2. Methods

2.1 Study area

The Cantabrian mountains are a range running W-E through northwest Spain, with altitudes surpassing the 2500 m a.s.l. The range is considered as a transition biogeographical area between Eurosiberian and Mediterranean regions (Loidi et al., 2015), influenced by Mediterranean climate in southern slopes and Temperate oceanic climate in northern slopes (Fig1 a). Therefore, we can find a complex array of communities adapted to both climates. At finer scales, studies in the area have found that in the highest altitudinal (alpine?) belt, topographic heterogeneity creates a mosaic of microclimatic conditions (Fig1 b) that determine changes in compositional (Jiménez-Alfaro et al., 2014) and functional diversity (García-Gutiérrez et al., 2018). Our study focuses, specifically, on alpine grasslands (E4.3 and E4.4 EUNIS codes) found above 1900m a.s.l, dominated mostly by Poaceae and Cyperaceae but also rich in Hemicryptophytes and Chamaephytes.

To study how microclimatic conditions variation might influence germination in this transitional range, we established two study communities: (1) a Mediterranean alpine community within Valles de Omaña and Luna Biosphere Reserve (Southern slope os the Cantabrian mountains, 42.910731 / 42.891192 N and -6.043621 / -6.107621 E) with extremely acid siliceous bedrock (3.8 – 4.8 PH range, unpublished data from 40 field samples) and (2) a Temperate alpine community within Picos de Europa National Park (Northern slope of the Cantabrian mountains, 43.168822 / 43.201078 N and -4.826706 / -4.830672 E) with calcareous bedrock (PH range?).

Mean annual temperatures are 8.08˚C and 5.13 ˚C and temperature annual range are 21.77 ˚C and 30.66 ˚C for the Mediterranean and Temperate community, respectively (values from own field data). Precipitation is highly variable in both communities: 2022 annual precipitation is 755mm (if we took the mean 2010-2022 = 1058mm from Barrios de Luna) or 1335 mm (from SENRA noromet) and 866 mm (Vega de Liordes, noromet)/ 3004.79mm (Vega de Urriellu, mean 2008-2014).

Interfaz de usuario gráfica, Aplicación

Descripción generada automáticamente

Fig 1. A: Map of northwest Spain with the Cantabrian Range´s precipitation gradient and the location of our two communities: Mediterranean and Temperate. B: Pictures correspond to real microclimatic gradient in both our communities.

Talk about differences between microhabitats here or in introduction?

2.2 Fieldwork:

For each community we established 4 collection sites, treated as different populations, between 1900-2200 m a.s.l and separated at least 500m. Next, we recorded all vascular plant species from a central representative plot of 3m radius and those were the target species of our experiment (22 sp. in Mediterranean and 45 sp. in Temperate).

The field goal was to collect 200 seeds from each target species. During August-September 2021 we gathered ripe seeds directly from the mother plants according to species maturity peak in the field. Sampling took place within a 50m radius from the central plot and seeds were collected from at least 20 different individuals. At the end of sampling season, in the Mediterranean community we obtained enough seeds from 21 target sp., two population for each except in 2 species. In the Temperate community we obtained seeds from 34 target sp. and decided to add 4 species also abundant in the community (total of 38 sp.), for 19 species we were able to collect 2 populations. Only three species (*Jurinea humilis*, *Thymus praecox* and *Silene ciliata*) were present in both communities, with 2 populations in each community.

2.3 Lab work

2.3.1 Experimental conditions

To test our hypothesis, we performed a continuous germination phenology/timing experiment to mimic 1 year of natural temperature regimes in alpine communities. We took 10 years of hourly soil temperatures data (2008-2019 field data collected with soil thermometers in our study area) and transformed it into weekly maximum temperature (Tmax) and minimum temperature (Tmin). We also used the registered temperatures to calculate the number of days with snow cover, estimated as days with no temperature variation between night and day (Zhang et al., 2005). Then, we chose the two extremes regimes in snow cover period and temperatures to mimic the microclimatic variability in our study area. The final programs consisted of weekly temperature modifications with monthly photoperiod variation. Each regime was programed in an Aralab incubator (Aralab climatic chamber Fitoclima S600 PL, equipped with 4 led modules 11W 350mA at 20%, photon flux?). Both incubators run simultaneously from July 2021 to September 2022, from now warmer regime with no snow period referred as “Fellfied incubator” and cooler regime with long snow period referred as “Snowbed incubator”. We used Fitolog 9000 software (version 9308,Aralab Pharmaceutical Stability software) to program the microclimatic regimes, it allowed us to use ramp setting for gradual temperature changes along each day and to monitor the incubators programs remotely. In our experiment there were no water stress during growing season (defined as days when T min <2ºC).

Gráfico, Histograma

Descripción generada automáticamente

Fig 2. Incubators’ weekly temperature regimes based on 10 years field data from extreme snow over and temperature regimes in Picos de Europa (located within our study region in northwest Spain). Both incubators were programed with the weekly mean of maximum and minimum soil temperatures, in orange the incubator mimicking “Fellfield” conditions and in blue the incubator mimicking “Snowbed” conditions. Vertical lines mark germination timing traits calculated in our study (Autumn, Spring and Summer). Horizontal lines represent the length of winter conditions (Tmean <3) in both fellfield and snowbed incubators, 126 and 168 days respectively. Additionally, photoperiod was modified every month according to our study region.

Description of main filters/stress at each season (boreal chapter seeds book)??

