# Intraspecific germination variability in water-limited alpine/High Mediterranean Mountains environments.

Abstract

## 1. Introduction

P1: Importance of intraspecific variability for local acclimatization and adaptation

P2: Regeneration niche, germination in alpine environments, then in High Mediterranean mountains then what is known about bWP. Introduce importance of microtopographical gradients

The importance of the seed regeneration niche has been gaining attention during the last years (e.g. xx) after being overlooked for decades (i.e. Briceño et al 2015,(Larson & Funk 2016; Jiménez-Alfaro et al. 2016). There is a consensus that temperature and moisture are two of the main abiotic factors driving plant reproduction (Bewley et al. 2013,(Körner 2021)) and specifically seed germination (Baskin & Baskin 2014). Thus, integrating temperature and water availability is a standard and useful approach (i.e. hydrothermal time) to characterize the germination niche Allen et al. 2000; Bradford 2002; Bewley et al. 2013). Nevertheless, germination studies in the alpine environment, mainly from temperate regions, have been mostly focused on the effect of temperature/warming (e.g. Mondoni et al. 2012; Hoyle et al. 2015; Fernández-Pascual et al. 2021) because moisture is not considered a limiting factor in those regions (Ref). Nevertheless, the climate in the High Mediterranean mountains is characterized by a two-month drought period in summer (ref), but very few studies have tackled how germination responds to water stress (ref), although it is known to be very sensitive to this kind of stress (Fischer & Turner 1978). Low water availability inhibits several physiological processes that lead to delayed or even impeded germination (Baskin & Baskin 2014), Sumner and Venn 2021). Nevertheless, most information about drought effects on germination proceeds mainly from studies on arid ecosystems (e.g. Cochrane et al., 2014; Gelviz-Gelvez 2020; Yi 2019) and there, responses vary notably depending on species (Kos & Poschlod, 2008) and also within species (Yi 2019). A study by Evans and Etherington (1990) found a sharp decrease in germination with water potentials between -0.57 and -0.7 Mpa.

Climate warming poses a future threat especially for temperate alpine environments, due to unpredictable precipitation and earlier snowmelt which could result in water stress during summer (Hanssen- Baeuer et al., 2017). Therefore, High Mediterranean mountains are a perfect example to study effects of water stress that will be more extent in the near future.

In particular, variation in seed germination and dormancy in response to water deficit requires scrutiny (Bernau 2020). Rosback 2022 found that “germination is triggered by high soil moisture levels (e.g. after snowmelt or summer rainfalls)… because small seeds (like alpine seeds) do not contain sufficient nutrients for rapid production of deep roots in a drying environment (Oberbauer and Miller 1982).

Mesoscale heterogeneity of alpine landscape; microclimatic conditions; species adaptations (similar to move along intro)

P3: Detailed sampling at supopulations level within microtopographical gradients. General descriptions of the methods, objectives, questions and hypotheses.

Most studies on alpine germination are focused on population and community levels (e.g. Cao et al., 2018; Cavieres & Arroyo, 2000; Giménez-Benavides, Escudero, & Pérez-García, 2005; Liu et al., 2011, 2018; Shimono & Kudo, 2005; Wagner & Simons, 2009), but very few have investigated responses at subpopulation level (ref). Intraspecific variation in germination responses to abiotic gradients has received less attention (Gya et al., 2023).

Last paragraph of introduction

Here we study intraspecific germination in adaptation to water stress in high Mediterranean mountains dry grasslands. We focus in to understudied topics (1) germination in water-limited High Mediterranean Mountains/alpine environments and (2) intraspecific variability of germination responses to water stress at the microscale level. Our specific research questions are: (1) Will seeds from warmer (i.e., drier) subpopulations germinate better under higher water stress levels? Thus, is there intraspecific local adaptation to the realized niche from adult plants? To calculate hydrotime models we need non-dormant ds, however, some oromediterraneous species are known to have some level of dormancy alleviated by after-ripening. No previous studies (to our knowledge) addressed this question in Dianthus langeanus, thus a second questions arises that needs to be answered first (2) Does the seeds' ripening stage (fresh vs. after ripened seeds) modify their response to water stress? We hypothesize that we will not find germination differences between ripening stages (results: fresh seeds have higher variability of germination responses and germinate worse) and that seeds from warmer/drier subpopulations will germinate better and faster at higher levels of water stress (results YES). To test our hypothesis we conducted a growth chamber experiment to investigate subpopulation-level intraspecific variation of germination to water stress. The results can help us understand how germination will respond to future climate change scenarios where precipitation is predicted to become more unpredictable and concentrate in intense episodes.

