	5.2	–
<i>Chaetanthera microphylla</i>	Asteraceae	A	100	100 ^{ns}	33.3	33.3 ^{ns}
<i>Diplolepis mucronata</i>	Asclepiadaceae	S	0	0 ^{ns}	–	–
<i>Galium</i> sp.	Rubiaceae	PH	0	100 ^{***}	–	33.3*
<i>Lupinus microcarpus</i>	Fabaceae	A	16.7	23.3 ^{ns}	3.7	12.8*
<i>Madia sativa</i>	Asteraceae	A	2	100 ^{***}	7.0	33.3 ^{***}
<i>Mutisia sinuata</i>	Asteraceae	S	4	100 ^{***}	2.6	33.3*
<i>Nicotiana corymbosa</i>	Solanaceae	A	0	20 [*]	–	2.9
<i>Rhodophiala rhodolirion</i>	Amaryllidaceae	PH	1.3	100 ^{***}	2.2	33.3*
<i>Sanicula graveolens</i>	Apiaceae	PH	0	100 ^{***}	–	33.3
<i>Schizanthus hookeri</i>	Solanaceae	PH	0	44.4 ^{**}	–	33.3
<i>Senecio eruciformis</i>	Asteraceae	S	2	85.6 ^{***}	1.9	15.3*
<i>Sisyrinchium arenarium</i>	Iridaceae	PH	0	0 ^{ns}	–	–
<i>Stachys albicaulis</i>	Lamiaceae	PH	0	23.3*	–	28.1
Mid-Andean belt						
<i>Anarthrophyllum gayanum</i>	Fabaceae	C	5.2	8.9 ^{ns}	3.9	8.7 ^{ns}
<i>Astragalus looseri</i>	Fabaceae	PH	7.7	16.7 ^{ns}	5.7	6.6*
<i>Calceolaria biflora</i>	Calceolariaceae	PH	0	23.3*	–	2.5
<i>Calceolaria purpurea</i>	Calceolariaceae	PH	0	0 ^{ns}	–	–
<i>Chaetanthera euphrasioides</i>	Asteraceae	A	0	24.4*	–	33.3
<i>Euphorbia collina</i>	Euphorbiaceae	PH	0	0 ^{ns}	–	–
<i>Laretia acaulis</i>	Apiaceae	C	0	6.7 ^{ns}	–	3.6
<i>Latace andina</i>	Amaryllidaceae	PH	0	18.9*	–	33.3
<i>Microsteris gracilis</i>	Polemoniaceae	A	92.7	100 [*]	3.2	33.3*
<i>Schizanthus grahamii</i>	Solanaceae	A	0	0 ^{ns}	–	–
<i>Taraxacum officinale</i>	Asteraceae	PH	72	49.3 ^{ns}	4.4	5.3*
<i>Tristagma bivalve</i>	Amaryllidaceae	PH	0	13.3*	–	33.3
<i>Tristagma</i> sp.	Amaryllidaceae	PH	0	5.6*	–	13.9
High-Andean belt						
<i>Azorella madreporica</i>	Apiaceae	C	0	2.2 ^{ns}	–	2.5
<i>Caiophora coronata</i>	Loasaceae	PH	34.5	54.5 ^{**}	4.8	5.1 ^{ns}
<i>Calandrinia caespitosa</i>	Montiaceae	PH	0	2.2 ^{ns}	–	1.6
<i>Cerastium arvense</i> *	Caryophyllaceae	PH	54.5	56.7 ^{ns}	6.1	3.0*
<i>Chaetanthera euphrasioides</i>	Asteraceae	A	0	0 ^{ns}	–	–
<i>Cistanthe</i> sp.	Montiaceae	PH	0	47.8 ^{**}	–	3.3
<i>Draba gilliesii</i>	Brassicaceae	PH	1.3	13.3 ^{***}	0.9	5.6*
<i>Erigeron andicola</i>	Asteraceae	PH	36.5	90 [*]	5.8	6.2 ^{ns}
<i>Gamochaeta</i> sp.	Asteraceae	PH	0	0 ^{ns}	–	–
<i>Loasa sigmoidea</i>	Loasaceae	A	0	3.3 ^{ns}	–	2.9
<i>Melosperma andicola</i>	Plantaginaceae	S	0	0 ^{ns}	–	–
<i>Montiopsis potentilloides</i>	Montiaceae	PH	12.5	40.7 ^{**}	10.1	10.3 ^{ns}
<i>Nassauvia pinnigera</i>	Asteraceae	PH	0	16.7*	–	8.8
<i>Nastanthus ventosus</i>	Calyceraceae	PH	0	0 ^{ns}	–	–
<i>Nicotiana corymbosa</i>	Solanaceae	A	0	0 ^{ns}	–	–
<i>Noccaea magellanica</i>	Brassicaceae	PH	0	2 ^{ns}	–	5.6
<i>Phacelia secunda</i>	Boraginaceae	PH	0	0 ^{ns}	–	–
<i>Pozoa coriacea</i>	Apiaceae	PH	0	0 ^{ns}	–	–
<i>Taraxacum officinale</i> *	Asteraceae	PH	37	40.7 ^{ns}	6.3	5.7 ^{ns}

