**Ageing / Longevity protocol**

Abstract (IAVS 2022):

In alpine landscapes, topographic roughness determines local environmental conditions along microhabitats such as fellfields vs snowbeds, which are supposed to act as local refugia under climate change. A functional ecological approach is still needed for understanding how these small-scale drivers modify the regeneration niche in alpine communities. Here we focused on seed longevity, a plant trait generally used to assess long-term ex-situ conservation of seeds, but with implications in seed persistence in the soil of natural habitats. We hypothesized that seed longevity of co-occurring species differs as a response of species preferential microniches, meaning that we can identify species groups which are consistently more short-lived than others. We analysed seed longevity of 25 species occurring in alpine communities from the Cantabrian Mountains (southern Europe) in two study systems (calcareous and siliceous) above 1900 m a.s.l. Seeds were exposed to laboratory-controlled accelerated ageing and then regularly sampled for germination tests. Initial viability (Ki), deterioration rate (r-1) and time taken for viability to fall to 50 % (p50) were estimated using probit analysis and microniche effects tested by GLMM in R. Our results show that seed longevity responses vary greatly across species, with p50 ranging from 3 up to 42 days. Seed longevity were ecologically and phylogenetically constrained, with some plant families adapted to cold and wet microhabitats (i.e., snowbeds) consistently showing short-lived seeds. Such results highlight that survival and persistence of alpine species facing climate change may depend on species microhabitat. While low average temperatures of alpine climates contribute to protect seeds from deterioration, some species might be particularly threatened with climate warming, especially those from cool-wet environments. The large variation of seed longevity, here observed within the alpine zone, may also have important implications for ex situ conservation.

Objective:

We want to obtain longevity traits from ex-situ experiments. Specifically obtain seed survival curves, Initial viability (Ki), deterioration rate (r-1) and time taken for viability to fall to 50 % (p50)

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 dominant/subordinate species be more resistant to ageing?

Hypothesis

* Fellfield specialist and snowbed specialist will respond differently to ageing
* Dominant species will be more adapted to the conditions they live in than subordinate species

Materials and Methods

Study area

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

* Villabandín (M2): siliceous bedrock and Mediterranean mountain climate,
* Picos (M1): calcareous bedrock and temperate mountain climate.

Gráfico

Descripción generada automáticamente con confianza media

*Figure 1. Distribution of plot data stored in a database on alpine grasslands in the Cantabrian Range, and mountain areas (M1, M2) selected for field studies. Grey areas show altitudes >1600 m.*

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.

Field sampling/Seeds

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.

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.