## 2. Methods

### 2.1. Study system

This study focuses on *Dianthus langeanus* Wilk. (Caryophyllaceae), a wild carnation endemic to the mountain systems of the northwestern Iberian Peninsula (Fig 1A). *D. langeanus* (Wilk Caryophyllaceae) mainly lives in open dry grasslands on acid soils (Fig 1B), where it can be locally abundant. Previous experiments indicate that this species has high seed production (Fig 1C) and high and fast germination responses at 20ºC (move along paper). We studied wild populations of *D. langeanus* in the northern limit of its distribution, in the Valles de Omaña and Luna Biosphere Reserve, in the southern ranges of the Cantabrian Mountains (Fig 1A, red square). The Cantabrian Mountains run E-W in northern Spain along 480 km in parallel to the Cantabrian Sea … . This mountain hub encloses summits above 2,500 m a.s.l and the treeline climbs up to 1600m a.s.l (ref TFM Jorge). It is considered a transitional biogeographical hub between the Eurosiberian and Mediterranean regions (Jiménez-Alfaro et al. 2021), influenced by Mediterranean climate on the southern slopes and temperate climate on the northern slopes.

### 2.2. Field sites

We established a systematic sampling across four summits above 2000 m a.s.l. (Fig 2 upper panel) inside the distribution area of *D. langeanus* and was highly abundant. In each summit we established a central representative plot (3m radius) where we did a floristic inventory and buried, at 5 cm deep, a Microlog SP3 datalogger, with hourly records of soil temperature and water potential (datalogger MicroLog SP3, EMS Brno, Czech Republic; accuracy in temperature measurements: +/- 0.3 ºC from -40 ºC to 60 ºC; water potential measurements with two Delmhorst gypsum sensors measuring range from -0.1 to -15 bars (permanent wilting point); records every hour). The recording period for the Microlog SP3 went from June 2021 up to now. To measure the spatial microenvironmental gradients we then established 20 additional plots (1m2) per each summit. 5 in each cardinal direction and separated by 10 m (cross design, Fig 2 lower panels), where we also did floristic inventories and buried, also at 5 cm deep, iButton dataloggers (Thermochron, iButton, Newbury, UK; accuracy: +/- 0.5 ºC from -10 ºC to +65 ºC, resolution: 0.5 ºC, records every four hours). The recording period for the iButtons went from 12 July 2021 to 29 May 2022 (321 days). In total, we collected floristic data for 84 plots and environmental data for 78 plots (one MicroLog SP3 in Cañada was damaged, and 5 iButtons could not be recovered).

All sampling summits were located on acidic bedrock (soil pH 3.8 – 4.8). *D. langeanus* was present in 47 out of 84 plots. In the subpopulations where *D. langeanus* was present, local community richness ranged from 3 to 14 species (average of 8 species) (Fig 2 lower panels). This *D. langeanus* communities were dominated by Hemicryptophytes (*Festuca summilusitana* (Franco and Rocha Afonso), Poaceae; and *Luzula caespitosa* (J. Gay ex E. Mey.) Steud, Juncaceae) and the most frequent accompanying species were (*F. summilusitana*, *L. caespitosa*, *Sedum brevifolium* DC, *Neoschischkinia truncatula* subsp. *durieui* (Boiss. & Reut. ex Willk.) Valdés & H.Scholz and *Armeria duriaei* Boiss). Soil climate was typically Mediterranean, with a 2-month drought period in summer (Fig 3A). The growing season stretched from April to November with a mean annual soil temperature of 8 ºC. Soil temperatures reached up to 40 ºC in summer and went down to -4 ºC in winter. ADD days with water stress in the growing season and mean of days with snow (high variations according to orientation of slope). To confirm previous literature reports which state that warmer soils are also drier we took our GDD measures and plotted it against the water potentials values at the microsite level. We used Microlog SP3 data collected for our four summits in 2022 and 2023 to test if, as expected, there was a positive relationship between GDD and water potential. We confirmed that in our study area, summits and years with higher GDD correlated with higher values of accumulated water potential (ΣΨ, R2=0.69, Fig 3B).

