**Ageing / Longevity protocol**

Objective:

We want to obtain longevity traits from ex-situ experiments. Specifically obtain seed survival curves, standard deviation of the frequency distribution of seed deaths in time (r) and p50 from our target species.

Ideally we will do also burying experiments in the field to compare both ageing responses in the seed bank.

Questions:

Is seed longevity correlated with other plant/seed traits?

Do we observe a difference between species from siliceous/calcareous sites?

Do we observe differences between typical snowbed vs fellfield species?

Or does the seed survival mostly relate to their filogeny?

Will seeds from subordinate species be more resistant to ageing?

Materials and Methods

Study area

Cantabrian range in northwest Spain. Our study area is divided in two mountain areas (see map/fig. ):

* Villabandín: siliceous bedrock and Mediterranean mountain climate,
* Picos: calcareous bedrock and temperate mountain climate.

At each mountain areas we stablished 4 sites, all locations above 1900 m.a.s.l. with a natural alpine grassland community.

Mean temperatures and precipitation of the area.

Seeds

Mature seeds were collected during August-September 2021 and were held at room temperature and humidity (22º C and 35% RH) until January 2022 when the experimental procedure began.

Based on their abundance in our focal alpine communities, this study focus on 34 accessions from 25 different species. 9 species with 2 populations; 3 with populations from different bedrocks (in red), 6 with 2 populations from the same bedrock. 15 accessions from siliceous bedrock; 19 accessions from calcareous bedrock.

|  |  |  |  |
| --- | --- | --- | --- |
| **Specie** | **Family** | **Bedrock** | **snow/fell** |
| Androsace villosa | Primulaceae | Calcareous | s |
| Androsace villosa | Primulaceae | Calcareous | s |
| Arenaria erinacea | Caryophyllaceae | Calcareous | n |
| Armeria duriaei | Plumbaginaceae | Siliceous | n |
| Carex sempervirens | Cyperaceae | Calcareous | s |
| Cerastium sp. | Cariophyllaceae | Siliceous | s |
| Dianthus langeanus | Cariophyllaceae | Siliceous | s |
| Festuca summilusitana | Poaceae | Siliceous | f |
| Gentiana verna | Gentianaceae | Calcareous | s |
| Gentianella campestris | Gentianaceae | Calcareous | s |
| Gypsophila repens | Caryophyllaceae | Calcareous | s |
| Gypsophila repens | Caryophyllaceae | Calcareous | s |
| Helianthemum canum | Cistaceae | Calcareous | s |
| Helianthemum canum | Cistaceae | Calcareous | s |
| Koeleria vallesiana | Poaceae | Calcareous | n |
| Minuartia recurva | Caryophyllaceae | Siliceous | s |
| Minuartia verna CF | Caryophyllaceae | Calcareous | n |
| Neochischkinia truncatula | Poaceae | Siliceous | n |
| Pedicularis pirenaica | Scrophulariaceae | Calcareous | s |
| Pedicularis pirenaica | Scrophulariaceae | Calcareous | s |
| Phyteuma hemisphaericum | Campanulaceae | Siliceous | s |
| Phyteuma hemisphaericum | Campanulaceae | Siliceous | s |
| Plantago alpina | Plantaginaceae | Siliceous | f |
| Plantago alpina | Plantaginaceae | Calcareous | n |
| Sedum anglicum | Crassulaceae | Siliceous | f |
| Sedum brevifolium | Crassulaceae | Siliceous | f |
| Silene acaulis | Caryophyllaceae | Calcareous | f |
| Silene acaulis | Caryophyllaceae | Calcareous | f |
| Silene ciliata | Caryophyllaceae | Siliceous | s |
| Silene ciliata | Caryophyllaceae | Calcareous | f |
| Solidago virgaurea | Compositae | Siliceous | f |
| Spergula morisonii | Caryophyllaceae | Siliceous | f |
| Thymus praecox | Labiatae | Siliceous | f |
| Thymus praecox | Labiatae | Calcareous | f |

According to previous results obtained for Mondoni et al. (2011) alpine seeds are short lived therefore we adapted the reduced seed longevity protocol develop by Davies et al. (2016).

