**Abstract** (350 words max)

1. Intraspecific variation exists in a wide range of biological processes, promoting species adaptation to environmental changes. Plant reproduction by seed relies on seed germination, an irreversible physiological process regulated by environmental temperature and water availability. Intraspecific variation in the thermal thresholds for germination is widespread in seed plants and has been the subject of abundant research. However, much less is known about intraspecific variation in water thresholds for seed germination. Despite that the extent and scale of intraspecific variation in the germination base water potential (ψb i.e. the minimum amount of water required for germination) can be of high ecological significance in water-limited ecosystems, it has never been tested at microclimatic scales.

2. We tested the hypothesis that water thresholds for seed germination show functional intraspecific variation along local microclimatic gradients in water-limited Mediterranean alpine ecosystems of the Iberian Peninsula (SW Europe). We sampled 18 subpopulations of *Dianthus langeanus* (Caryophyllaceae), separated by 10 m intervals, and with contrasting field-measured microclimatic conditions. We measured germination responses to water stress using polyethylene glycol (PEG) solutions. We fitted hydro-time models to calculate the germination ψb of each subpopulation and tested the expectation that seeds collected from warmer and drier subpopulations had lower ψb (i.e. their germination was more drought-tolerant).

3. We found significant differences between subpopulations' ψb (i.e. intraspecific variation in germination responses to water stress). Seeds from warmer and drier subpopulations had lower ψb, indicating a more drought-tolerant germination. These results suggest that intraspecific variation in the ψb for germination could potentially have functional significance, even at environmental microscales (c. 10 m).

4. Synthesis: Our results indicate that the germination base water potential is a functional trait with potential implications for individual phenology, reproduction, and fitness in water-limited ecosystems. This functional intraspecific variation in base water potential highlights the adaptation potential of seed germination to both current and future climate scenarios.

**Key words**

Germination base water potential; Hydro-time models; Intraspecific variability; Microclimate; Microscale; Plant regeneration; Seed germination thresholds; Water-limited ecosystems.

Optional translated abstract (Spanish)

1. La variación intraespecífica es una parte imprescindible de un amplio abanico de procesos biológicos y es la base la adaptación de las especies a los cambios ambientales. La reproducción de las plantas se basa en la germinación de las semillas, un proceso fisiológico irreversible que es regulado por la temperatura y disponibilidad de agua en el ambiente. La variación intraespecífica enfocada a los límites termales para la germinación han estado objeto de abundante investigación. Sin embargo, los límites hídricos para la germinación han sido mucho menos estudiados. La distribución y magnitud de la variación intraespecífica en el potencial hídrico base para la germinación (ψb i.e. la cantidad mínima de agua requerida para germinar) puede tener un alto significado ecológico en ambientes con limitación de agua, pero su funcionalidad nunca ha sido testada en microescala.

2. En este estudio testamos la hipótesis que los límites hídricos para la germinación muestran variación intraespecífica funcional a lo largo de microgradientes locales en ambientes alpinos mediterráneos con estrés hídrico de la Península Ibérica (SW Europa). Muestreamos 18 subpoblaciones de la especie *Dianthus langeanus* (Caryophyllaceae), separadas a intervalos de 10m, y con condiciones microclimáticas contrastantes registradas en campo. Medimos las respuestas de germinación al estrés hídrico usando soluciones de polietilenglicol (PEG). Ajustamos modelos “hydro-time” para calcular ψb para la germinación en cada subpoblación y examinamos la predicción de que semillas de subpoblaciones con condiciones más cálidas y secas tendrán ψb más bajos (i.e. su germinación es más tolerante a la sequía).

3. Encontramos diferencias significativas entre los ψb de las subpoblaciones, i.e. hay variación intraespecífica en la respuesta al estrés hídrico. Semillas de subpoblaciones con condiciones más cálidas y secas tuvieron ψb más bajos, lo que significa que su germinación es más tolerante a a la sequía. Estos resultados apoyan la hipótesis que la variación intraespecífica en ψb para germinación tiene un significado funcional, incluso a microescala (aprox. 10m).