### 2.3. Microclimatic indices

We used the records of our dataloggers to calculate soil microclimatic indices as in Paper picos. For comparison, we homogenized the data between the two data sources (MicroLog SP3 and iButtons) by keeping the same recording frequency (every four hours) and the time period with records for all loggers (the 321 calendar days from 12 July 2021 to 29 May 2022). We calculated bioclimatic indices based on WorldClim standard bioclimatic variables (Fick & Hijmans 2017), together with other variables relevant for describing alpine microtopographical gradients. We selected 6 temperature-related indices: (1) bio1 = annual mean temperature; (2) bio2 = mean diurnal range, i.e. the mean of the monthly differences between maximum and minimum temperatures; (3) bio7 = temperature annual range; i.e. the difference between the maximum temperature of the warmest month and the minimum temperature of the coldest month; (4) snow = the number of days of snow cover, when the soil temperature is around 0 ºC, calculated for the period in which the maximum temperature was < 0.5 ºC and the minimum temperature was > -0.5 ºC; (5) FDD = freezing degree days, i.e. the sum of daily mean temperatures for days in which the mean temperature was below 0 ºC (Choler 2018); and (6) GDD = growing degree days, i.e. the sum of daily mean temperatures for days in which the soil mean temperature at five cm deep was above 5 ºC (Körner 2021). For easier interpretation of FDD, we transformed the values from negative to positive, so higher values represent more freezing. To identify the main gradients of microclimatic variability, we conducted a principal component analysis (PCA) including all bioclimatic indices (Fig 3C). Axis 1 of the PCA explained 64% of the variance and ordered summits along a gradient of thermicity, towards which the greatest contribution was made by GDD (23.4) and bio1 (23.5). GDD was highly correlated with bio1, bio2 and bio7 (> 70%, details in Supplementary xxx). Thus, we decided to use GDD as the single best descriptor of microclimatic variability for further analyses.

### 2.4. Seed collection

We sampled seeds of *D. langeanus* from each plot where the species was present (Fig 2 lower panels). We collected mature fruits (capsules) at the time of natural dispersal (August 7-8th, 2023). In each subpopulation, within a 2m radius from the datalogger, we sampled at least 20 randomly selected mother plants following standard protocols for sampling seeds of wild populations (ENSCONET, 2009). In total, we sampled 47 plots with *D. langeanus* but only were able to collect enough seeds for experiments (> 600) from 18 of them, hereafter called “subpopulations”. Immediately after collection, we manually cleaned the seeds and kept them at room conditions (22 ºC and 35 % RH) until the start of the germination experiments. For each subpopulation used in subsequent experiments, we measured dry seed mass by weighing 10 individual seeds from each subpopulation after the seeds had spent 3 months drying with silica gel (Mettler Toledo, modelo, precision 0.1 mg). We did not find previous information about the species' water requirements for germination; consequently, we tested it across a large gradient of water potential to identify the base water potential required for germination.

### 2.5. Germination experiments

We wanted to measure germination responses to water stress in functionally significant ecological conditions, i.e. using fresh seeds at the time of dispersal. However, although our previous experiments indicated high germination in relatively fresh *D. langeanus* seeds, we also expected that the seeds could show some light level of physiological dormancy and that they could require dry after-ripening to release this dormancy. Since we wanted to calculate hydro-time models, and these models require working with non-dormant seed lots, we decided to repeat the experiments with two seed storage treatments: fresh seeds (10 days after collection, hereafter called “fresh”) and after ripened seeds (45 days after collection, hereafter called “after ripened”). For each storage treatment, we used 12 subpopulations, as seed numbers allowed: 6 subpopulations were repeated for both treatments, 6 subpopulations were used only for the fresh treatment, and 6 subpopulations were used only for the after ripened treatment (Table 1).