Procedure:

Seed were already at room temperature, we checked their humidity using Hygropalm 3 display unit (Rotronic Instrument UK Ltd, Crawley, UK) with results between 30 and 35%. To elevate the moisture content of the seeds before to ageing all the samples were rehydrated for 5 days (the amount of days in rehydration solution was shorter due to the small size and short-live of alpine seeds) at 47% RH at 20ºC (See Hay et al. 2008) in open glass vials placed over a non-saturated solution of LiCl in distilled water held in a sealed 300 x 300 x 130 mm electrical enclosure box (Ensto UK Ltd, Southhampton, UK). The seed equilibrium, humidity (eRH) was checked using a Hygropalm 3 display unit (Rotronic Instrument UK Ltd, Crawley, UK) before transferring the glass vial to the ageing conditions.

Once that eRH was in equilibrium samples were transferred to a second electrical enclosure box, with a non-saturated solution of LiCl at 60% RH placed in an incubator whitout light at 45+/- 2 ºC.

For each accession, we placed 175 seeds inside the glass vials. Following Davies protocol removal time intervals were 2, 10, 15 and 15 and 30 days followed for a 4-weeks germination experiment. However in our study, to adapt the protocol to the number of seed available, we remove 3 (pseudo)replicates of 14 seeds at each interval (modifying the number of seeds used in Davies’ protocol).

For our germination experiment we apply GA3 to all accessions with Petri dishes 1% agar + 250 ml/L of GA3 (following Kew Royal Botanic Garden Technical Information sheet\_13a .Once a week we score the germination experiments we had running removing seedlings when the radicle is at least 2mm long.

Germination conditions were set with alternating temperatures 22/12 at 12/12 photoperiod (cistaceae accessions scarified with sand paper previous to rehydration period)

Data collection:

Tables for data collection will vary depending on the statistic software

Andrea uses Genstat and data should be recording following

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Code |  | | | | | | | | Specie | |  | | | | | | |
| Week | 1 | | | 2 | | | 3 | | | 4 | | | cut test | | | sown | total germination |
| Ageing day | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | live | mouldy | empty |
| 2 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 10 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 15 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 30 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

\*Problem: no open source programe, seems to analize the species one by one. Could be time consuming in our study with 34 different accessions to compare. Check more in detail with Andrea

I we could use R the table should look like this (need to investigate more)

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Code | Ageing | Week | Replicate | N germinated | Mouldy | Empty | Sown | Total germination |
| 240821-01 | 2 | 1 | 1 |  |  |  |  |  |
| 240821-01 | 2 | 1 | 2 |  |  |  |  |  |
| 240821-01 | 2 | 1 | 3 |  |  |  |  |  |

Data Analysis

Probit analysis (regression analysis type)

V = Ki – p/ʋ

V: viability after p days

Ki: initial viability

p: days in ageing solution

ʋ: time for viability to decline 1 NED (Normal equivalent deviates)

Other measures:

* P50: estimate of the time for viability to fall to 50% (obtained from seed survival curve or P50= Ki x ʋ
* Seed survival curves: plot seed viability (here referred to % germination) vs ageing period (days)

References:

Davies RM, Newton RJ, Hay FR and Probert RJ. 2016. 150-seed comparative longevity protocol- a reduced seed number sceening method for indentifying short-lived seed conservation collections. Seed Science & Technology 44:1-16

Hay FR, Adams J, Manger K and Probert R. 2008. The use of non-saturated lithium chloride solutions for experimental control of seed water control. Seed Science & Technology 36: 737-746.

Davies R, Di Sacco A & Newton R. Kew Royal Botanic Gardens.Germination testing: procedures and evalutation. Technical Information Sheet\_13a.

Mondoni A, Probert RJ, Rossi G, Veggini E & Hay FR. 2011. Seeds of alpine plants are short lives: implicatios for long-term conservation. Annals of Botany 107: 171-179

EXTRA

Equipment specifications (Following Kew recommendations)

|  |  |  |
| --- | --- | --- |
| Description | Model/Product | Supplier |
| Seed containers | • 2 ml clear Wheaton-style vial - VGA-220-012C  • 5 ml clear Wheaton-style vial - VGA-220-121U  • Glass Petri dishes 60 x 12 mm - PDS-100-011U | Fisher Scientific Ltd: www.fisher.co.uk |
| Sealable box | Electrical Enclosure Box Cubo 0 (conforming to  IP67): 300 x 300 x 132 mm  • ABS base - OABP303010B  • Clear lid - OPCT303003L | Ensto UK Ltd: www.ensto.com |
| Stand to hold seed samples above LiCl solution inside box | Fisherbrand incubation tray in polypropylene blue:  250mm x 240 mm - FB55681 | Fisher Scientific Ltd: www.fisher.co.uk |
| Fan-assited oven capable of reaching 60°C | LEEC KIF Compact | Jencons-PLS: www.jencons.co.uk |
| Lab-based hygrometer | HC2-AW sensor with USB interface, connected to  laptop/PC running HW4-E software. Range: 0 to  100% RH, -40 to 85 °C. | Rotronic Instruments (UK) Ltd:  www.rotronic.com |
| Statistical analysis software | • Genstat version 12.1  • Origin version 8 | VSN International: www.vsni.co.uk  Origin Lab: www.originlab.com |

