**TITLE:** Intraspecific variability in germination under water stress shows a seed functional response to soil microclimatic gradients.

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**Author contributions**

BJA obtained the funding. EFP, BJA and CE conceived the idea and designed the methodology. CE and DCT collected the data. CE and EFP analysed the data. CE led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

**Data availability statement**

Raw data and R script for analysis are available in GitHub (XXX)

**Conflict of interest statement**

None declared

**Abstract** (350 words max)

1. Intraspecific variation plays a crucial role in a wide range of biological processes and under global change, shaping plant adaptation and regeneration. In alpine habitats, despite the topographic-derived microclimate that might buffer the detrimental effects of climate change, plant communities are suggested to be highly vulnerable as the warming rate increases and the patterns of water availability become more unpredictable. Whilst warming effects on germination have been better studied, we still lack information about the responses to water stress. Particularly, which is the extent and functionality of intraspecific variability of germination base water potential (i.e. minimum water threshold for germination) along local water availability microclimatic gradients.

2. To address this question, we used as a model species *Dianthus langeanus*, an endemic wild carnation characteristic from drought-limited Mediterranean alpine communities in the NW Iberian Peninsula. We sampled 18 subpopulations with contrasting field-measured environmental conditions at the microscale level. We tested germination responses using polyethylene glycol (PEG) solutions to simulate water stress and by fitting hydro time germination models analysed them with GLMM. We repeated the experiments with two storage treatments: fresh and after ripened seeds (10 vs 45 days after collection, respectively) to ensure the use of non-dormant seeds. Additionally, we investigated the relationship between seed mass and base water potential.

3. We found significant differences between subpopulations' base water potential. Seeds from warmer and drier subpopulations had lower base water potential (i.e. more water stress tolerant). Interestingly, with only a month difference between germination trials, seeds drastically changed their germination responses showing opposite patterns according to the storage treatment. We also found that heavier seeds had lower germination base water potential.

4. Synthesis: Our results indicate that germination base water potential is a functional trait with important consequences for individual fitness and it exhibits intraspecific variation across microclimatic gradients of water availability. Such findings suggest either ongoing processes of local adaptation or a wide phenotypic plasticity and a potential capacity for adaptation to both current and future climate scenarios. Our study further highlights the notable ecological implications of rainfall timing for regeneration in alpine water-limited environments.

**Key words**

Alpine; Mediterranean; Microclimate; Microscale; Intraspecific variation; Seed germination; Plant regeneration.

Optional translated abstract (Spanish)

## 1. Introduction

Intraspecific variability is defined as the genotypic or phenotypic differences exhibited among individuals and populations of the same species (Albert et al. 2010). This variation plays a key role in a wide range of biological processes, from individual fitness to population dynamics, species interactions, community assembly and ecosystem properties (Westerband et al. 2021). Intraspecific variability has been hypothesized to be a response to heterogeneous environments (Van Kleunen & Fischer 2005) and an essential condition for plants to adjust to novel environmental conditions (Jump et al. 2009). The adjustment comes from two non-exclusive mechanisms: (1) adaptive evolution (i.e. local adaptation) and (2) phenotypic plasticity (i.e. acclimatisation) (Nicotra et al. 2010; Reed et al. 2011; Fernández-Pascual & Jiménez-Alfaro 2014). Adaptive evolution, a long-term process of genotypic changes, broadens a species’ potential niche. However, locally adapted populations face limitations in survival conditions, rendering them more susceptible to local threats when dispersal and gene flow are restricted (Atkins & Travis 2010; Valladares et al. 2014), especially under current global change (Peterson et al. 2018). In this situation, phenotypic plasticity may be the key to accelerate plant responses to new conditions (Matesanz et al. 2010; Nicotra et al. 2010; Reed et al. 2011; Walck et al. 2011), acting as a buffer against environmental changes (Lande 2009; Chevin et al. 2010).