4. Síntesis. Nuestros resultados indican que el ψb para germinación es un carácter funcional con importantes consecuencias para la fenología, reproducción y fitness de los individuos en ambientes con limitada disponibilidad hídrica. Estos hallazgos resaltan el potencial de adaptación de la germinación a condiciones climáticas actuales y futuras.

## 1. Introduction

Intraspecific variation is defined as the genotypic or phenotypic differences exhibited among individuals and populations of the same species (Byars, Papst and Hoffmann, 2007; 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 variation 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). When intraspecific variation shows predictive patterns of variation along mechanistically related environmental gradients, it is understood that this variation can have a functional role in individual and population responses to the environment (de Bello *et al.*, 2021)

The plant life cycle begins with seed germination, an irreversible ecophysiological process driven by moisture and temperature (Bewley et al. 2013). In seasonal climates, successful regeneration is limited to a specific timeframe, where germination responses to moisture and temperature serve to match germination to this timeframe (Anderson et al. 2012, Escobar et al. 2018, Rosbakh et al. 2022).This dependency on moisture and temperature means that germination can be highly sensitive to changes in these two environmental factors (Walck *et al.*, 2011). Thus, the adaptation or acclimatization of plant regeneration to ongoing climate change will likely depend on intraspecific variation in seed germination responses to moisture and temperature (Cochrane *et al.*, 2015). However, compared to temperature (Orrù *et al.*, 2012; Fernández-Pascual *et al.*, 2013; Fernández-Pascual, Mattana and Pritchard, 2019), there are fewer studies on how the germination of wild species responds to changes in environmental moisture or water stress (Bernau *et al.*, 2020; Sumner and Venn, 2021). Current assumptions about germination responses to drought are centred on the species level (Kos and Poschlod, 2008; Yi *et al.*, 2019; Gelviz-Gelvez *et al.*, 2020), and the extent of intraspecific variation in germination responses to water stress is largely unknown (Gya *et al.*, 2023). Furthermore, it is unknown if this variation has functional significance (de Bello *et al.*, 2021).

A potential functional role for intraspecific variation in germination responses to water availability is the occurrence of germination during the water-available periods, maximising the favourable period for seedling establishment (Escobar, Silveira and Morellato, 2018). Optimizing the timing of germination in a water-limited system could increase the time for development of a root system capable of surviving summer drought, having a direct effect on seedling survival and overall plant fitness (Giménez-Benavides, Escudero and Iriondo, 2007). Recent findings support that within a single species, populations from arid conditions show advanced reproductive phenology: earlier flowering (Anderson *et al.*, 2012), germination (Christie *et al.*, 2022; Mira *et al.*, 2023) and seedling emergence (Dickman *et al.*, 2019). Such intraspecific variation in phenological and reproductive allocation traits is key for climate adaptation (Kurze, Bareither and Metz, 2017) (Anderson *et al.*, 2012). For instance, results from Dickman *et al.*, (2019) show rapid shifts in regeneration trait means and variance in response to drought, increasing the chance of population survival.

In alpine ecosystems (i.e. areas above the treeline; Körner, 2021), current climate change is producing earlier snowmelt (Vorkauf *et al.*, 2021) and increasing summer drought (Kotlarski *et al.*, 2023). However, the high topographic complexity (Scherrer and Körner, 2011) of alpine systems creates microclimatic gradients that could buffer the effect of climate warming in alpine communities (Körner and Hiltbrunner, 2021; Jiménez-Alfaro *et al.*, 2024). Therefore, it can be expected that alpine plants show intraspecific variation in their seed regeneration traits. This variation may be especially critical in alpine systems influenced by Mediterranean climatic conditions, which can be water-limited by a period of 1-2 months of summer drought (Sumner and Venn, 2021).