* non-native species

germination responses.

We selected three months for stratification because it is the minimum amount of time that seeds experience snow cover in the field, especially those from the low elevation vegetation belt. In addition, other studies have proved that this period is enough to promote germination in alpine species with physiological dormancy requiring cold stratification to initiate germination (e.g., Schwiendbacher et al., 2011; Sommerville et al., 2013; Hoyle et al., 2015; Fernández-Pascual et al., 2017). Although 4 °C is a higher temperature than those recorded below the snow cover (that usually fluctuate around 0 °C, Körner, 2003), we

chose this temperatures because it is an efficient and widely used treatment to the break the seed dormancy in many species (Bewley et al., 2013; Baskin and Baskin, 2014). In addition, several studies addressing the role of cold stratification in the seed germination of alpine species have used temperatures between 3 and 5 °C for the cold stratification allowing our results to be comparable with those studies (e.g. Kaye, 1997; Liu et al., 2011; Schwiendbacher et al., 2011; Sommerville et al., 2013; García-Fernández et al., 2015; Fernández-Pascual et al., 2017).

For controls, we also followed standard protocols where other group

of 200 clean seeds per species was air-dried for 48 h. Then, they were placed into paper bags (without substrate), and stored in plastic boxes (20 × 15 × 6 cm) at room temperature (20 °C) for three months, allowing the maintenance of low moisture content in the seeds as it occurs when they are dispersed from the mother plants (Bewley et al., 2013). We used these control conditions because we wanted to assess the importance of to experience constant low temperature and not to compare germination responses mimicking sites with and without snow cover.

2.3. Germination assays

After three months, cold stratification and control boxes were open to initiate seed germination assays. For each species and experimental condition, 50 seeds were placed in a Petri dish between two layers of wet filter paper Petri dishes (4 replicates per species and condition) were arranged in a growth chamber set with a thermoperiod of 20°/10 °C (day/night) and a photoperiod of 14/10 h (light/dark). We chose these germination conditions because they had produced good results in seed germination experiments with other alpine plant species from many different mountains (Chabot and Billings, 1976; Cavieres and Arroyo, 2000; Liu et al., 2013; Sommerville et al., 2013; Fernández-Pascual et al., 2017). During the germination assay seeds were checked every two days for germination, and germinated seeds were removed from the Petri-dishes to reduce mistakes. Seeds were scored as germinated if the radicle and at least 1 mm of the green epicotyls or cotyledons were visible (Schütz, 2002). Germination assays lasted 45 days.

After the end of the germination assay, non-germinated seeds were placed on moist filter papers at room temperature and sliced along its longitudinal axis with a scalpel. Both seed sections were incubated in a 0.1% aqueous solution of Tetrazolium chloride for 12 h in darkness to assess their viability (Hendry and Grime, 1993). Seeds showing a strong red-stained embryo were considered viable (Appendix A in Supplementary material).