To test the seed germination responses to water stress, we performed laboratory experiments using polyethylene glycol solutions to simulate different water potential scenarios (PEG). PEG solutions maintain relatively steady and precise the desired osmotic potentials to study germination thresholds (Bewley et al. 2013). Since we could not find previous information about the species water potential requirements for germination, we performed a previous pilot study that showed zero germination at -1.4 and -1.6 MPa. Thus, we excluded those levels and selected five water potential treatments for the final experiment: 0, −0.2, −0.4, −0.6, −0.8, −1 and −1.2 MPa.

For each treatment combination (7 water potential treatments x 2 storage treatments x 12 subpopulations) we sowed four Petri dishes with 25 seeds each (except in the -1 and -1.2 MPa water potential treatments, where we expected low germination, and we sowed only 2 dishes with 25 seeds each). We used 90 mm Ø Petri dishes with two layers of filter paper (Filtros Anoia S.A. paper for germination assays, Ref.518G085). To each dish, we added 5 ml of either (a) distilled water or (b) a PEG 6000 solution prepared according to Michel & Kaufmann (1973) and Vilella et al. (1991) to reach osmotic potentials of −0.2, −0.4, −0.6, −0.8, −1 and −1.2 MPa at 20 ºC (the experimental temperature). We sealed Petri dishes with parafilm to avoid evaporation of the solutions and to maintain constant water potentials throughout the experiment.

Seeds were incubated in conditions simulating late summer days in the field, when germination has been seen to happen (paper move-along): constant 20°C with a daily photoperiod of 12-12h light/dark. It must be noted, that we used constant 20ºC rather than a more realistic diurnal alternating regime in order to maintain the stability of water stress conditions for the PEG solutions. Conditions were programmed in an incubator (Aralab climatic chamber Fitoclima S600 PL, equipped with four led modules 11W 350mA).

We monitored germination, defined as radicle emergence > 1.5mm, for 28 days: daily until the cumulative germination curve flattened (day 21) and then every two or three days until the end of the experiment. We removed germinated seeds during the scoring and, once the experiments were finished, we cut non-germinated seeds under a binocular loupe and classified them as viable, dead or empty. Seeds with firm and white embryos were considered viable, i.e. potentially germinable (Baskin and Baskin 2014). Subsequent analyses only consider germinated and germinable seeds.

### 2.6. Data analysis

### All analyses were done in R (R core Team 2022) using the packages glmmTMB (Brooks et al., 2017) for fitting Generalized Linear Mixed Models (GLMMs) and seedr (Fernández-Pascual and González-Rodríguez, 2020) for fitting hydro time models. Model fit and residuals were visually checked using the DHARMa package (Hartig, 2022). Data visualization was created with packages ggplot2 (Wickham, 2016) and patchwork (Pedersen 2023) with the wesanderson palette (Ram and Wickham 2023).

To test if final germination varied as a function of water potential and storage time, we fitted GLMMs with binomial distribution. Final germination proportion was the response variable. Explanatory fixed factors were the storage and water potential treatments. Random factors included subpopulation nested within summit. Model formula: Final germination (germinated, viable - germinated) ~ storage \* water potential + (1|summit/subpopulation), family = binomial.

To calculate the water potential germination thresholds of each subpopulation, we fitted hydrotime models. For each subpopulation, the model returned the base water potential (ψb), i.e. the lower water stress threshold beyond which no germination is possible. Then, we modelled base water potential as a function of the subpopulation’s microclimate (measured as GDD, see above) using GLMMs with Gaussian distribution. Explanatory fixed factors were the storage treatment and the subpopulation’s GDD. The summit was included as a random factor (and not subpopulation, as before, since in this case each subpopulation provided one data point for the model). Model formula: ψb ~ storage \* GDD + (1|summit), family = Gaussian. We found a significant interaction storage \* GDD, thus consequently, we tested each storage treatment separately to check if base water potential varied according to GDD in fresh and after-ripened seeds. Model specification: ψb ~ GDD + (1|summit), family = Gaussian.