In the plant life cycle, environmental changes first influence plant regeneration from seeds, a key process that determines the ability of plant populations to migrate or persist (Walck et al. 2011; Orrù et al. 2012; Baskin & Baskin 2022). Seed germination is an ecophysiological process driven by moisture and temperature (Bewley et al. 2013) and, thus, it is highly sensitive to changes in these two environmental factors (Walck et al. 2011). Intraspecific variability in seed germination responses to moisture and temperature will be key for the adaptation or acclimatization of plants to ongoing climate change (Cochrane et al. 2015). However, compared to temperature (Orrù et al. 2012; Fernández-Pascual et al. 2013; Fernández-Pascual et al. 2019), fewer studies have tackled how the germination of wild species responds to changes in water stress (Bernau et al. 2020; Sumner & Venn 2021). Current assumptions about germination responses to drought are species-based (Kos & Poschlod 2008) mainly from arid ecosystems (Yi et al. 2019; Gelviz-Gelvez et al. 2020). However, It is largely unknown whether intraspecific variability affect germination responses to water stress as recently suggested by Yi et al. (2019); and whether this variability has functional significance along local gradients (Gya et al. 2023). A promising approach to study seed responses to moisture and water stress is the application of developmental threshold models (Donohue et al. 2015), specifically, the modelling of the seed germination niche using hydro-time models (Allen et al. 2000; Bradford 2002; Bewley et al. 2013). In the hydro-time framework, for germination to happen, water availability in the environment must surpass a specific threshold (i.e. the base water potential, ψb). Each seed in a population has its own value of ψb, and therefore calculating this parameter and its variation allows to test the sources and mechanisms of variation among individuals (i.e. intraspecific variability in seed responses to water stress) (Donohue et al. 2015).

Addressing seed germination responses to water stress may be useful for understanding the effects of global warming in alpine ecosystems, with a general trend to earlier snowmelt and increasing summer drought (Möhl et al. 2023). The high topographic complexity observed at fine scales in alpine ecosystems (Scherrer & Körner 2011) provides a mosaic of microclimatic conditions (Körner 2021) with sharp gradients within few centimetres (Graham et al. 2012). Microclimatic gradients of temperature and moisture have been suggested to buffer the effect of climate warming in alpine communities (Körner & Hiltbrunner 2021)(Jiménez-Alfaro et al. 2024). It is therefore expected that the ability of plant populations to cope with microclimatic gradients under climate change will depend on the intraspecific plasticity of seed germination. The study of alpine plant regeneration have mostly focused on the effects of temperature and warming (e.g. Mondoni et al. 2012; Hoyle et al. 2015; Fernández-Pascual et al. 2021). However, soil moisture needs to be also considered in alpine regions (Körner 2021) and it has been shown to specifically trigger germination in the Caucasus (Rosbakh et al. 2022). This may be especially critical in alpine systems influenced by Mediterranean climatic conditions, which are influenced by a period of 1-2 months of summer drought (Sumner & Venn 2021) with implications on plant regeneration (Giménez-Benavides et al. 2005; Giménez-Benavides et al. 2018; Mattana et al. 2022) and growth (Albrecht et al. 2024).

In this study, we address the intraspecific variability on germination responses to water stress along a microclimatic gradient in a drought-limited Mediterranean alpine system. We focused on an endemic and locally abundant species adapted to these conditions, *Dianthus langeanus* Wilk. (Caryophyllaceae). The primary hypothesis is that germination responses to water stress will show intraspecific variability along local gradients of water availability. In particular, we expect lower base water potentials for germination in seeds collected from warmer and drier subpopulations (i.e. higher germination tolerance to water stress). According to previous information of the germination of Mediterranean species (Mattana et al. 2022), fresh seeds of the study species might show some degree of physiological seed dormancy that requires dry after ripening to be alleviated. Thus, a secondary hypothesis is that seed storage in dry after ripening conditions will modify seed dormancy and thus germination responses to water stress. Additionally, we also accounted for seed mass, another relevant seed trait that has been proven to modulate germination responses (Bond et al. 1999; Pons & Fenner 2000; Fernández-Pascual et al. 2019; Fernández-Pascual et al. 2021). Despite the contradictory evidence that seed mass has shown in response to drought both positive responses for smallest seeds (Kikuzawa & Koyama 1999; Merino-Martín et al. 2017; Gya et al. 2023) and also positive responses to largest seeds (Kidson & Westoby 2000; Gelviz-Gelvez et al. 2020), for our tertiary hypothesis we expect that seed mass modulates the responses in alpine habitats, and particularly in Mediterranean systems.