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, Meyer and Khan, 2000; Bradford, 2002; Bewley *et al.*, 2013). Within the hydro-time framework, germination occurs when 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, therefore, calculating this parameter and its variation allows testing the sources and mechanisms of variation among individuals (i.e. the intraspecific variation in seed responses to water stress) (Donohue *et al.*, 2015). In this study, we used hydro-time models to measure the intraspecific variation of germination responses to water stress with the aim to describe how germination base water potential varies along local microclimatic gradients in a drought-limited Mediterranean alpine ecosystem. We focused on an endemic and locally abundant species adapted to these conditions, *Dianthus langeanus* Wilk. (Caryophyllaceae). We hypothesised that germination responses to water stress would show functional intraspecific variation along local gradients of water availability, expecting lower ψb for germination in seeds collected from warmer and drier subpopulations (i.e. germination more drought-tolerant).

## 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 and ripe seeds are dispersed during August (Fig. 1C). Seed production is high, usually > 10 seeds per capsule and up to 250 seeds per individual. 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 °C. 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 2500 m a.s.l. and the treeline in acid soil climbs up to 1650 m a.s.l. (González Le Barbier et al., 2024, under revision). It is 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 oceanic 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 °C from -40 °C to 60 °C; 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 April 2024 (raw data available in GitHub repository); in June 2022, two extra Microlog SP3 were buried in each summit to cover spatial microclimatic variation in colder and warmer extremes. Within each Microlog SP3, the gypsum sensors were only separated by 5 cm, and when checking the data both values were very similar thus, we decided to calculate a mean WP measurement per each Microlog SP3 logger. Additionally, to avoid misleading WP measures we focused only on the growing season period in 2022 and 2023, i.e. from April to September, avoiding periods with daily main temperatures below 0 °C. This was done because frozen soil also gives negative WP values as plants suffer a “physiological drought” which is mostly unrelated to plant growth, seed production and germination. 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, Jiménez- Alfaro et al. 2024). We also sampled species composition in these plots and buried, at 5 cm deep, iButton dataloggers (Thermochron, iButton, Newbury, UK; accuracy: +/- 0.5 °C from -10 °C to +65 °C, resolution: 0.5 °C, records every four hours). The recording period for the iButtons went from 12th July 2021 to 29th May 2022 (321 days, raw data available in GitHub repository). In total, we collected floristic data from 84 plots and environmental data from 78 plots (one MicroLog SP3 was damaged the first year, and five 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 graminoid 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 end of March to November with a mean annual soil temperature of 8 °C. Monthly maximum and minimum soil temperatures reached up to 40 °C in summer and went down to -4 °C 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). 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 six 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 °C, calculated for the period in which the maximum temperature was < 0.5 °C and the minimum temperature was > -0.5 °C; (5) FDD = freezing degree days, i.e. the sum of daily mean temperatures for days in which the mean temperature was below 0 °C (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 °C (Körner 2021). For easier interpretation of FDD, we transformed the values from negative to positive, so higher values represent more freezing conditions (see Table S1 in Supporting Information).

Some studies that have approached the interrelationship between temperature and water availability in the soil showed that drier soils also become warmer (Seneviratne *et al.*, 2010), however to our knowledge very few have been done at a microscale level (Graham *et al.*, 2012). Therefore, we used Microlog SP3 data collected for seven subpopulations in 2022 and 2023 to test using a linear model if, as expected, there was a positive relationship between GDD and cumulative water potential i.e. warmer years are also drier years at local scale. The significant results (R2 = 0.66, P < 0.01, Fig. 3B) were used to confidently extrapolate this assumption to all subpopulations sampled. See the discussion for further considerations about this assumption.

To identify the main gradients of microclimatic variation, 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 Table S2). Therefore, we decided to use GDD as the single best descriptor of microclimatic variation 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 °C 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 three 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. At the same time, based on previous information on Mediterranean alpine species (Mattana *et al.*, 2022), we expected that fresh *D. langeanus* seeds could show some degree level of physiological dormancy and that they could require dry after-ripening to release this dormancy. From a previous germination phenology experiment, mimicking field temperatures, we observed that the focal species germinated rapidly to 100% in the first autumn after sowing (September-November) without needing a cold stratification period. Except for this previous phenology experiment, no prior information about dormancy alleviation was available for our study species. Hydro-time models (Bradford, 2002), can be affected by seed dormancy, thus we repeated the experiments with two seed storage treatments to ensure working with non-dormant but relatively fresh 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: six subpopulations were repeated for both treatments, six subpopulations were used only for the fresh treatment, and six subpopulations were used only for the after ripened treatment (Table 2).

