2. Methods

2.1 Study area

The Cantabrian mountains run E-W through northwest Spain with altitudes surpassing 2500 m a.s.l. This mountain range is considered as a transitional biogeographical area between Eurosiberian and Mediterranean regions (Loidi et al., 2015), influenced by Mediterranean/continental climate in southern slopes and Temperate/oceanic climate in northern slopes (Fig1 a). The particularity of its location facilitates the coexistence of a complex array of communities and species adapted to both climates. A recent review quantified the richness of the Cantabrian mountains in 2338 native species and subspecies (Jiménez-Alfaro et al., 2021). At finer scales, studies in the area found that topographic heterogeneity creates a mosaic of microclimatic conditions (Fig1 b) whichin turn determine changes in compositional (Jiménez-Alfaro et al., 2014) and functional diversity (García-Gutiérrez et al., 2018) in the highest altitudinal belt. 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 (Fig 1a): (1) Mediterranean alpine community within Valles de Omaña and Luna Biosphere Reserve (southern slope of 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42.910731 / 42.891192˚ N and -6.043621 / -6.107621˚ E) with extremely acid siliceous bedrock (3.8 – 4.8 PH range, unpublished data from 40 field samples) and (2) Temperate alpine community within Picos de Europa National Park (northern slope of the Cantabrian mountains, 43.168822 /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 (1058mm if we took the mean 2010-2022 from Barrios de Luna) or 1335 mm (from SENRA noromet) /3004.79mm (Vega de Urriellu, mean 2008-2014). Snowfall usually begins late November in both communities, but it ends at different time periods, end march in Mediterranean and mid-May in Temperate (unpublished field data from soil temperature sensors).

Interfaz de usuario gráfica, Aplicación

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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 49 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 chosen at random. 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 38 target sp., and for 19 sp we were able to collect 2 populations. Only three species (*Jurinea humilis*, *Thymus praecox* and *Silene ciliata*) were present at both communities, with 2 populations in each community.

2.3 Lab work

2.3.1 Experimental climatic 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 (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 extreme regimes in snow cover period and temperatures to mimic the microclimatic variability. The final experimental programs consisted of weekly temperature modifications (fig 2) with monthly photoperiod variation. Each experimental program was configured using Fitolog 9000 software (version 9308, Aralab Pharmaceutical Stability software) in Aralab incubators (Aralab climatic chamber Fitoclima S600 PL, equipped with 4 led modules 11W 350mA at 20%, photon flux?). Fitolog software allowed us to use ramp setting for gradual temperature changes along each day and to monitor the incubators programs remotely. Both incubators run simultaneously from July 2021 to September 2022, the incubator with the warmer temperatures and no snow period program will be referred as “fellfied incubator” and the incubator with cooler temperatures and long snow period program will be referred as “snowbed incubator”. In our experiment there were no water stress during growing season (defined as days when T mean >3 ˚C).

Gráfico, Histograma

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Fig 2. Weekly temperature programs configured in the two incubators. Programs based on 10 years field data from Picos de Europa (located within our study region in northwest Spain). Both incubators were configured with the weekly mean of maximum and minimum soil temperatures. In orange the fellfield incubator mimicking “Fellfield” conditions and in blue the snowbed incubator mimicking “Snowbed” conditions. Vertical lines mark germination timing traits calculated in our study (Autumn, Spring and Summer, see table 1). Horizontal lines represent the length of winter conditions (Tmean <3 ˚C) in both fellfield and snowbed incubators, with a length of 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 temperature regimes and the length and climatic conditions during the winter period (defined as the period with Tmean <3 ˚C). 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 after winter 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 during growing season was around 3-4˚C each week (more detailed information about weekly programs in Appendix table Xx) reaching a maximum mean temperature of 18.5˚C in fellfield incubator and 15.5˚C in snowbed incubator both during last week of August.

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 (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 available) with germination filter paper (Filtros Anoia S.A. paper for germination assays, Ref.518G085). Filter papers were kept soaked adding 2-3 ml of distilled water every 2-3 weeks and sealed with parafilm to maintain the moisture content, avoiding water stress during growing season.

First sowing took place between weeks 36 and 41 (except for 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.