The main differences between incubators were the length and climatic conditions during the winter period (defined as the period with Tmin below 2 degrees). In fellfield incubator we programmed below 0˚C temperatures with daily temperature and photoperiod variation for 126 days while in snowbed incubator we programmed 0˚C constant and darkness for 168 days (see winter period in fig 2). Consequently, the growing season also differed between the two incubators with 172 days in fellfield incubator and 122 days in snowbed incubator. Mean temperature differences between our two incubators was around 3-4˚C each week (more detailed information about weekly programs in Appendix table Xx) reaching a maximum mean temperature (Tmean) of 18.5˚C in fellfield incubator and 15.5˚C in snowbed incubator.

2.3.2 Experiment settings

After field collection seeds were cleaned manually and sown within 20 days of collection. We followed a sequential sowing according to species maturity peak in the field. Each population (in total N= 97) was sowed and placed simultaneously in both incubators, with four replicates of 25 seeds in 9 cm diameter Petri dishes (numbers were modified in populations with fewer seeds) with germination filter paper (Fanoia paper for germination assays Ref.518G085). Filter papers were kept soaked adding 2-3 ml of distilled water every 1-2 weeks, avoiding water stress during growing season.

First sowing took place between weeks 36 and 41 (except 2 annual species sowed in week 31). By week 40 some species had already germinated more than 65% or were highly affected by fungus (N= 38 populations), thus we decided to make a second sow in week 42 to be able to keep track of germination timing across a natural year.

Germination scores were done every 2 weeks before winter and weekly after winter based on the results of a recent metanalysis (Fernández-Pascual et al., 2021), where most alpine species required cold stratification and warm cues to germinate.

Germination was recorded when radicle was >1mm long and germinated seeds were removed. The experiment was terminated after 14 months (July 2021 - September 2022) for a total of 24 to 28 scorings, and the remaining seeds were cut open under the binocular loupe to assess visually if they were empty, infected, or looked normal. Seeds with white and firm embryos were considered viable (Baskin & Baskin, 2014). Empty seeds were not included for further germination analysis.

2.4 Statistics

There is still few information about germination phenology traits, here we propose a set of tentative functional germination metrics calculated from the raw scoring data (see table 1). We decided to remove from analysis species with 0 germination and those that had less than 25% of viable seeds (in case our experimental conditions were not able to break dormancy or seed quality was not adequate).

We performed the analysis of the raw data by fitting generalized mixed models with Bayesian estimation (Markov Chain Monte Carlo generalized linear mixed models, MCMCglmm) using the R package MCMCGLMM (Hadfield, 2010). To model germination timing traits (Total, Autumn, Spring, Summer germination and Germination under winter conditions) we used binomial MCMCglmms (family = multinomial2) while for t50 and Environmental heat sum we used gaussian MCMCglmms (family = gaussian). Analysis was run separately for each community, models had incubator as fixed factor and species identity, population and phylogeny as random factors. Phylogeny was included using a reconstructed tree for the 54 species, created with V.PHYLOMAKER R package (Jin & Qian, 2019). In all models we used weakly informative priors, with parameter-expanded priors for the random effects. Each model was run for 1 000 000 iterations, with an initial burn of 100 000 and a thinning interval of 100 (REF?). From the resulting posterior distributions, we calculated mean parameter estimates and 95% credible intervals (CI). To estimate phylogenetic signal of seed germinations over all variables we used Pagels’s lambda (λ) (M. Pagel, 1999). Additionally, to investigate the patterns in our study area a more complex model was run to include both incubator and community as fixed factors with special interest on their interaction (see results in appendix table xx).

Table 1: Description of the functional germination metrics calculated in our study along with their ecological significance and our specific predictions.

|  |  |  |  |
| --- | --- | --- | --- |
| Functional germination metrics | Description | Ecological significance | Incubators prediction |
| Germination rate | Cumulative germination by time passed (days). Germination speed (1/days) | Fast germination can be positive or negative: it means more time to grow before winter season but also higher vulnerability to early frosts. | Faster in fellfield incubator |
| Total germination | Total amount of seeds germinated from 31/07/2021 to 19/09/2022. | Higher final germination means high potential of regeneration by seeds. | Higher in fellfield incubator |
| Autumn germination | Germination at mid-November (from 31/07/21 to 12/11/21. | Germination of species without physiological dormancy. Strategy to germinate fast and grow before winter ‘s adverse conditions. | Higher in fellfield incubator |
| Spring germination (relative) | Germination at mid-June relative to end of autumn (from 13/11/21 to 16/06/22. | Germination of species with cold stratification requirement but no need for high temperatures. | Higher in fellfield incubator |
| Summer germination (relative) | Germination at mid-September relative to spring (from 17/06/22 to 19/09/22. | Germination of species with cold stratification and warm cued requirements for germination. | Higher in snowbed incubator |
| Germination  in winter conditions | Germination during winter period (from Tmin≤3ºC until Tmin >2ºC). | Germination of species able to germinate under snow. | Higher in snowbed incubator |
| t50 | Time to reach 50% germination. Calculated from linear model. Species under 50% germination were excluded from analysis (n= 72 populations). | Specific germination speed metric, highly comparable with other studies. | Higher in snowbed incubator |
| Environmental heat sum | Sum of degrees (Tmean) needed to reach t50. Species under 50% germination were excluded from analysis (n= 72 populations). | Number of degrees that species need to accumulate before germination. Strategy to avoid too early season germination after winter when frost events can still happen. | Equal number of degrees in both incubators. |