Additionally, we checked if base water potential varied as a function of seed mass by fitting GLMMs with gamma distribution (since the model did not fulfil Gaussian assumptions). Base water potential was used as the response variable and seed mass and storage treatment as the explanatory variables. Summit was included as a random factor. Model formula: ψb ~seed weight \* storage + (1|summit), family = Gamma. We did find a marginally significant relationship when both storage treatments were analysed separately: only in after ripened seeds we found a marginally significant negative relationship i.e. the heavier the seed the lower the base water potential for germination (details in Supplementary xxx).

## 3. Results

### 3.1 Effect of storage treatment and water potential on final germination proportions

Final germination was higher in after ripened than in fresh seeds (Fig 4A). With no water stress (i.e. distilled water treatment) fresh seeds only attained 70% germination, while germination of after ripened seeds was almost 100%. With increasing water stress, germination dropped below 50% at -0.2MPa in fresh seeds, but at -0.6 MPa in after ripened seeds. At -0.8MPa and below, germination was negligible in both fresh and after ripened seeds. Lower water potential also led to slower germination (Fig 4B). GLMMs confirmed that differences between storage and water potential treatments were statistically significant (p-value < 0.001 in both explanatory factors and significant interaction, Supplementary table xx).

### 3.2 Germination base water potential as a function of microclimate

We used Bradford’s hydrotime model to calculate the ψb for germination for 12 subpopulations in the fresh treatment and 12 populations in the after ripened treatment (Table 1). Values of ψb were higher (i.e. less water stress-tolerant) in the fresh than in the after ripened seeds (average -0.1 vs -0.4 in those 6 subpopulations that were sown at both storage times).

Given the significant interaction between storage treatment and microclimate (measured as GDD; z = 2.45, p-value < 0.05), we analysed the relationship between base water potential and GDD separately for fresh and after ripened seeds. For fresh seeds we found no significant relationship (Fig 4 left panel). On the contrary, after ripened seeds showed a significant relationship (z = -1.99, p-value <0.05) of decreasing ψb in subpopulations with higher GDD (Fig 4, right panel).

### 4. Discussion

P1: Interpretate main results answering our questions (some after ripening alleviated the dormancy thus germination was significantly higher/ subpopulations from warmer/drier microclimatic conditions had higher germinations in lower water potentials (extend and add ref) (slower germination with low water potential confirming results by Gya 202; Cochrane et al 2015, Vázquez-Ramírez and Venn 2021,)

P2: Relate interpretation of the results with literature. Intraspecific variation showed higher importance in oromediterraneous germination drivers (not considered in previous alpine research). Importance of short after ripening times can have major impact in seeds responses in the field CAREFUL comparing PEG results to field bc ..Camacho 21. Comment seed mass vs WP results

Low and spread out germination (Gya 2023) could be an indication of bet-hedging, a strategy well known for germination in habitats with high variability in climate (Evans and Dennehy, 2005). Bet hedging has been found to be a strategy that yields advantages during drought events (Evans and Dennehy, 2005, Lampei et al 2017). Variation in seed germination of a species under different environmental conditions can increase long-term reproduction of plant populations by temporally spreading risk and thus maximizing their fitness (Philippi & Seger, 1989; Simons, 2011; Venable, 2007).

From Gya 2023: drought led to lower germination percentage and slower germination. Seeds from the driest populations had higher germination percentage and shorter time to germination than seeds from the wettest populations, suggesting local adaptation to drought . Also evidence of local adaptation was found Bernau 2020 in Chile peppers, but in this study seeds from drier ecotones had slower a lower germination than landraces from wetter environments). (Contradictory results)

P3: Limitations of the study:

* Limited populations, clearer results if we could have tested more subpopulations
* Experimental conditions (constant T and 12h light) not realistic in the field. Camacho 21. Also add info from germination drivers exp
* Wp data limited to fewer plots, use of different loggers
* iButtons data from 21-22 but seed collected 23
* No field data/observations yet (although maybe we can incorporate some info about persistence exp

P4: future directions:

* Study more oromediterranean species responses to WP (germination drivers exp)
* Needed field information about field germination and seedling stress
* Potential of local adaptation to climatic changes
* Local adaptation vs phenological plasticity(need for a common garden experiment)