Based on the results of a recent metanalysis (Fernández-Pascual et al., 2021), most alpine species required cold stratification and warm cues to germinate, we decided to check germination every two weeks before winter, and weekly after winter until the end of the experiment.

Germination was recorded when radicle was >1.5mm long and germinated seeds were removed. The remaining seeds were cut open under the binocular loupe to visually assess 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. The experiment was terminated after 14 months (July 2021 - September 2022), for a total of 24 to 28 germination scorings.

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 during collection was not optimal).

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 (Germination rate, Total germination, Autumn germination, Spring germination, Summer germination and Germination under winter conditions), we used binomial MCMCglmms (family = multinomial2) while for derivative traits (t50 and environmental heat sum) we scaled the variables and used gaussian MCMCglmms (family = gaussian) (see table 1 for details). T50 was calculated by fitting a linear model between the two germination scores before and after reaching 50% germination. Environmental heat sum was calculating summing the degrees experienced (considering daily mean temperature) from sowing date to t50 date for each petridish.

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 we run a more complex model 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 for each metric. Traits were calculated for petridish and used as replicates per population.

|  |  |  |  |
| --- | --- | --- | --- |
| Functional germination metrics | Description | Ecological significance | Prediction |
| Germination rate | Cumulative germination by time passed (days). Germination speed (1/days) | Fast germination can be positive or negative: mean 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 at the end of the experiment (from 31/07/2021 to 19/09/2022). | Higher total 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. Rapid onset of germination following snowmelt | 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 Tmean≤3ºC until Tmean >3ºC). | Germination of species able to germinate at very low temperatures expanding their growing season and taking advantage of snowmelt. | Higher in snowbed incubator |
| t50 | Time to reach 50% germination. Species under 50% germination were excluded from analysis (n= 72 populations). | Specific germination speed metric, highly comparable with other studies in the field. | 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. |

3.Results

Description of the dataset

The final dataset used the raw scoring data of 54 species and 96 populations for all traits except t50 and environmental heat sum (n= 72 populations), representing 21 plant families. The total number of viable seeds used in the experiment was 16 120.

Traits responses

In general, germination rate was faster (p<0.001) in fellfield incubator for both communities (Fig 3) although, the pattern observed across the experiment differ between communities. In the Mediterranean community, 82% of species did not have dormant seeds and were able to germinate fast, reaching 45-55% of germination, with water available even when temperatures dropped lower than 10ºC in late autumn. After winter period we can observe a significant delay in snowbed incubator (p<0.001). In the Temperate community, only 55% of species, with lower values (5-25%) germinated before winter. After winter period germination peaked when temperatures increased, and we also observed a significant delay in snowbed incubator (p<0.001).

Gráfico, Gráfico de líneas

Descripción generada automáticamente

Fig 3. Cumulative germination curves throughout the experiment in our two communities. Left panel for Mediterranean community and right panel for Temperate community. Within each panel, orange curve accounts for fellfield incubator and blue curve accounts for snowbed incubator. Vertical lines stand for germination timing traits calculated (autumn, spring and summer from left to right, same as in figure 2) in each community.

Overall, warmer temperatures of fellfield incubator promoted higher total germination proportion. In the Mediterranean community differences were existent but not statistically significant (Fig 4, upper left panel, credible interval (CI) is crossing the zero-effect line), reaching 0.85 and 0.82 of total germination proportion for fellfield and snowbed incubators respectively (Fig 5, upper left panel). In the Temperate community incubators differences were statistically significant (Fig 4, upper right panel, credible intervals do not cross the zero-effect line), reaching 0.72 and 0.65 of total germination proportion for fellfield and incubators snowbed respectively (Fig 5, upper right panel).

For autumn germination we observed fellfield incubator having a positive effect and reaching significantly higher germination values in that period (Fig 4 and Fig 5 second row). In the Mediterranean community we registered a 0.55 germination proportion in fellfield compared to 0.4 in snowbed incubator and in the Temperate community it was 0.24 of germination proportion in fellfield compared to 0.07 in snowbed.

