2. Methods

2.1 Study area

The Cantabrian mountains are a range running E-W through northwest Spain, with altitudes surpassing 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). The particularity of the geographical location facilitate the coexistence of a complex array of communities and species adapted to both climates, a recent study 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 have found that in the highest altitudinal 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 (Fig 1a): (1) a 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42.910731 / 42.891192 N and -6.043621 / -6.107621 E) with extremely acid siliceous bedrock (3.8 – 4.8 PH range, unpublished data from 40 field samples) and (2) a Temperate alpine community within Picos de Europa National Park (Northern slope of the Cantabrian mountains, 43.168822 /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 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 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 extreme regimes in snow cover period and temperatures to mimic the microclimatic variability in our study area. The final experimental programs consisted of weekly temperature modifications with monthly photoperiod variation (fig 2). Each experimental program was configured using Fitolog 9000 software (version 9308, Aralab Pharmaceutical Stability software) in an Aralab incubator (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 warmer program and no snow period will be referred as “fellfied incubator” and the incubator with cooler program and long snow period 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

Descripción generada automáticamente

Fig 2. Incubators’ weekly temperature programs 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 configured 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 ˚C) 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 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 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 (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 >1.5mm 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 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.

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 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 derived traits (t50 and environmental heat sum) we scaled the variables and used gaussian MCMCglmms (family = gaussian) (see table 1 for details). 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 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.

|  |  |  |  |
| --- | --- | --- | --- |
| 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: can 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 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. | 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 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. |

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.

Trait x microclima

In general, germination rate was faster (p<0.001) in fellfield incubator for both communities (Fig 3) although, the patterns observed differ between communities. In Mediterranean communities, 82% of species did not have dormant seeds and were able to germinate fast with water available even with temperatures lower than 10ºC (reaching 45-55% of germination in both incubators). After winter period we can observe a clear and significant delay in snowbed incubator. In Temperate communities, only 55% of species germinated before winter with lower mean germination values (reaching 5-25% in snowbed and fellfield incubators, respectively). After winter period (cold stratification) we can notice that germination peaked when temperatures rose and we observe a similar delay in snowbed incubator.

Gráfico, Gráfico de líneas

Descripción generada automáticamente

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

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

For autumn germination we observed equivalent/homologous responses in both communities with fellfield incubator reaching significantly higher values (Fig 4 second row, where CI do not overlap with zero-effect line, and Fig 5 second row with mean values). In the Mediterranean community we registered a 0.55 germination proportion in fellfield compared to 0.4 in snowbed and in the Temperate community was 0.24 of germination proportion compared to 0.07 in snowbed.

In spring germination, we found contrasting results between communities. In the Mediterranean community there is no significant effect of incubator (Fig 4 left panel, CI overlap with zero-effect line) with both incubators reaching similar levels of germination proportion 0.28 and 0.3 in fellfield and snowbed, respectively (Fig 5). On the other hand, for the Temperate community, there is significantly higher germination in fellfield incubator, mean germination proportion of 0.4 (Fig 4 and 5, right panel third row).

Summer germination show homologous responses in both communities with snowbed incubator reaching higher germination proportion (Fig 4 fourth row, where CI do not overlap with zero-effect line, and Fig 5 fourth row with mean values). Specially noticeable in the Temperate community where it reach 0.37 germination proportion.

Germination under winter conditions was noticeable higher in snowbed incubator for both communities (fig 4 and fig 5 fifth row). Here we can observe a difference of more than 10 percentual points??.

When looking into t50 trait, both communities show homologous responses with higher values in snowbed incubator, meaning that it took more time to reach 50% germination in snowbed incubator in both communities.

Contradictory to all the other traits when considering Environmental heat sum we did not find any significant differences between incubators.

Trait x community

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 community. Nevertheless, the significant interaction term showed that the differences between incubators were higher in the temperate community for all traits (except total germination and germination in winter conditions) (see results from complex model in appendix table xx).

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, 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.

Fig 5. Mean trait values calculations for our traits at each incubator (orange = fellfield, blue = snowbed) in both our communities. 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.

How to calculate trait values: first mean of petridish x species, then mean of all species including a standard deviation measure for every incubator

Table 2. Present trait summary values to allow comparison. Test differences? Low detection power?

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Traits** | **Mediterranean** | | | | ***Temperate*** | | | |  |
|  | Fellfield | | Snowbed | | Fellfield | | Snowbed | |  |
|  | Mean | SD | Mean | SD | Mean | SD | Mean | SD |  |
| Germination rate |  |  |  |  |  |  |  |  |  |
| Total germination | 85.5 | ±24.3 | 83.3 | ±27.1 | 72 | ±33.9 | 66.7 | ±36.9 |  |
| Autumn germination | 53.5 | ±42.4 | 38.9 | ±41.6 | 23.9 | ±33.9 | 8.1 | ±20.7 |  |
| Spring germination | 29.1 | ±37.8 | 32.6 | ±40.9 | 39.8 | ±38.6 | 22.1 | ±31.3 |  |
| Summer germination | 2.87 | ±7.46 | 11.8 | ±24.4 | 8.38 | ±15.4 | 36.4 | ±35.6 |  |
| Winter temperatures germination | 3.81 | ±12.8 | 23 | ±35.8 | 2.12 | ±11 | 16 | ±26.5 |  |
| Cold germination | 24.9 | ±36.6 | 9.66 | ±20 | 32.5 | ±34.8 | 6.14 | ±12.5 |  |
| Warm germination | 3.3 | ±7.92 | 11.8 | ±24.4 | 13.6 | ±22.4 | 36.4 | ±35.6 |  |
| T50 | 82.7 | ±96.5 | 122 | ±108 | 164 | ±98.7 | 236 | ±79.4 |  |
| Environmental Heat Sum | 307 | ±469 | 241 | ±226 | 448 | ±359 | 426 | ±294 |  |
| Delay time to T50 | 41 | ±88.2 |  |  | 75.5 | ±88.4 |  |  |  |

Sort first responses by species, then take into account the whole community and then some notes about intraspecific variation.

Interesting points from preliminary results

* More than half of the species were non dormant (32/54). Out of the 32, 22 had higher germination or had higher fungi affectation and therefore were sowed a 2nd time (2 petri dishes of 25 at both conditions)
* We can observe large differences in germination (more than 30%) with only 3ºC variation between conditions after the same amount of time (ex: *Jurinea humilis*)
* 19 species germinated better in Fellfield conditions, 2 in snowbed conditions (*Spergula, Helictochloa*)
* 10 species had large germination differences between populations (ex: *Cerastium*) within the same bedrock type and between bedrock types.
* 12 species able to germinate with 9/6ºC max (2nd sowing)

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