2.4. Seed germination parameters and statistical analyses

For each replicate per species we calculated the final percentage of seed germination (FG) both for control and cold-stratified seeds. The FG was arcsine transformed to improve normality and stabilize variances. Statistical differences for FG between treatments were assessed for each species with one-way analysis of variance (ANOVA).

In addition, for each replicate per species a coefficient of velocity of germination (CVG) was calculated with and without cold stratification as follow:

$$CVG = 100[\sum N_i / \sum N_i T_i]$$

Where N_i is the number of seeds germinated on day i and T_i is the number of days elapsed from the start of the experiment (Scott et al., 1984). CVG increases as more seeds germinate in a shorter time, indicating how fast germination occurs (Scott et al., 1984). Given that assumptions for parametric tests were not met even after data transformations, significant differences for CVG between treatments were assessed with Mann-Whitney U tests.

To assess the altitudinal variation in the importance of cold stratification for germination we conducted a two-way ANOVA using the FG as the response variable, and vegetation belt (Low, Mid and High) and treatment (with and without cold stratification) as fixed factors. In the case of CVG, statistical differences were evaluated with a Kruskal-Wallis ANOVA by ranks. Further, three indicators of germination at the community level were calculated for each vegetation belt: 1) the percentage of species that did not require cold stratification for germination, 2) the percentage of species that germinated after a cold-stratification period, and 3) the percentage of species where seed germination was significantly increased (final percentage or speed of germination)

after cold stratification. These three indicators were statistically compared among altitudinal vegetation belts with multiple proportions tests (Zar, 1999).

3. Results

In the Low-Andean belt, eight out of the eighteen tested species showed some germination without cold stratification, with very low FG (< 10%) in four of them (Table 1). After cold stratification however, fifteen species germinated and only two showed FG < 10% (Table 1). Two species (*Diplolepis mucronata* and *Sisyrinchium arenarium*) did not germinate during the evaluated period neither with nor without cold stratification, whereas *Anarthrophyllum cumingii* germinated only without cold stratification. Eleven of the fifteen species that germinated after cold stratification significantly increased their FG compared with no-stratification (Table 1). Among the seven species that germinated both with and without stratification, six significantly increased their CVG after cold stratification (Table 1), indicating that in these species germination was faster in cold-stratified seeds.

In the Mid-Andean belt, only four out of the thirteen studied species showed seed germination without cold stratification, and only two of them with FG > 10% (Table 1). Seeds of ten species germinated after cold stratification, where seven of them showed a FG > 10% (Table 1). Only *Calceolaria purpurea*, *Schizanthus grahamii* and *Euphorbia collina*, did not germinate in any of the evaluated conditions. Viability tests showed that only the seeds of *E. collina* were non-viable (see Appendix A in Supplementary material). Cold stratification significantly increased the FG of six species (Table 1). Only three out of the four species that germinated both with and without stratification significantly increased their CVG after cold stratification (Table 1).

In the High-Andean belt, only six out of the nineteen species tested showed some seed germination without cold stratification (Table 1). After cold stratification, seeds of twelve species germinated (Table 1), although most of these species showed FG < 60% (Table 1). Seven species did not germinate during the germination assays (Table 1), viability test however revealed that all those seeds that did not germinate were viable (Appendix A in Supplementary material). Cold stratification significantly increased FPSG in seeds of six species, whereas CVG slightly increased in *Draba gilliesii* seeds but it decreased in *Cerastium arvense* seeds (Table 1).