Thus, the predicted warmer and dryer climate will favor germination of drought-tolerant species. Our results suggest that the species-specific germination responses to environmental conditions are important in structuring the desert steppe community and have implications for predicting community structure under climate change. Yi 2019 (desert species

GBIF.org (24 November 2023) GBIF Occurrence Download <https://doi.org/10.15468/dl.d2zyk2>

## 5. References

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Table 1. Bradford hydrotime model results from dr hydrotime function.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **Fresh** | | | |  | **After ripened** | | | |
| **Sub**  **population** | N treatments | theta | ψb | sigma | R2 | N treatments | theta | ψb | sigma | R2 |
| **A00** | 7 | 0.73 | 0.04 | 0.47 | 0.81 | 4 | 1.03 | -0.40 | 0.17 | 0.97 |
| **A02** |  |  |  |  |  | 5 | 1.50 | -0.55 | 0.24 | 0.96 |
| **A11** |  |  |  |  |  | 5 | 1.18 | -0.44 | 0.27 | 0.98 |
| **B00** | 6 | 0.95 | -0.06 | 0.41 | 0.88 |  |  |  |  |  |
| **B03** | 6 | 1.26 | 0.08 | 0.57 | 0.89 | 5 | 1.46 | -0.47 | 0.25 | 0.95 |
| **B07** | 5 | 0.78 | 0.07 | 0.41 | 0.88 |  |  |  |  |  |
| **B17** | 6 | 1.26 | -0.10 | 0.45 | 0.91 |  |  |  |  |  |
| **B19** |  |  |  |  |  | 4 | 1.09 | -0.35 | 0.25 | 0.96 |
| **B20** | 4 | 0.67 | -0.16 | 0.28 | 0.90 |  |  |  |  |  |
| **C00** | 6 | 0.87 | -0.17 | 0.32 | 0.90 | 5 | 1.14 | -0.43 | 0.22 | 0.95 |
| **C06** | 5 | 0.92 | -0.25 | 0.34 | 0.94 |  |  |  |  |  |
| **C18** |  |  |  |  |  | 5 | 1.09 | -0.37 | 0.24 | 0.95 |
| **C19** | 6 | 0.70 | -0.17 | 0.38 | 0.91 | 6 | 0.92 | -0.41 | 0.24 | 0.94 |
| **C20** |  |  |  |  |  | 5 | 1.20 | -0.44 | 0.23 | 0.94 |
| **D00** | 5 | 0.92 | -0.23 | 0.32 | 0.91 | 5 | 1.01 | -0.45 | 0.21 | 0.93 |
| **D11** |  |  |  |  |  | 5 | 1.54 | -0.48 | 0.30 | 0.90 |
| **D12** | 5 | 0.77 | -0.13 | 0.31 | 0.88 |  |  |  |  |  |
| **D19** | 5 | 0.94 | -0.16 | 0.35 | 0.93 | 5 | 1.29 | -0.42 | 0.28 | 0.91 |

Fig1: (A) Iberian Peninsula map, red square highlights our study system. Shadows according to *D. langeanus* potential distribution under current climatic conditions (adapted from Rocha et al., 2017); (B) Picture of high mountains dry grasslands in our study area; (C) *D. langeanus* flower and seed image.

Imagen de la pantalla de un celular con la imagen de una flor

Descripción generada automáticamente con confianza media

Fig 2. Upper panel: Location of the four summits included in our study. Lower panels: Spatial image of our sampling cross design in each of our four summits, at each square we registered botanical inventories and buried iButtons, coloured squares represents where *D. langeanus* was presents and seeds were collected.