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 conditions. PEG solutions maintain relatively steady and precise osmotic potentials to study germination water thresholds (Bewley *et al.*, 2013). From a pilot study, we found zero germination at -1.4 and -1.6 MPa, thus we 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 two 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 five 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 °C (the experimental temperature). We sealed Petri dishes with parafilm to avoid evaporation of the solutions and to maintain constant water potentials throughout the experiment. No condensation was noticeable during the experiment due to the daily checks, and each petri dish was not open for more than a few seconds a day. Although we did not tested the accuracy of water potential in the petri-dishes, we assume low and comparable effects of evaporation that might have impacted the absolute value of water potentials in the petri dishes. Nevertheless, the study focuses on the important patterns underneath the germination base water potential and the relative differences observed at subpopulations level.

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 20 °C 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 and 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 GitHub repository).

### 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, 2020). Data visualization was created with packages ggplot2 (Wickham, 2016) and patchwork (Pedersen, 2023) with the wesanderson palette (Ram and Wickham, 2023).

### To explore the dormancy levels of the seeds, i.e., whether final germination varied as a function of storage time (2 storage treatments: fresh and after-ripened seeds) and water potential (7 water potential levels), we fitted GLMMs with binomial distribution, in which germination proportion was the response variable. Storage treatments were carefully distributed across all subpopulations to ensure representation from all four submits in both storage treatments. Explanatory fixed factors were the storage and water potential treatments (N= 24). Random factors included subpopulation nested within summit in the model formula: Final germination (germinated, viable - germinated) ~ storage \* water potential + (1|summit/subpopulation), family = binomial. Analyses were repeated with the subset of subpopulations which had been sowed in both storage treatments and results remained consistent (see details in Table S3).

To test our primary prediction, i.e., whether ψb varied as a function of subpopulation microclimate, we calculated the water potential germination thresholds of each subpopulation by fitting Bradford’s hydro-time models (Bradford, 1990) with the seedr package. For each subpopulation, the seedr package returned the estimate of ψb for the 50th germination percentile, i.e. the lowest water potential beyond which germination does not occur in the seed occupying the median position in the population distribution of ψb values, which is assumed to follow a normal distribution.

The seedr package replicates the calculations of Bradford’s theoretical model (Bradford, 1990) to fit the hydro-time model using three variables: the value of the water potential treatment (), the time since the beginning of the experiment (t) and the cumulative germination proportions (g). The calculations are as follows: (1) cumulative germination proportions are aggregated at the treatment level across replicates (e.g. Petri dishes); (2) cumulative germination proportions undergo the probit transformation; (3) time (t) and experimental water potentials () are transformed into a base water potential variable by applying the formula

were is the hydro time constant; (4) a linear model is adjusted as follows

; (5) an iterative optimization process is used to find the value of that results in the maximum *R2* for the linear model; (6) the base water potential (ψb) of the 50th percentile is calculated as:

; and (7) the standard deviation () of ψb across the seed population is calculated as:

The figures underlying calculations

For three of the subpopulations of the fresh seed storage treatment (A00, B03 and B07), some replicates (i.e. petri dishes) did not reach 50% germination in water, resulting in positive ψb being calculated.For further analysis, these positive values were assigned a maximum value of 0, following Bradford’s interpretation that the Wb can have a maximum value of 0. Then, we modelled ψb 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, N= 18) in the model formula: ψb ~ storage \* GDD + (1|summit), family = Gaussian. We found a significant interaction between storage andGDD, meaning that the relationship between GDD and ψb was different between both storage treatments. To test the strength of that relationship, we tested each storage treatment. Model formula: ψb ~ GDD + (1|summit), family = Gaussian. Analyses were repeated with the subset of subpopulations which had been sowed in both storage treatments and results remained consistent, details can be checked at Table S4.