When species were grouped by vegetation belt, there was not effect of vegetation belt on the final seed germination ($F_{2,127} = 2.2$, $P = 0.34$), but cold stratification significantly affected seeds germination ($F_{2,127} = 18.1$, $P < 0.0001$; Fig. 1). Whilst the mean FG of cold-

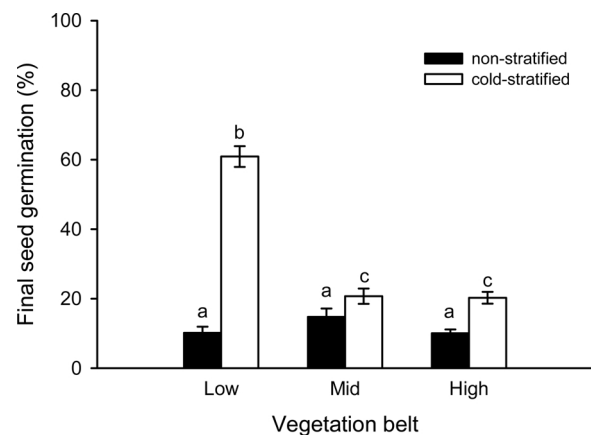


Fig. 1. Final Percentage of seed germination of alpine plant species from the central Chilean Andes. Values are shown as mean \pm 2 standard error ($n = 13$ –19 species). Vegetation belts correspond to **Low-Andean**, 2100–2500 m; **Mid-Andean**, 2600–3200 m; and **High-Andean**, 3200–3600 m above sea level. Lowercase letters indicate significant differences ($P < 0.05$).

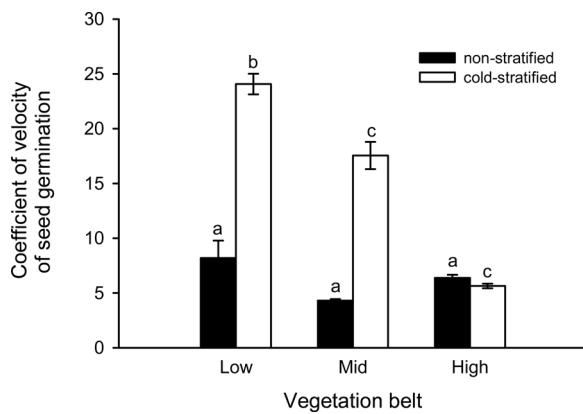


Fig. 2. Coefficient of velocity of seed germination of alpine plant species from the central Chilean Andes. Values are shown as mean \pm 2 standard error ($n = 13$ –19 species). Vegetation belts correspond to **Low-Andean**, 2100–2500 m; **Mid-Andean**, 2600–3200 m; and **High-Andean**, 3200–3600 m above sea level. Lowercase letters indicate significant differences ($P < 0.05$).

stratified seeds did not differ between species from the Mid- and High-Andean belts, those from the Low-Andean belt showed a significantly greater FG (c. three-fold; Fig. 1). Likewise, while the CVG of non-cold stratified seeds was similar among vegetation belts ($H_{2,56} = 2$, $P = 0.44$), after cold stratification the germination of seeds decreased with elevation ($H_{2,56} = 6.8$, $P < 0.05$; Fig. 2).

The percentage of species that germinated without cold stratification was similar among the three altitudinal vegetation belts (35% on average; Fig. 3a). In contrast, the percentage of species that germinated after a cold-stratification period was 32% greater in the Low- compared to the High-Andean belt, whereas the Mid-Andean belt showed no difference with the two others vegetation belts (Fig. 3b). Likewise, the percentage of species that increased their seeds germination after a cold-stratification period was almost twice in the Low- compared to the High-Andean belt, whilst the Mid-elevation belt showed no differences with the two others (Fig. 3c).

4. Discussion

Although one third of the studied species showed seed germination without cold-stratification, after three months of cold stratification a higher amount of species showed germination, and there were important increases in the final number of seeds germinated. Thus, our results suggest that, in general, a cold and wet period is important for promoting seed germination in alpine plant species from the central Chilean Andes. This is in line with several other studies that have shown that a cold-stratification period is important for seed germination of different alpine plant species (Cavieres and Arroyo, 2000; Schütz, 2002; Shimono and Kudo, 2005; Giménez-Benavides et al., 2005; Sommerville et al., 2013; García-Fernández et al., 2015; Hoyle et al., 2015; Fernández-Pascual et al., 2017). In the field, the cold stratification of the seeds naturally occurs when they are in the soil covered with snow during the winter, experiencing constant temperatures around 0–1 °C. Although we chose a higher temperature for our cold-stratification (4 °C) compared to that the seeds may experience in field, stratification temperatures of 3–5 °C are widely used in seed germination studies because they break the dormancy in many different species (Bewley et al., 2013; Baskin and Baskin, 2014). Although temperatures close to 0 °C would have been more realistic and has been used for cold-stratification of seeds in studies with alpine plant species (Schütz, 2002; Shimono and Kudo 2005; Milbau et al., 2009), the majority of studies addressing the role of cold stratification in the seed germination of alpine species have used temperatures between 3 and 5 °C for the cold stratification, (e.g. Kaye, 1997; Giménez-Benavides et al., 2005; Liu et al., 2011; Schwienbacher et al., 2011; Sommerville et al., 2013;