Seed containers: for our seed size glass vials/petri dishes of 2-5 cm diameter is enough to contain up to 200 seeds (adjust according number of seeds and seed size). For the germination experiment after ageing we will use plastic petri dishes divided in 3 parts (pseudo-replicates)

Sealable box: Andrea has 4 sealable boxes, I think we should have enough with 2 (one for rehydration process, one for ageing process)

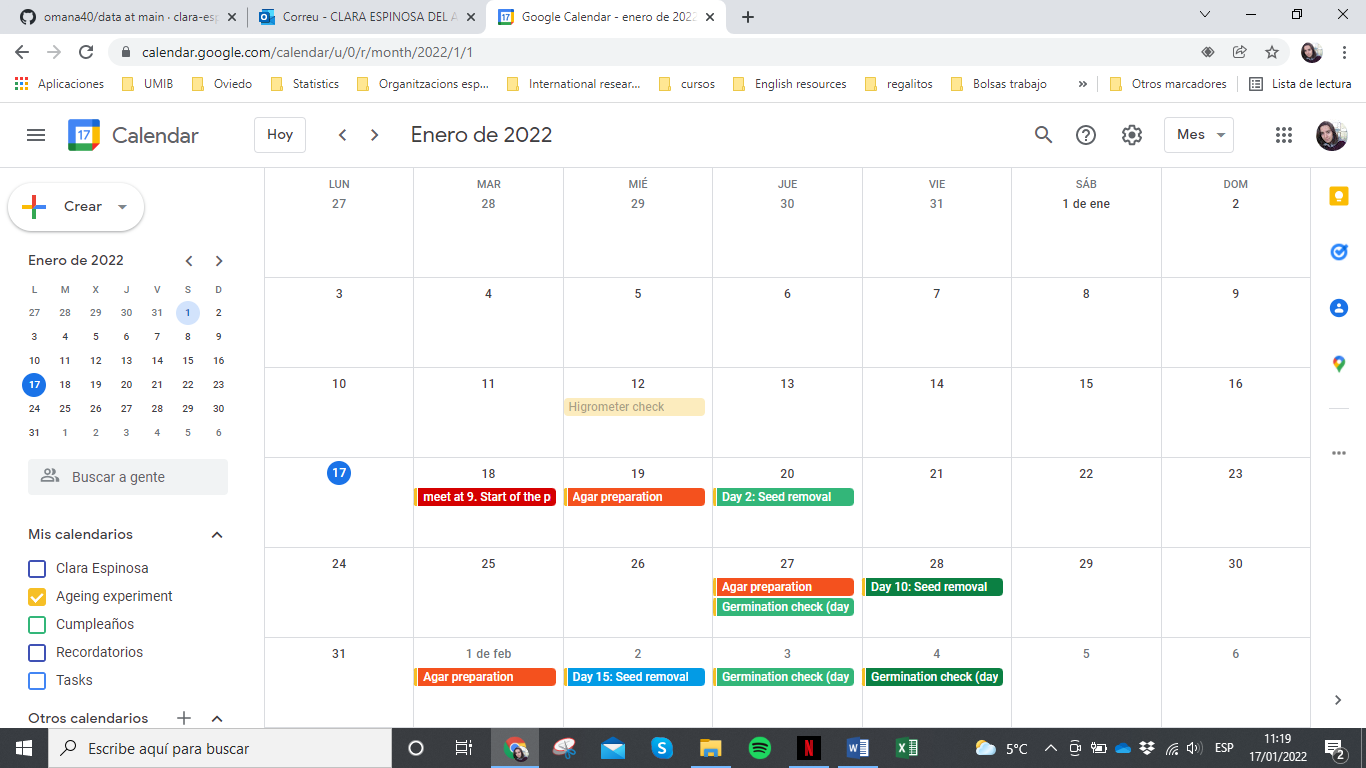
Box stands: 2 per sealable box (can be stack)