## 2. Material and Methods

### 2.1. Study system

*Dianthus langeanus* Wilk. (Caryophyllaceae) is a wild carnation endemic to the mountain systems of the northwestern Iberian Peninsula (Fig. 1A). *D. langeanus* mainly lives in open dry grasslands on acid soils (Fig. 1B), where it can be locally abundant. Flowering onset occurs in early June (Fig. 1C), and ripe seeds are dispersed during August. Seed production is high, usually >10 seeds per capsule and up to 250 seeds per individual (own data, not published). Germination occurs mainly during end-summer/early autumn at high rates and with high success when water is available at temperatures between 10 and 22 ºC (previous exploratory experiment data not shown). Here, 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 Cantabrian Mountains (Fig. 1A). The Cantabrian Mountains run E-W in northern Spain along 480 km in parallel to the Cantabrian Sea. This mountain system includes summits above 2,500 m a.s.l and the treeline in acids soil climbs up to 1650m a.s.l (González Le Barbier et al., 2024 JVA). It is considered a transitional biogeographical hub between the Eurosiberian and Mediterranean regions (Jiménez-Alfaro et al. 2021), influenced by the Mediterranean climate on the southern slopes and the temperate climate on the northern slopes.

### 2.2. Field sampling

We established a systematic sampling across four nearby summits above 2000 m a.s.l. (Fig. 2) where *D. langeanus* is highly abundant. In each summit, we established a central representative plot (3 m radius) where we did a floristic relevé, recording species composition, and buried, at 5 cm deep, a Microlog SP3 datalogger, with hourly records of soil temperature and soil water potential (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 to November 2023 (add data as supplementary?). To measure the spatial microenvironmental gradients we established 20 additional plots (1m2) per each summit: five plots in each cardinal direction with a 10 m separation (cross design, Fig. 2). We also sampled species composition in these plots and buried, 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 12th July 2021 to 29th May 2022 (321 days, add data as supplementary?). In total, we collected floristic data from 84 plots and environmental data from 78 plots (one MicroLog SP3 was damaged, and 5 iButtons could not be recovered).

*D. langeanus* was present in 47 out of 84 plots (Fig. 2). In the plots where *D. langeanus* was present, local community richness ranged from 3 to 14 species (average of 8 species). The communities with *D. langeanus* were dominated by the hemicryptophytes *Festuca summilusitana* Franco and Rocha Afonso (Poaceae) and *Luzula caespitosa* J. Gay ex E. Mey. Steud (Juncaceae). The most frequent accompanying species were *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. Monthly maximum and minimum soil temperatures reached up to 40 ºC in summer and went down to -4 ºC in winter (Fig. 3A).

### 2.3. Microclimatic indices

We used the records of our dataloggers to calculate soil microclimatic indices as in Jiménez-Alfaro et al. 2024 (JVA): First, we homogenized the data between the two data loggers (MicroLog SP3 and iButtons) by keeping the same recording frequency (every four hours) and the same time period with records for all loggers (the 321 calendar days from 12th July 2021 to 29th May 2022). We calculated bioclimatic indices based on WorldClim standard bioclimatic variables (Fick & Hijmans 2017), together with other variables relevant for describing alpine micro topographical 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 conditions (Supplementary Table 1).

We used Microlog SP3 data collected for seven subpopulations in 2022 and 2023 to test (linear model) if, as expected, there was a positive relationship between GDD and cumulative water potential (ΣΨ, R2=0.69, P < 0.01, Fig. 3B) i.e. warmer years are also drier years. The significant results were used to confidently extrapolate this assumption to all subpopulations sampled.

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 the 78 plots 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, bio7 and FDD (> 70%, details in Supplementary Table 2). Therefore, 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). We collected mature fruits (capsules) at the time of natural dispersal (August 7-8th, 2023). In each plot, we sampled at least 20 randomly selected mother plants within a 2 m radius from the datalogger, 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 seeds) 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, New classic SG – Model ML1052E/01, precision 0.1 mg).

### 2.5. Germination experiments

We wanted to measure germination responses to water stress in 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 degree level of physiological dormancy and that they could require dry after-ripening to release this dormancy. Since we wanted to calculate hydro-time models (Bradford 2002) and no prior information about dormancy alleviation was available for out study species; we repeated the experiments with two seed storage treatments to ensure working with non-dormant seed lots: 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 (PEG, an inert water-binding polymer) solutions to simulate different water potential scenarios. PEG solutions maintain relatively steady and precise osmotic potentials to study germination water 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 seven 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 Villela et al. (1991) to reach desired osmotic potentials 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 described to happen in a previous exploratory experiment (not shown): 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 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 > 1.5 mm, 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 & Baskin 2014). Subsequent analyses only consider germinated and germinable seeds. A total of 14,246 viable (germinated + germinable) *D. langeanus* seeds were used in this study (raw data is available in supplementary Table 3).