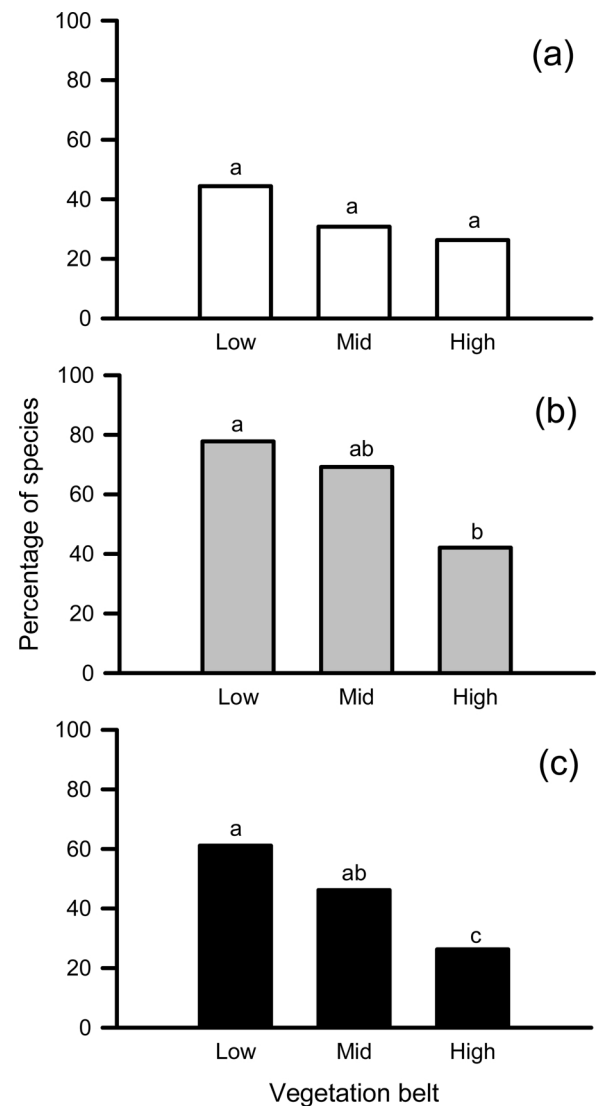


Fig. 3. Altitudinal variation of the importance of cold stratification for seed germination in alpine plant species of the central Chilean Andes. Vegetation belts correspond to **Low-Andean**, 2100–2500 m; **Mid-Andean**, 2600–3200 m; and **High-Andean**, 3200–3600 m above sea level. a) Percentage of species whose seeds did not require cold-stratification for germination; b) Percentage of species whose seeds germinated after cold-stratification; and c) Percentage of species whose seeds significantly increased their germination after cold-stratification. Different lowercase letters indicate significant differences ($P < 0.05$).

García-Fernández et al., 2015) allowing our results to be comparable with all of them. Further, as the maximum density of pure water is reached at 4 °C, stratification temperatures between 0 and 5 °C may have similar effects on the germination of seeds. Thus, it seems likely that a cold stratification period *sensu lato* is more important to trigger germination than its temperature *per se*. Nevertheless, a note of caution has to be considered as for both stratified and control seeds we did not use temperatures as those that seeds experience in the field. Thus, further studies are needed considering field temperatures from exposed and snow protected areas to gain insights on the importance of different duration of cold stratification and the mechanisms involved.