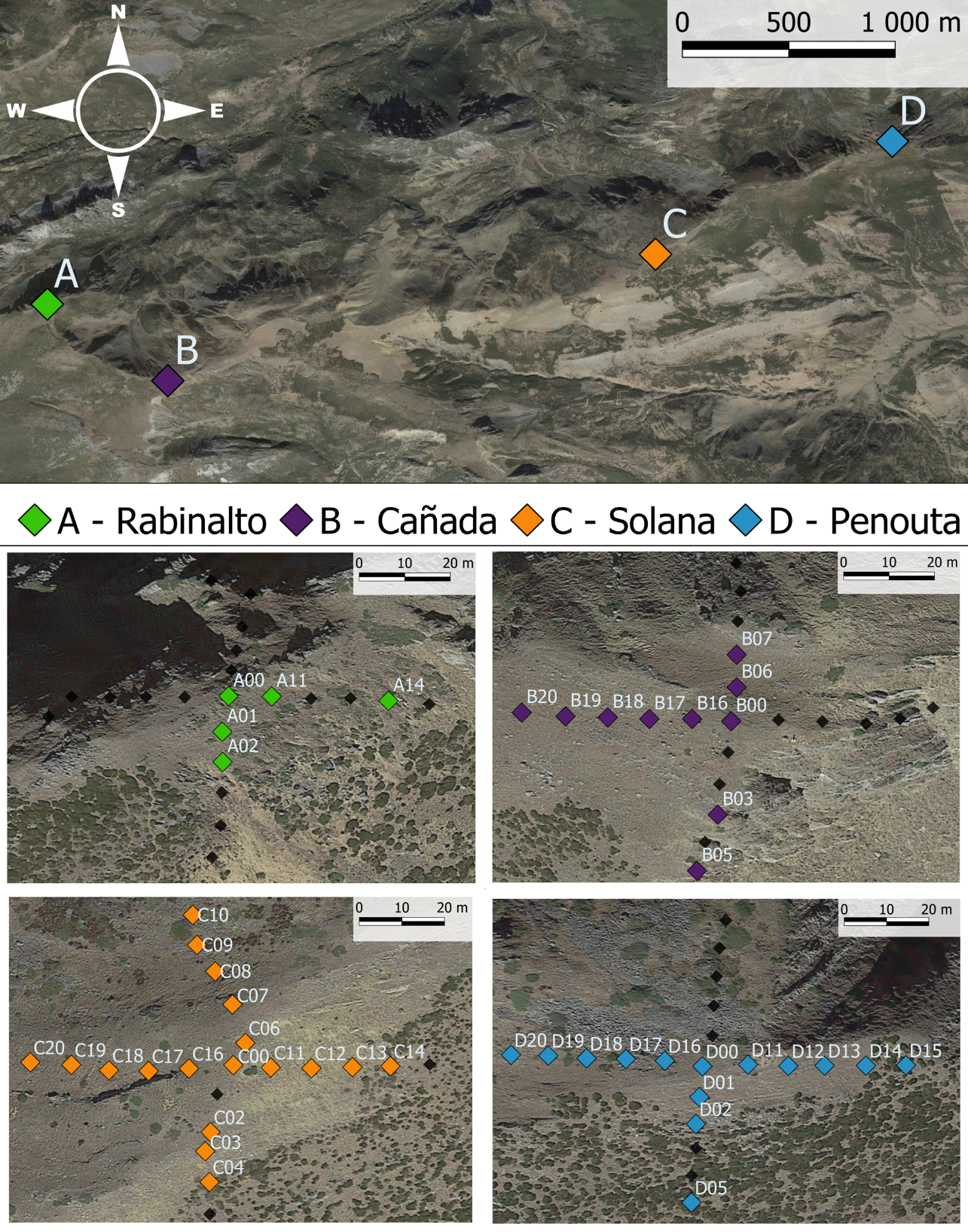


Fig 3. (A) Climogram of our study area, based on three of our summits Microlog SP3 data from July 2021 to June 2022. Lines in red represent monthly mean maximum and minimum temperatures; bars in grey represent the monthly mean of maximum ψb in Mpa. (B) Correlation graph between GDD and absolute sum of ψb registered. We used data from the growing season (April-November) of 2022 and 2023 in three of our summits, Cañada data is not complete and thus removed from visualization. (C) Principal Component Analysis of all 78 plots with environmental data, filtered according to iButtons recording specifications, each colour represents plots of a different summit.