### 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 & González-Rodríguez 2020) for fitting hydro time models. Model fit and residuals were visually checked using the DHARMa package (Hartig 2020). Data visualization was created with packages ggplot2 (Wickham 2016) and patchwork (Pedersen 2023) with the wesanderson palette (Ram & Wickham 2023).

To test our primary prediction, i.e., whether base water potential varied as a function of subpopulation microclimate, we calculated the water potential germination thresholds of each subpopulation by fitting hydrotime models with seedr package. For each subpopulation, the model returned the base water potential (ψb), i.e. the lower water potential 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 specific 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) in the model formula: ψb ~ storage \* GDD + (1|summit), family = Gaussian. We found a significant interaction storage \* GDD, 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.

To test our secondary prediction, i.e., whether final germination varied as a function of storage time and water potential, we fitted GLMMs with binomial distribution, in which germination proportion was the response variable. Explanatory fixed factors were the storage and water potential treatments. Random factors included subpopulation nested within summit in the model formula: Final germination (germinated, viable - germinated) ~ storage \* water potential + (1|summit/subpopulation), family = binomial. Finally, to test our last prediction, 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.

## 3. Results

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

We used Bradford’s hydrotime model to calculate the ψb for germination in the 12 subpopulations of the fresh treatment, and in the 12 populations of 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) (Table 1). Given the significant interaction between storage treatment and microclimate (measured as GDD; model 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) (detailed model results in supplementary Table 3).

### 3.2 Final germination proportion as a function of storage treatment and water potential

Final germination was higher in after ripened than in fresh seeds (Fig. 5A). With no water stress (i.e. distilled water treatment, WP treatment = 0) fresh seeds only attained around 70% germination, while germination of after ripened seeds was almost 100%. With increasing water stress, germination dropped below 50% at -0.2 MPa in fresh seeds, whereas, in after ripened seeds, water stress needed to reach -0.6 MPa to cross the same germination threshold. At -0.8 MPa and below, germination was negligible in both fresh and after ripened seeds. Lower water potential also led to slower germination (Fig. 5B). GLMMs confirmed that differences between storage and water potential treatments were statistically significant (p-value < 0.001 in both explanatory fixed factors and significant interaction detailed model results in supplementary Table 4). Individual subpopulations cumulative germination curves can also be checked at supplementary Fig 1.

### 3.3 Germination base water potential as a function of seed mass

The measured seed mass obtained from *D. langeanus* subpopulations was not significantly different between those populations assigned to fresh and after ripened storage treatment (seed mass summary in supplementary table 5), neither we found a relationship between subpopulations preferential GDD and seed mass (supplementary Fig 2). However, the base water potential was modulated by seed mass only if we consider the responses of after ripened seeds (p-value = 0.058). The more disperse responses observed in fresh seeds did not allow to find any relationship (Fig 6).

### 4. Discussion

Our study confirms that subpopulations of *D. langeanus* in warmer and drier conditions have lower base water potentials for germination, indicating that germination responses to water stress show intraspecific variability along local microclimatic gradients of water availability. The lower base water potential (i.e. ability to germinate with less water available) observed in warmer and drier microclimatic conditions suggests either a potential local adaptation or a wide phenotypic plasticity at the microscale. Although intraspecific trait variability has been previously stated to be strongly driven by microenvironmental heterogeneity (Westerband et al. 2021), this is the first time that within-population variation at the microscale level has been reported for regeneration traits in alpine areas. The fact that this variability aligns with a gradient of water stress supports that the base water potential is a functional trait with important consequences for individual fitness and species occurrence patterns at the local scale (https://doi.org/10.1073/pnas.141544211).