The importance of cold stratification for the seed germination of alpine species of the central Chilean Andes varied across the altitudinal vegetation belts, but in contrast to our expectations, the positive effect of cold stratification on seeds germination did not increase with altitude. The percentage of species showing seeds germination after the cold stratification period decreased from 83% at the Low-Andean to 63% at the High-Andean vegetation belt. Moreover, the positive effect

of the cold stratification in the final percentage of germination and the coefficient of velocity were greater on seeds of species from the lower Andean vegetation belt. Several not mutually exclusive explanations can be suggested to account for this interesting altitudinal trend.

On one hand, it has been shown that a cold stratification period can synchronize germination (Schütz, 2002; Baskin and Baskin, 2014). One of the main sources of seedlings mortality in alpine habitats is drought (Bell and Bliss, 1980; Stöcklin and Bäumler, 1996; Forbis, 2003; Briceño et al., 2015). The central Chilean Andes are located in a Mediterranean-type climate zone where the lower elevations of the Andes experience the long summer drought that characterizes this climate type zone, and where this drought is an important factor of seedling mortality (Cavieres et al., 2006, 2007). The frequent occurrence of summer storms at high elevations implies that the summer drought is much less severe at higher elevations (op. cit). Hence, faster and more abundant seed germination immediately after snow melts might assure a longer period with soil water availability enabling seedlings to develop deeper roots and to attain a critical biomass to successfully cope with the dry summer conditions (see also Giménez-Benavides et al., 2005). In our study area, seedlings density decreased through the growing season, but only at the lower elevations (see Appendix B in Supplementary material), where this seedlings mortality coincides with a decrease of the soil water potentials below -3 MPa towards the second half of the growing season (Jan–Mar; Sierra-Almeida et al., 2009). Although seedlings emergence in early spring could increase mortality risk due to the freezing temperatures that are frequent in the first part of this period, seedlings of the great majority of these species are able to resist very low temperatures (Sierra-Almeida et al., 2010). Therefore, cold stratification requirements would be crucial for species at low elevations because it allows seed germination to occur immediately after the snow-melts when soil moisture is high, increasing the chances of a successful seedlings establishment despite the occurrence of freezing temperatures.

On the other hand, the low positive effect of cold stratification on seed germination found on species from high-Andean belt could be related with additional requirements for seed germination. Although cold stratification produced seed germination in 63% of the species at the High-Andean belt, the average germination was lower than 20%, and no changes in the coefficient of velocity were observed after cold stratification. Interestingly, the seeds of these species that did not germinate remained viable (Appendix A in Supplementary material), suggesting a deeper dormancy that was not broke after three months of cold stratification. Many of the studies that report increased seed germination of alpine species after cold stratification have used stratification periods of 1–3 months (e.g., Shimono and Kudo, 2005; Giménez-Benavides et al., 2005; Sommerville et al., 2013). Nonetheless, several studies have found that species from sites where the snow cover lasts for long periods need longer cold stratification periods for germination than species from sites with shorter duration of snow cover (Meyer and Monsen, 1991; Meyer et al., 1989, 1990, 1995; Schütz and Rave, 1999; Cavieres and Arroyo, 2000), although other studies have found no relationship (Schütz and Milberg, 1997; Schütz, 2002). The three months of cold-stratification used in this study corresponded to the average duration of snow cover in the Low- and Mid-Andean vegetation belt. Snow duration in the High-Andean belt can span from 3 to 5 months, suggesting that longer cold-stratification periods may enhance germination. However, Cavieres and Arroyo (2000) studied the seed germination of *Phacelia secunda* populations at 2900 and 3400 m a.s.l. in the same study area, and found that there were no important differences in the germination after 3 or 5 months of cold stratification in this species. In addition, an assessment with six species from the High-Andean belt that were exposed to three and five months of cold stratification and then exposed to the same germination assay conditions reported here, showed that only in two species a longer stratification period increased germination (see Appendix C in Supplementary material). Hence, it seems unlikely that longer stratification periods could enhance