Additionally, we wanted to control for seed mass as a covariate, since it is a trait that can modulate germination responses (Bond, Honig and Maze, 1999; Pons and Fenner, 2000; Fernández-Pascual, Mattana and Pritchard, 2019; Fernández-Pascual *et al.*, 2021). Evidence on germination responses to drought controlled by seed mass are contradictory with both positive responses for the smallest seeds (Kikuzawa and Koyama, 1999; Merino-Martín *et al.*, 2017; Gya *et al.*, 2023) and also positive responses to the largest seeds (Kidson and Westoby, 2000; Gelviz-Gelvez *et al.*, 2020). Firstly, we checked if there were any preexisting differences in seed mass between storage treatments, which was not the case. We then checked if germination ψb varied as a function of seed mass only, and again no significant relationship. Finally, we checked if germination ψb varied as a function of seed mass and GDD by fitting GLMMs with gamma distribution (since the model did not fulfil Gaussian assumptions) and found no significant relationship between neither of the terms. We found a positive correlation between seed mass and subpopulations GDD, nevertheless this did not translate into other differences (detailed model information can be found in supplementary tables/figures xxx)

## 3. Results

Our results confirmed that fresh seeds (i.e. 10 days after collection) had some degree of dormancy. Final germination was higher in after ripened than in fresh seeds (Fig. 4A). 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. 4B, see Figure S1 for subpopulation-level cumulative germination curves). GLMMs confirmed significant effects of storage, water potential treatments and their interaction (p < 0.001, see Table S3 for full model details).

We used Bradford’s hydro-time 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 2). 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 six subpopulations that were sown at both storage times) (Table 2).

Given the significant interaction between storage treatment and microclimate (measured as GDD; model z = 2.45, *p* < 0.05), we analysed the relationship between ψb and GDD separately for fresh and after ripened seeds. For fresh seeds we found no significant relationship (Fig. 5 left panel). On the contrary, after ripened seeds showed a significant relationship (z = -1.99, *p* <0.05) of decreasing ψb in subpopulations with higher GDD, i.e. more water-stress tolerance in subpopulations from drier and warmer plots, as per our primary prediction (Fig. 5, right panel) (detailed model results in Table S4).

### 4. Discussion

Our study confirms that subpopulations of *D. langeanus* in warmer and drier conditions have lower ψb for germination, indicating that germination responses to water stress show intraspecific variation along local microclimatic gradients of water availability. Although intraspecific trait variation has been previously stated to be strongly driven by microenvironmental heterogeneity (Westerband, Funk and Barton, 2021), this is the first time that within-population variation at the microscale level has been reported for regeneration traits. The fact that this variation aligns with a gradient of water stress supports that ψb might be a functional trait with potential implications for individual fitness and species occurrence patterns at the local scale.

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, Mceachern and Cowan, 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 and Wiener, 2000) which has been interpreted as a type of bet-hedging in face of unpredictable disturbances (Venable and Brown, 1988; Gremer and 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 variation and found advantageous during drought events (Evans and Dennehy, 2014; Lampei, Metz and Tielbörger, 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.

Maternal or transgenerational effects, here included with seed mass as a proxy, have been previously reported to have an important role in seed germination responses (reviewed in (Roach and Wulff, 1987). In our study, seed mass did not significantly affect germination responses nor germination ψb, although we did find a correlation between subpopulations from warmer microhabitats having also heavier seeds. Our results suggest a differential level of maternal influence, potentially habitat or scale related; while other results reported in the literature show contradictory results with both lighter (Kikuzawa and Koyama, 1999; Merino-Martín *et al.*, 2017; Gya *et al.*, 2023) and heavier (Kidson and Westoby, 2000; Gelviz-Gelvez *et al.*, 2020) seeds showing positive response to drought.