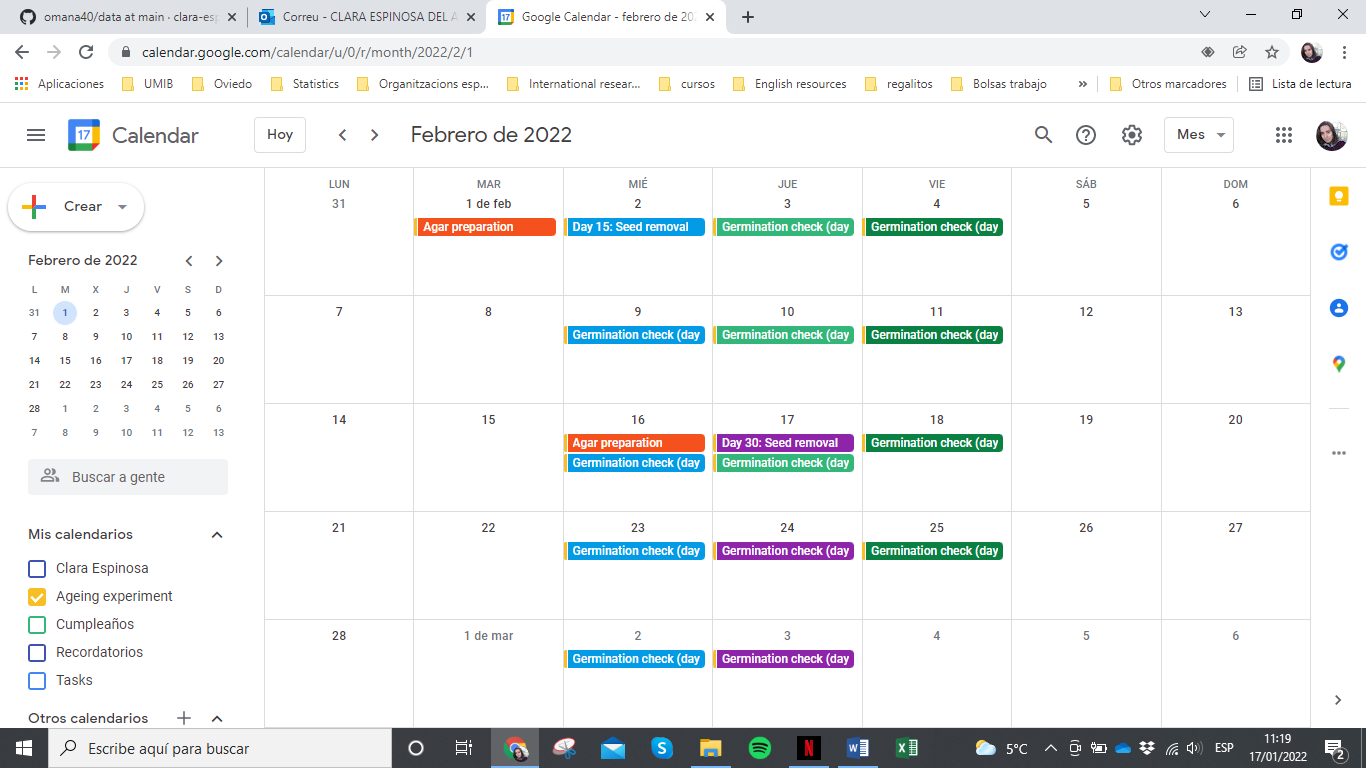
Lab-based hygrometer: Andrea uses Rotronic to assess the HR of the seeds (same we were considering) and to check TºC and HR inside sealable boxes uses: TINYTAG TV-4500 VIEW 2 (color azul) protección IP65 (185 € sin IVA) <https://e-berman.info/producto/tv-4500/>