For spring germination, the Mediterranean community showed no significant effect of incubator (Fig 4, third row left panel CI crossing zero-effect line) with both incubators reaching similar levels of germination proportion (0.28 and 0.3 germination proportion in fellfield and snowbed, respectively, see Fig 5 third row left panel). On the other hand, for the Temperate community, there is significantly higher germination in fellfield incubator with a mean germination proportion of 0.4 almost doubling snowbed incubator (Fig 4 and 5, right panel third row).

Summer germination trait showed reached higher germination values in snowbed incubator (Fig 4 and Fig 5 fourth row). In the Mediterranean community it reached 0.12 germination compared to 0.03 in fellfield incubator. In the Temperate community, differences between incubators were more noticeable, snowbed incubator reached 0.37 germination proportion compared to 0.08 in fellfield incubator.

Germination in winter conditions was noticeable higher in snowbed incubator for both communities (fig 4 and fig 5 fifth row). Constant 0ºC and darkness seemed to favour germination proportion with 0.2 germination in the Mediterranean community and 0.14 in the Temperate community, five times higher than their respective values in fellfield incubator.

When looking into t50 trait, we found significant higher values in snowbed incubator (fig 4 sixth row), meaning that it took more time to reach 50% germination in the cooler incubator. The average t50 time was 83 and 122 days in the Mediterranean community and 164 and 236 in the Temperate community for fellfield and snowbed incubator respectively (fig 5 sixth row).

To conclude with the traits, we found Environmental heat sum marginally significant different (p=0.04) between incubators in the Mediterranean community with 304 degrees in fellfield compared to 238 degrees in snowbed incubator (fig 5 last row). No differences were found between incubator in the Temperate community (fig 4 last row right panel).

To sum up the Mediterranean community showed no significant differences between incubators for total germination and spring germination traits (see fig 4, left panel, CI are crossing the zero-effect line). However, we did find significant differences between incubators in the other traits. Germination rate, Autumn germination and Environmental heat sum were higher in fellfield incubator while Summer germination, Germination in winter conditions and t50 were higher in snowbed incubator (see fig 5 left panel).

The Temperate community showed significant differences between incubators in all traits (see fig 4, right panel, CI are not crossing the zero-effect line) except in Environmental heat sum. Germination rate, Total, Autumn and Spring germination were higher in fellfield incubator whereas summer germination, Germination in winter conditions and t50 were higher in snowbed incubator (see fig 5, right panel).

Community patterns comparison

We observed homologous responses for all our traits (although not all significant) in both communities except for Spring germination. The effect size was always larger in the Temperate community (see Fig 4) suggesting that the differences found between incubators were bigger.

When testing differences between communities with the complex model, we found a that the temperate community had a significant slower germination rate, lower autumn germination and bigger t50. The rest of the traits did not statistically differ between communities. Nevertheless, a significant interaction term in Germination rate, Autumn, Spring, Summer germination, t50 and Environmental heat sum confirmed that germination differences between incubators were bigger in the temperate community for all traits (see detailed results from complex model in appendix table xx).

Phylogenetical signal

Lambda signal (Pagel 1999) was significant for Germination rate, Total and Autumn germination, and Germination in winter conditions. For Spring, Summer germination, t50 and Environmental heat sum traits no significant signal was detected (same results for both communities, see appendix table xxx).

Diagrama

Descripción generada automáticamente

Fig 4. Effect of incubator snowbed regime (blue = snowbed) on Total germination, Autumn germination, Spring germination, Summer germination, Germination in winter conditions, t50 and Environmental heat sum for both our communities (left panel for Mediterranean community and right panel for Temperate community), according to the MCMC-glmm analysis of raw data. Dots indicate the posterior mean of the effect size and whiskers its 95% credible interval. The vertical dashed line marks zero effect. When the credible intervals cross the zero-line, effect is not significant.

Diagrama

Descripción generada automáticamente

Fig 5. Mean trait values calculations for our traits at each incubator (orange = fellfield, blue = snowbed) in both our communities: left panel or Mediterranean community and right panel for Temperate community. Errors bars in germination timing traits (Total, Autumn, Spring, Summer, Winter) are binomial confident intervals. Errors bars in t50 and Environmental heat sum are standard error measures. Notice the different scales in y-axis.

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