The higher germination we observed in after ripened seeds across all water potential treatments supports that a low level of dormancy in fresh *D. langeanus* seeds is alleviated by a short period of after-ripening (35 days). Seeds drastically changed their germination responses in a month, suggesting notable ecological implications of rainfall timing (Levine et al. 2011) in alpine water-limited environments. If rain episodes occur concurrently with dispersal, or shortly thereafter, the dormant part of the seed population will fail to germinate despite the moistened soils and favourable temperatures. Our results indicate a type of developmental delay (Tuljapurkar 1990; Tuljapurkar & Wiener 2000) which has been interpreted as a type of bet-hedging in face of unpredictable disturbances (Venable & Brown 1988; Gremer & Venable 2014), such as potential dry-autumn years that could result in high seedling mortality. Bet-hedging has been observed in other habitats with high climate variability and found advantageous during drought events (Evans & Dennehy 2014; Lampei et al. 2017). If rain episodes happen a month after dispersal, when drought risk can be predicted to be lower due to the closeness of winter, most of the seed population will be able to germinate, and to respond appropriately to microscale soil water stress. These results highlight how a short after ripening period can have a major functional impact in seeds regeneration in the field.

We also confirmed that seed mass may influence base water potential. Nevertheless, our results with *D. langeanus* indicate that the effect of seed mass only becomes apparent in after ripened seeds, where subpopulations with heavier seeds showed lower base water potentials, corroborating results by (Kidson & Westoby 2000; Gelviz-Gelvez et al. 2020). More research is needed to disentangle if there is a general role of seed size as a response to drought or if is species specific (Gelviz-Gelvez et al. 2020). More investigations are also required to clarify if relationships between seed size and germination under water stress might differ among ecosystems (Yi et al. 2019).

### 4.1 Ecological implications

The functional significant of after ripening and base water potential demonstrates the importance of water limitation in alpine germination, a factor which has been generally ignored in previous alpine research and which is expected to become more incident in the future (Kotlarski et al. 2023), especially in biogeographically transitional mountains such as the southern European mountain systems. Unexpectedly, the base water potential for germination in *D. langeanus* (average across after-ripened populations = -0.48 MPa)is relatively high in comparison to other species (i.e. germination tolerance to water stress seems relatively low). Our results on base water potential for *D. langeanus* are comparable to other studies (e.g., temperate Britain) in which a sharp decrease of germination was reported under water potentials between -0.57 and -0.7 MPa (Evans & Etherington 1991). However, those values contrast with lower base water potentials reported for Mediterranean ruderal species (e.g., -0.8 to -1.9, Frischie et al. 2018; Jiménez-Alfaro et al. 2018), indicating that our study system is less limited by water than typical low-altitude Mediterranean systems. The relatively high base water potential of *D. langeanus* could be a way to ensure that germination only goes forward with intense rainfall episodes, i.e. a best-bet strategy to match germination to the most favourable environmental window (Pausas et al. 2022).

We note that the intraspecific variability detected in this study cannot be attributed solely to either local adaptation or phenotypic plasticity. The persistence of plant populations is shaped by a dynamic and complex feedback between phenotypic plasticity and local adaptation (Kinnison & Hairston 2007), and previous studies showed that adaptive evolution of phenotypic plasticity is possible in nature, even at small spatial scales (Van Kleunen & Fischer 2005). To disentangle their effects, reciprocal and common garden experiments are needed (e.g., Potvin & Tousignant 1996). Nevertheless, it is clear that the intraspecific variation detected in our study area do not follow a random pattern. This is in line with several studies in alpine areas which suggest that local adaptation processes are taking place in the seed regeneration niche (Giménez-Benavides et al. 2007; Mondoni et al. 2009).

Although our study supports the functional significance of germination water potential as a relevant seed trait, we must acknowledge some limitations to our conclusions. First, our environmental data is constrained to 2021- 2022 while seeds were collected in 2023. Therefore, we must assume that relative microclimatic differences between subpopulations remain comparable across years, and that our GDD measures are a valuable proxy for the environmental thermicity-drought gradient. Second, it was not possible the seed collection from some subpopulations due to the insufficient presence of the species in some plots. Nevertheless, our statistical models still detected significant relationships within our subpopulation data (n = 18). Second/third, the constant germination temperatures used in the experiments are not realistic in field conditions, but they were necessary to maintain the stability of water potential solutions. Moreover, our preliminary data indicated that the focus species has a wide germination niche without significant differences between constant and alternating temperatures, reaching up to 70% germination even in darkness. Third/fourth, the translation of laboratory PEG results into field behaviour should be done carefully (Camacho et al. 2021). In the field, soil water availability is affected by dynamic soil hydraulic conductivity, which in turn depends on soil textural properties (Camacho et al. 2021). It would be important to confirm our results with field emergence data, but it must be considered that maintaining such controlled water potential treatments in the field would be extremely difficult if not impossible with current technologies.