The potential functional significance of ψb (and after-ripening) advocates for the importance of drought in driving the timing and success of germination in water-limited ecosystems. This is also the case in alpine systems, highlighting the importance of alpine drought, 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 ψb for germination in *D. langeanus* (average across after-ripened populations = -0.48 MPa)is relatively high in comparison to some other species (i.e. germination tolerance to water stress seems relatively low). Our results *D. langeanus* are comparable to studies performed with temperate floras (e.g., Britain) in which a sharp decrease of germination was reported under water potentials between -0.57 and -0.7 MPa (Evans and Etherington, 1991). However, our values contrast with lower ψb reported for Mediterranean ruderal species (e.g., -0.8 to -1.9, Frischie et al. 2018; Jiménez-Alfaro et al. 2018), perhaps because our study system is less limited by water than typical low-altitude Mediterranean systems. Alternatively, the relatively high ψb 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). Interestingly, we found a relatively narrow range of base water potential (from -0.35 to -0.55 MPa), with 75% of values falling into the range of -0.40 to -0.48 MPa. From the field data collected, we were able to measure how much time it takes to go from -0.55 to -0.35 MPa and results showed very little (few hours) or non-existent differences. This indicates that rain episodes make the soil surpass the germination base water potential range limits very rapidly. These results suggest a more limited ecological significance of base water potential in the field, even though this species has been proven to germinate within few hours in the laboratory.

The lower ψb (i.e. more drought tolerance) observed in warmer and drier microclimatic conditions suggests either a potential local adaptation or a wide phenotypic plasticity at the microscale. However, we note that our experimental design does not allow to disentangle both processes and future 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 does not follow a random pattern, i.e. it potentially has functional significance. 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, Escudero and Iriondo, 2007; Mondoni *et al.*, 2009). The persistence of plant populations is shaped by a dynamic and complex feedback between phenotypic plasticity and local adaptation (Kinnison and Hairston, 2007), both processes aimed at adjusting to new environmental conditions (Nicotra *et al.*, 2010; Reed, Schindler and Waples, 2011; Fernández-Pascual and Jiménez-Alfaro, 2014). Our results are also in line with previous studies that showed adaptive evolution of phenotypic plasticity in nature, even at small spatial scales (Van Kleunen and Fischer, 2005). Under climate change scenarios, phenotypic plasticity may be the key to accelerate plant responses to new conditions (Matesanz, Gianoli and Valladares, 2010; Nicotra *et al.*, 2010; Reed, Schindler and Waples, 2011; Walck *et al.*, 2011), acting as a buffer against environmental changes (Lande, 2009; Chevin, Lande and Mace, 2010) and showing adaptive responses to drought within a few years (Dickman *et al.*, 2019).

Although our study supports the potential functional significance of germination water potential as a relevant seed trait, we must acknowledge some caveats 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 drought gradient, which is not unreasonable since in the study system soil climate largely depend on stable factors such as slope, exposure and soil physical properties. Temperature influences soil moisture through several coupled pathways, generating an increment of evapotranspiration rates (i.e. higher moisture loss) but also reducing the amount of snow and snow-related water supply in the soil (Seneviratne *et al.*, 2010). This relationship of heat and drought has been corroborated at larger scales (Seneviratne *et al.*, 2010) but very few studies have tested it at local scales (Graham *et al.*, 2012). Second, 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, 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 technology. A more feasible and desirable idea, would be to record field emergence continuously measuring soil field water potential and compare those values with the ones we obtained in the lab.

Some studies like Hardegree et al. (2018) suggest that wet-thermal models (i.e. keeping the water potential below the optimal across several temperature treatments) might be a precise and less time-consuming tool to expand hydro-thermal model applications for non-dormant native plant species. However our results suggest that this wet-thermal results should be taken with caution in topographically complex and water-limited environments where subpopulations germination base water potential might change. Future research should extend our understanding of intraspecific variation in germination responses to water stress to other species and ecosystems, including different degrees of environmental water-limitation. In addition, we need complementary studies with reciprocal sowing and common garden experiments to disentangle the effects of phenological plasticity and local adaptation, as well as directly test the potential functional significance. To improve our mechanistic understanding and move forwards our knowledge, it would be interesting to collect seeds from the extremes of the gradient, do reciprocal sows in a greenhouse and simulate rainfall episodes of diverse intensity while measuring soil base water potential and checking for germination. Finally, our understanding needs to be expanded to include the whole seed regeneration spectrum. Germination base water potential can influence different stages of the regeneration niche. For example, the time differences to reach base water potential, thus the time when germination could start will influence seedling development, a stage considered highly vulnerable (Leck, Simpson and Parker, 2008), generating a potential mismatch between suitable environmental conditions and proper developmental stage. The developmental mismatch could then lead to a reduced reproductive success (Anderson *et al.*, 2012). Another regeneration stage is soil seed persistence, during which seeds undergo cyclical rehydration cycles that influence seed viability in the soil (Long *et al.*, 2011), and it would be interesting to test if their germination base water potential could be also be related to these rehydration times and persistence.