germination on seeds of the species from the high-Andean belt, suggesting that seeds of these species need other factors to overcome dormancy. Among the factors that overcome dormancy, physical scarification has been found to increase germination in some alpine species (Amen, 1966; Baskin and Baskin, 2014). For instance, Bell and Amen (1970) reported the presence of germination inhibitors in the seed testa of *Luzula spicata* and *L. parviflora*, which were liberated after mechanical scarification through repeated freeze/thawing of water in the testa (cryofracturation). Dorne (1981) found that seed dormancy in seeds of the alpine *Chenopodium bonus-henricus* was imposed by phenols present in the testa, which also required a combination of low temperature, humidity and cryoturbation to disable its inhibitory action on germination. Cavieres and Arroyo (2001) also suggested that scarification via cryoturbation could be involved in the dormancy release of *Phacelia secunda* seeds from very high elevations in the Chilean Andes. Further work is needed to unravel the dormancy mechanisms involved in species from the highest elevations of the central Chilean Andes.

As the species of the High Andean belt showed low germination compared to the Low Andean belt but the ungerminated seeds remained viable, our results also suggest that deep seed dormancy becomes more important towards higher elevations. Similar results have been reported by Bu et al. (2007) who studied the seed germination of 570 alpine plant species collected at different elevations in the Tsinghai-Tibet plateau, China, reporting a negative correlation between germination percentage (but still viable seeds) and elevation. It has been proposed that seed dormancy would be advantageous in habitats spatially and temporary unpredictable such as alpine habitats (Cavieres, 1999; Schwienbacher et al., 2011; Jaganathan et al., 2015). In alpine habitats, seed germination depends on the seeds produced in the previous growing seasons (Körner, 2003). Unseasonably cold years and/or short growing seasons can produce strong reductions in seed output, while also decreasing seed germination and seedling establishment (Bliss, 1985; Galen and Stanton, 1999). If a long and favorable growing season was preceded by a very short growing season where species did not produce seeds, only species that remained dormant in the soil will have the possibility to germinate and recruit (Cavieres, 1999; Jaganathan et al., 2015). Thus, the deep dormancy found here in the higher elevations species as well as in other alpine species (e.g., Schwienbacher et al., 2011; Baskin and Baskin, 2014) suggests that this strategy is very important to cope with the interannual fluctuation of the growing season length in high-elevation environments.

In conclusion, our exploratory study suggest that cold stratification is important to release seed dormancy in alpine plant species from the high-Andes of central Chile, and it seems to be particularly important in most of the species from lower elevations, increasing their final seed germination and the velocity of this process. This strategy seems to be related with a synchronization of seed germination with a period when soil moisture is not limiting for seedling growth. Moreover, the lower percentage of species that germinate after cold stratification, the small positive effect of cold stratification on the seed germination, and the high viability of the seeds that no germinated in these species suggest that break dormancy mechanisms other than cold stratification are required. However, further research is needed to unravel the dormancy mechanisms of these high elevation species and their importance in assuring a successful establishment in the high-Andes of central Chile. In addition, germination trials that properly simulate the environmental conditions that seeds experience in the field (e.g. duration of snow cover, temperature to which seeds are exposed post-dispersal, etc.) are needed to increase our understanding of the mechanisms involved in this important process (see Briceño et al., 2015 for further suggestions). Global climate change is causing important changes in the snow cover duration in several alpine areas around the world (Beniston, 1997, 2012; Gobiet et al., 2014), threatening those alpine species that requires cold stratification to initiate their seed germination, and indirectly favoring those with no such requirement (Sommerville et al., 2013). The species inhabiting the lower elevations of alpine areas are

those that naturally experience shorter snow cover periods, and those where the major changes in snow cover duration have been observed during the last decades, and also those where the major changes are expected (Beniston, 1997, 2012; Gobiet et al., 2014). Thus, major impacts on the natural regeneration process of the species in the Low-Andean vegetation belt may be expected with the current trends in climate change.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ppees.2017.09.005>.

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