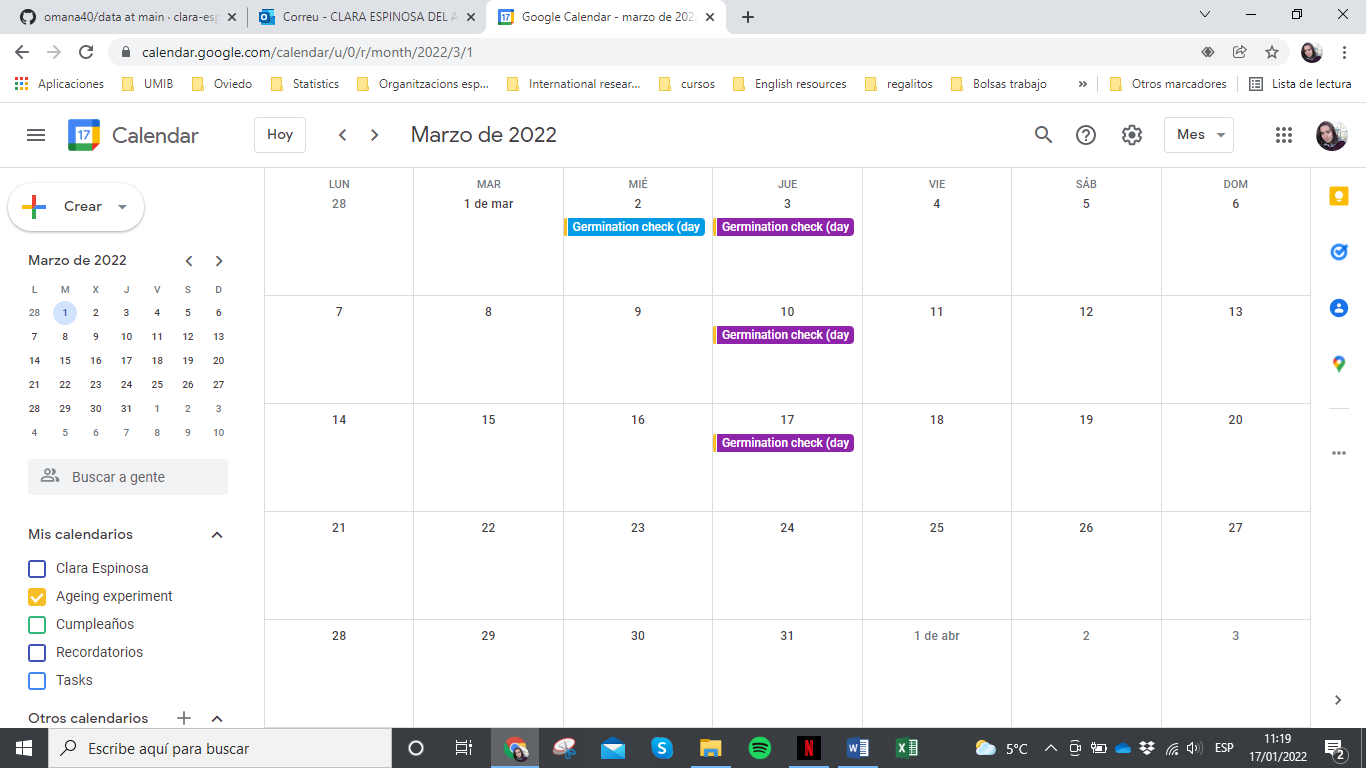
Statistical analysis software: Genstat es de pago, su doctorante había intentado hacer las seed survival curve mediante R pero no había encontrado una manera “sencilla”

Technical requirements:

* All samples with at least 150 seeds (high quality) for the reduced protocol (Davies et al. 2016, 150-seed comparative longevity protocol). We will modify it to remove the same amount of seed at each time interval and have replicates), we would ideally use 180 seeds, but it will be adapted to seed availability (175)
* High viability seed lots (>85% germination)
* Known germination requirements: temperatures, photoperiod and extra manipulation (scarification, cold stratification…)
* To be sure, after discussing it with Borja and Eduardo, we should apply GA3 to all samples before germination tests.
* One of the species (2 accessions) belonging to Cistaceae family will need to be scarified beforehand.

Calendar: 





Protocol synthesis

1. Seeds at room temperature for at least 24 h
2. Place seeds in glass vials as a monolayer (1 vial/accession)
3. 14 days in the rehydration solution (47% RH at 20ºC) [385 g of LiCl in 1L of distilled water, is an exothermic reaction thus leave stabilize for 24h inside he incubator before adding the seed containers] \*For alpine seed which are already short-lived we will decrease it to only 5 days to avoid any ageing during this preparatory stage\*
4. Time intervals in the ageing solution (60% RH at 45ºC) [300 g of LiCl in 1L of distilled water, is an exothermic reaction thus leave stabilize for 24h inside the oven)
   1. Remove seeds after 2, 10, 15 and 30 days
   2. 42 seeds/accession each time divided in 3 pseudo replicates
5. Germination tests to monitor every week for each time interval.

Petri dishes with agar at 1% + GA3 (250 mg/L, add when agar T<50ºC). 1 L of agar approx. 33 Petri dishes of 90 mm of diameter

1. Cut test to check any seed left after 4 weeks of germination test

\*Both rehydration and ageing solution can cause irritation to skin and mucoses, manipulates with gloves always\*