### 4.2 Future research

Future research should extend our understanding of intraspecific variability in germination responses to water stress to other species and ecosystems, including different degrees of environmental water-limitation. In addition, complementary studies with reciprocal sows and common garden experiments will help to disentangle the effects of phenological plasticity and local adaptation. Finally, our understanding needs to be expanded to include the whole seed regeneration spectrum, including soil seed persistence and seedling emergence responses to microclimatic conditions under current and future scenarios.

### 4.3 Conclusions and Implications

Base water potential is a functional trait with important consequences for individual fitness and it can show intraspecific variability along local microclimatic gradients of water availability. More research focused on the seed regeneration niche and seed traits can detect critical sources of potential local adaptation or phenotypic plasticity which could potentially help buffer global change effects. In particular, and according to our results, the increasing unpredictability of precipitation in future climatic scenarios will have notable ecological implications for regeneration in alpine water-limited environments.

## 5. References

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**Table 1**. Bradford hydrotime model results for the studied subpopulations in fresh and after-ripened conditions. The detailed location of subpopulation codes is shown in Figure 2. N treatments = number of water potential treatments that could be included in the model; theta = hydrotime constan; ψb = Base water potential (median); sigma = sigma of the base water potential; R2 = adjustment of the model.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Fresh | | | | | After ripened | | | | |
| Subpopulation | 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 |

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

Descripción generada automáticamente con confianza baja

**Figure 1.** Study system. (A) Distribution of *D. langeanus* in the Iberian Peninsula (dark areas, adapted from Rocha et al., 2017); the red square highlights our study system. (B) One studied community with *D. langeanus* in Mediterranean alpine acidic grasslands of Sierra de Villabandín, Cantabrian Range, Spain. (C) Detail of*D. langeanus* flowers and seeds.

Mapa

Descripción generada automáticamente

**Figure 2**. Field sites. Upper panel: Location of the four summits included in our study. Lower panels: Aerial image of our sampling cross design in each of the four summits, at each diamond we registered floristic relevés and buried environmental data loggers. Coloured squares represent subpopulations where *D. langeanus* was present; black squares sites where *D. langeanus* was absent.

Gráfico, Histograma

Descripción generada automáticamente

**Figure 3**. Climate of the study sites. (A) Climatic diagram of our study area, based on Microlog SP3 data from July 2021 to June 2022 from three of the four investigated summits. Lines in red represent monthly averages of the daily maximum and minimum temperatures; bars in grey represent the monthly averages of the maximum water stress in MPa (-1.5 is considered the wilting point). (B) Positive correlation between GDD and cumulative water stress 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 was removed from the visualization. (C) Principal Component Analysis ordination of the microclimatic indices for the 78 plots with environmental data. Each colour represents plots from a different summit.

Gráfico, Gráfico de dispersión

Descripción generada automáticamente

**Figure 4**. Germination base water potential as a function of microclimatic conditions measured in 12 subpopulations of *Dianthus langeanus* in NW Spain. Germination base water potential (Wb) was calculated using the hydro-time model. Microclimate was measured as growing degree days (GDD) above 5 ºC. P-values obtained from GLMMs as explained in the methods.

Gráfico, Gráfico de líneas

Descripción generada automáticamente

**Figure 5**. Germination responses to water stress in fresh and after-ripened seeds. (A) Mean final germination proportion from both storage treatments in every water potential treatment (n subpopulations = 12 in both cases). (B) Cumulative germination curves from all subpopulations (N=12) for both storage treatments.

Gráfico, Gráfico de dispersión

Descripción generada automáticamente

**Figure 6**. Germination base water potential as a function of subpopulation seed mass measured in 12 subpopulations of *Dianthus langeanus* in NW Spain. Germination base water potential (Wb) was calculated using the hydro-time model. Seed mass was measured per 10 individual seeds with precision scale. P-values obtained from GLMMs as explained in the methods. Coloured points represent each subpopulation summit.