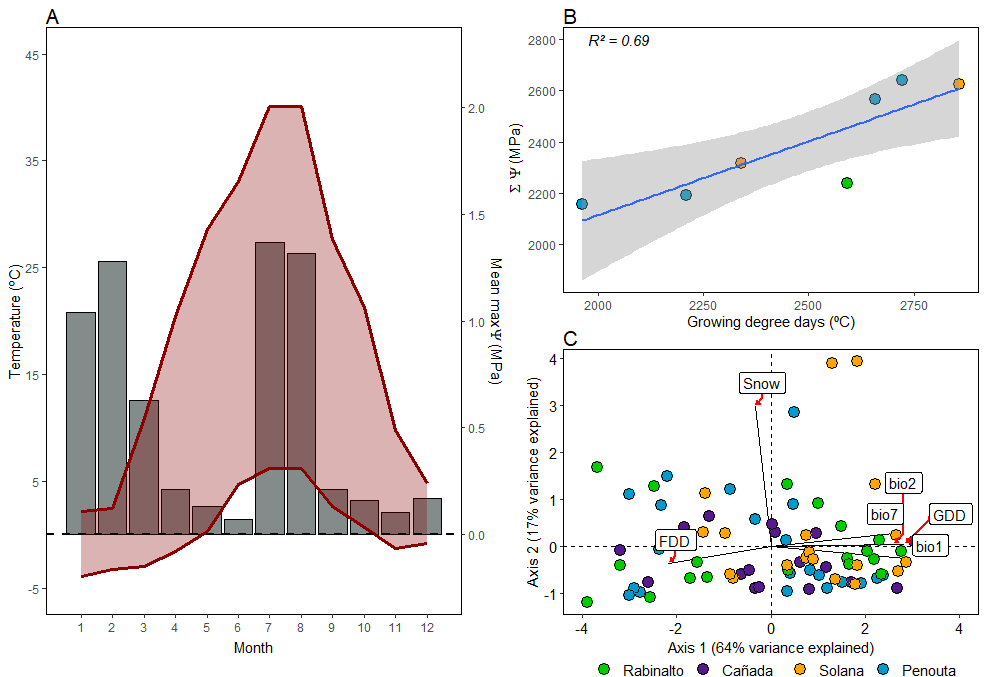


Fig 4. (A) Mean final germination proportion for both storage treatments in every water potential treatment (n subpopulations = 12 in both cases). Bottom panel (B): Cumulative germination curves from all subpopulations (N=12) for both storage treatments.

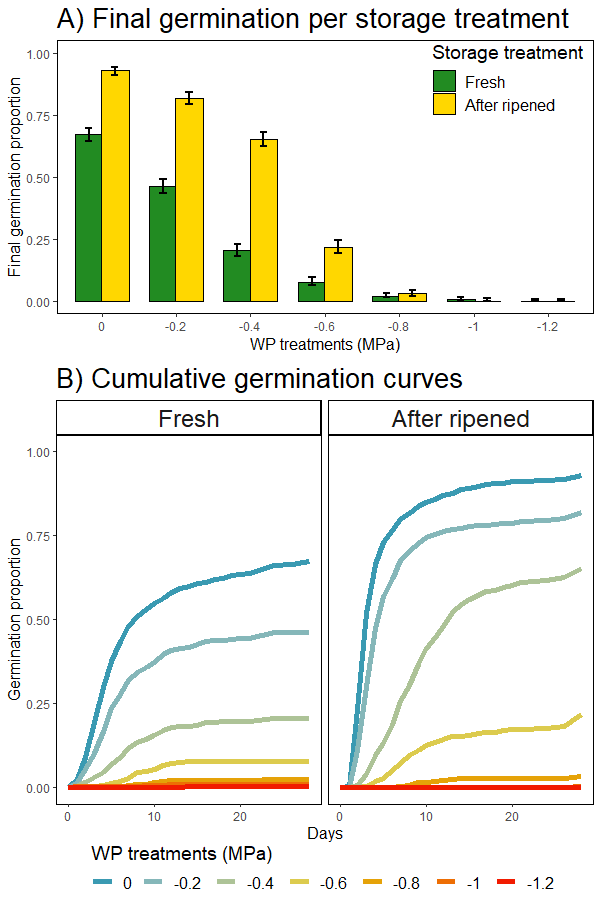


Fig 5. Base water potential calculated using seedr (Bradford method) for each subpopulation and their correlation with each subpopulation GDD. P-values from fitting a glmmTMB as explained in Methods.

