# Wild species intraspecific germination variability in water-limited alpine environments.

General goal:

Study intraspecific germination adaptation to water stress in Mediterranean high mountain grasslands.

Novelty:

Germination in water-limited alpine environments is understudied.

Interspecific responses to water stress in wild species.

Research questions:

1. Will seeds from warmer/drier subpopulations germinate better under higher water stress levels? Intraspecific adaptation to realized niche from adult plants, transgenerational effects into the regeneration niche?
2. Will the ripening stage (fresh vs. after-ripening) modify their response to water stress? Physiological responses to explore bet-hedging strategies?

Hypothesis:

1. Seeds from warmer/drier subpopulations will germinate better and faster at higher levels of water stress.
2. Fresh seeds will have higher variability of germination responses and will germinate worse.

Approach/General methods

Seed collection of 1 specialist/strict alpine species, *Dianthus langeanus*, from Mediterranean high mountain grasslands. Seed collection the 7th-8th of August 2023, from 16 different collection sites (considered as subpopulations). Collections sites where previously iButtons were buried for 11 months (from 12/7/2021 to 29/05/2022) and we have temperatures registered every 4 hours. Additionally, we have hourly temperature and water potential data from 7 plots (A00, B00, B07, C00, C18, D00, D12).

Fresh seeds germination trial: (A00, B00, B03, B07, B17, B19, C00, C06, C19, D00, D19, D12). Seeds from 12 subpopulations were subjected to 7 water stress treatments (h2O, -0.2, -0.4, -0.6, -0.8, -1, -1.2 MPa, following standard typical with PEG 6000). 4 Petri dish 90mm diameter with 25 seeds for each WP treatment (100 seeds x treatment). Seeds sowed in two layers of germination paper. Added 5ml of PEG solutions and sealed Petri dishes with parafilm to avoid evaporation of the solution.

After-ripening germination trial: (A00, A02, A11, B03, B19, C00, C18, C19, C20, D00, D11, D19) Seeds from 12 subpopulations were subjected to 7 water stress treatments (h2O, -0.2, -0.4, -0.6, -0.8, -1, -1.2 MPa, following standard typical with PEG 6000). 4 Petri dish 90mm diameter with 25 seeds for each WP treatment (100 seeds x treatment, number were adapted in subpopulations with lower seeds). Seeds sowed in two layers of germination paper. Added 5ml of PEG solutions and sealed Petri dishes with parafilm to avoid evaporation of the solution.

6 populations were subjected to both fresh and after-ripening sowing (A00, B03, C00, C19, D00, D19)

## 2.Methods

### 2.1 Study system

Our sampling sites are located above 2000 m a.s.l in the southern slope of the Cantabrian Mountains, a mountain range running E-W in northern Spain and within the Valles de Omaña and Luna Biosphere Reserve. The climate in the study system is characterized by a 2-month dry period in summer (average annual precipitation of 1050 mm, mostly accumulated in spring and autumn). The growing season stretches from March to October with a mean annual soil temperature of 8ºC. Grazing impact is also restricted to wild populations of Cantabrian chamois. The sampling sites are located above very acidic bedrock (pH 3.8 – 4.8, own non-published data). Local community richness ranges from 20 to 30 species dominated mostly by *Poaceae* and *Cyperaceae*, but they are also rich in Hemicryptophytes and Chamaephytes.

We followed a systematic sampling across 4 summits with a central representative plot where we buried a temperature and water potential sensor (Microlog ref). The recording period for the Microlog SP3 went from XXXX to XXXX. We also stablished 20 additional plots, 5 in each cardinal direction separated by 10m (cruces) where we buried iButtons (Thermochron, iButton, Newbury, UK; accuracy: +/- 0.5 ºC from -10 ºC to +65 ºC, resolution: 0.5 ºC, records every 4 hours). The recording period for the iButtons went from 12/7/2021 to 29/05/2022 (xx days).

Each plot is considered as a different subpopulation of Dianthus langanus.

### 2.2 Soil Bioclimatic indices (from Picos paper)

We used microclimatic data of the temporal and spatial surveys to calculate soil bioclimatic indices. For comparison, we homogenized the data of the temporal survey at four-hour intervals, keeping the same 330 calendar days covered by the spatial survey. In total, we obtained 40 data points for the temporal survey (four sites x 10 years) and 80 for the spatial survey (four sites x 20 plots). We calculated bioclimatic indices based on standard variables used by WorldClim (Fick & Hijmans 2017), together with other variables with a relevant function on alpine topographic gradients. The selected variables were: (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 soil temperature is around 0 ºC, calculated for the period in which the maximum temperature was < 0.5 ºC and the minimum temperature was > -0.5 ºC; (5) FDD = freezing degree days, i.e. the sum of daily mean temperatures for days in which the mean temperature was below 0 ºC (Choler 2018); and (6) GDD = growing degree days, i.e. the sum of daily mean temperatures for days in which the soil mean temperature at five cm deep was above 5 ºC (Körner 2021). For FDD, we transformed the values from negative to positive, so higher values represent more freezing. To identify the main gradients of microclimatic variability at both temporal and spatial scales, we conducted a principal component analysis (PCA) with the full set of bioclimatic indices.

### 2.3 Seed sampling

The study focuses on *Dianthus langeanus*, a wild species endemic to grasslands in high Mediterranean mountains, very abundant in our study area with high seed production. On the 7th and 8th of August 2023 we collected the mature seeds directly from the mother plants, following standard protocols for sampling seeds of wild populations to maximize intraspecific genetic diversity (ENSCONET 2009). Sampling took place within a 2m radius of the datalogger location, we collected seed from at least 10-20 individuals chosen at random. The goal was to collect 600 seeds from each subpopulation. We were able to collect enough seeds from 18 plots/subpopulations. For only 6 of them more than 1200 seeds were collected.

### 2.4 Germination trials

We decided to divide the subpopulations into 3 groups: one group of 6 subpopulations only sowed immediately after collections, another group of 6 subpopulations sowed after 1.5 months in room conditions (22ºC and 35% RH approx) and a final group of 6 subpopulations sowed both immediately and after 1.5 months in room conditions.

All subpopulations were subjected to 7 water stress treatments (h2O, -0.2, -0.4, -0.6, -0.8, -1, -1.2 MPa, following standard tipical with PEG 6000). 4 Petri dish 90mm diameter with 25 seeds for each WP treatment (100 seeds x treatment, number were adapted in subpopulations with lower seeds). Seeds sowed in two layers of germination paper. Added 5ml of PEG solutions and sealed Petri dishes with parafilm to avoid evaporation of the solution.

All seeds were exposed to constant 20ºC temperature treatment with 12-12h photoperiod in Aralab incubators (Aralab climatic chamber Fitoclima S600 PL, equipped with 4 led modules 11W 350mA, using Fitolog 9000 software version 9308, Aralab Pharmaceutical Stability software).

The germination experiment run for 28 days with daily germination scores until day 21 and afterwards every 2-3 days until the end of the experiment.

### 2.5 Data Analysis

All analysis were done in R software (ref), using seedR package (ref) and GerminaR package (ref). visualization was done with ggplot package (ref)