In conclusion, our results indicate that ψb is, potentially, a functional trait with important consequences for reproductive timing and success and, ultimately, for individual fitness. Nevertheless, more research is needed to understand the impact of this trait across habitats, and in relation with other adult and regenerative traits. The finding that ψb shows intraspecific variation along microscale climatic gradients of water availability indicates that seed germination has a high potential to adapt to climatic changes, highlighting the capacity of seeds to integrate environmental signals to produce a fine scale regulation of germination events in time and in space. This plasticity can be a valuable buffer against global change effects, allowing plant populations to cope with the increasing unpredictability of precipitation in future climatic scenarios.

## 5. References

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## Figure captions

**Figure 1.** Study system description. (A) Distribution of Dianthus 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 Mountains, Spain. (C) Detail of D. langeanus flowers and seeds.

**Figure 2**. Field sites location. 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. In each diamond, we registered floristic relevés, buried environmental data loggers and collected *Dianthus langeanus* seeds when available. Coloured squares represent subpopulations where *D. langeanus* was present; black squares sites where *D. langeanus* was absent.

**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 (left y-axis); bars in grey represent the monthly averages of the maximum water stress in MPa (right y-axis). (B) Positive correlation between GDD and cumulative water stress (ΣΨ) in two different years at each site. We used data from the growing season (April-September) of 2022 and 2023 in our four summits. (C) Principal Component Analysis ordination of the microclimatic indices for the 78 plots with environmental data. Each colour represents plots from a different summit.

**Figure 4**. Germination responses to water stress in fresh (i.e. dormant) and after-ripened (i.e. non-dormant) seeds. Figures show the values averaged across all subpopulations (n = 12). (A) Mean final germination proportions and binomial confidence intervals in both storage treatments and every water potential treatment. (B) Cumulative germination curves in both storage treatments and every water potential treatment.

**Figure 5**. Seed germination ψb as a function of microclimatic conditions (GDD). Results are shown separately for fresh (i.e. dormant) and after-ripened (i.e. non-dormant) seeds (n = 12, subpopulations in each storage treatment). Germination ψb was calculated using the hydro-time model. Microclimate was measured as growing degree days (GDD) above 5 °C. P-values obtained from GLMMs as explained in the methods.

## Supporting information

Supporting Figure 1: Subpopulations cumulative germination curves

Supporting Figure 2: Subpopulations Bradford Hydrotime representation

Supporting Table 1: Subpopulations bioclimatic indices

Supporting Table 2: PCA summary from Figure 3C

Supporting Table 3: Germination model results

Supporting Table 4: Base water potential model results

## Tables

**Table 1.** Replication statement

|  |  |  |
| --- | --- | --- |
| **Scale of inference** | **Scale at which the factor of interest is applied** | **Number of replicates at the appropriate scale** |
| Subpopulation | Seed | 25 seeds x 4 Petri dish x 2 storage treatments x 12 subpopulations  25 seeds x 4 Petri dish x 7 Water Potential treatments x 18 subpopulations |

**Table 2**. Bradford hydro-time 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 = hydro-time constant, represents the difference in time between a specific water potential and the base water potential needed for germination; ψb = Base water potential ( estimate for the 50th germination percentile); sigma = represents the standard deviation of the base water potential estimate across the percentiles of seed populations i.e how much seeds within seed populations differ in base water potential. Giving a quantitative estimate of the uniformity or synchrony in germination timing among seeds in the population ( (Bradford, 2002).; R2 = adjustment of the linear model used to calculate the ψb .

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 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 |