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).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Code** | **Specie** | **Habitat** | **Family** | **Site** | **Bedrock** | **snow/fell** |
| 300821-03 | Androsace villosa | specialist | Primulaceae | Hoyo | Calcareous | s |
| 310821-11 | Androsace villosa | specialist | Primulaceae | Cazadores | Calcareous | s |
| 300821-08 | Arenaria erinacea | specialist | Caryophyllaceae | Hoyo | Calcareous | n |
| 240821-01 | Armeria duriaei | specialist | Plumbaginaceae | Rabinalto | Siliceous | n |
| 310821-13 | Carex sempervirens | specialist | Cyperaceae | Cazadores | Calcareous | s |
| 280721-02 | Cerastium sp. | not-specialist | Cariophyllaceae | Rabinalto | Siliceous | s |
| 110821-01 | Dianthus langeanus | specialist | Cariophyllaceae | Penauta | Siliceous | s |
| 240821-02 | Festuca summilusitana | specialist | Poaceae | Rabinalto | Siliceous | f |
| 130921-06 | Gentiana verna | specialist | Gentianaceae | Hou | Calcareous | s |
| 300921-03 | Gentianella campestris | not-specialist | Gentianaceae | Hoyo | Calcareous | s |
| 300821-10 | Gypsophila repens | not-specialist | Caryophyllaceae | Hoyo | Calcareous | s |
| 310821-08 | Gypsophila repens | not-specialist | Caryophyllaceae | Cazadores | Calcareous | s |
| 300821-05 | Helianthemum canum | not-specialist | Cistaceae | Hoyo | Calcareous | s |
| 310821-05 | Helianthemum canum | not-specialist | Cistaceae | Cazadores | Calcareous | s |
| 310821-09 | Koeleria vallesiana | specialist | Poaceae | Cazadores | Calcareous | n |
| 310821-10 | Minuartia CF | specialist | Caryophyllaceae | Cazadores | Calcareous | n |
| 120821-01 | Minuartia recurva | specialist | Caryophyllaceae | Cañada | Siliceous | s |
| 240821-07 | Neochischkinia truncatula | not-specialist | Poaceae | Rabinalto | Siliceous | n |
| 130921-04 | Pedicularis pirenaica | specialist | Scrophulariaceae | Hou | Calcareous | s |
| 310821-04 | Pedicularis pirenaica | specialist | Scrophulariaceae | Cazadores | Calcareous | s |
| 240821-10 | Phyteuma hemisphaericum | specialist | Campanulaceae | Cañada | Siliceous | s |
| 250821-07 | Phyteuma hemisphaericum | specialist | Campanulaceae | Penauta | Siliceous | s |
| 240821-04 | Plantago alpina | specialist | Plantaginaceae | Rabinalto | Siliceous | f |
| 300821-15 | Plantago alpina | specialist | Plantaginaceae | Hoyo | Calcareous | n |
| 240821-09 | Sedum anglicum | specialist | Crassulaceae | Cañada | Siliceous | f |
| 240821-11 | Sedum brevifolium | not-specialist | Crassulaceae | Cañada | Siliceous | f |
| 130921-07 | Silene acaulis | specialist | Caryophyllaceae | Hou | Calcareous | f |
| 140921-03 | Silene acaulis | specialist | Caryophyllaceae | Boches | Calcareous | f |
| 230921-01 | Silene ciliata | specialist | Caryophyllaceae | Penauta | Siliceous | s |
| 300821-11 | Silene ciliata | specialist | Caryophyllaceae | Hoyo | Calcareous | f |
| 240821-08 | Solidago virgaurea | not-specialist | Compositae | Rabinalto | Siliceous | f |
| 150721-01 | Spergula morisonii | not-specialist | Caryophyllaceae | Cañada | Siliceous | f |
| 120821-06 | Thymus praecox | not-specialist | Labiatae | Rabinalto | Siliceous | f |
| 300821-07 | Thymus praecox | not-specialist | Labiatae | Hoyo | Calcareous | f |

Procedure:

We checked seeds 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 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 in the glass vials inside the enclosure boxes. Following Davies protocol removal time intervals were 2, 10, 15 and 15 and 30 days followed for a 4-weeks germination experiment. In our study, we sow 3 (pseudo)replicates of 14 seeds at each time interval.

For our germination experiment we sow 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 scored the germination experiments we had running, removing seedlings when the radicle was 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

\*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 will use R software where different packages are built for germination data (GerminaR package) and so we can also add phylogenetic information into our analysis (GLMM)

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)

\*Dose-response curve\* binary data weibull model?

Preliminary results:

Unbalanced dataset for plant family representation. 13 families but only 2 (Caryophyllaceae and poaceae) with +2 species.

P50:

* Siliceous and calcareous values are similar but we observe a wider range in calcareous
* Specialist have lower p50 mean value than not specialist
* Specialist + calcareous have lower p50 mean value value than not specialist + siliceous
* Snowbed + calcareous have lower p50 mean value than neutral + siliceous
* Snowbed + specialist have lower p50 mean value

Slope:

* Siliceous and calcareous values are similar but we observe a wider range in calcareous
* Snowbed have a higher negative slope i.e. faster viability decrease, but also higher variation
* Specialist have higher negative slope
* Calcareous + snowbed have higher negative slope
* Specialist + snowbed have higher negative slope
* Calcareous + specialist have higher negative slope

Ki:

* Siliceous have higher values than calcareous
* Specialist have higher variability and higher values than not specialist
* Specialist + siliceous have higher Ki
* Neutral microhabitat have higher Ki
* Calcareous + neutral AND siliceous+fellfield have higher Ki

Curves:

* Some population have almost exact response (e.g. Pedicularis pyrenaica) while other have noticeable variation (e.g. Silene acaulis, likely because this particular species have a wide distribution range and might have wider adaptation capacity)
* Calcareous have higher variability and faster decrease than siliceous
* Specialist have higher variability and faster decrease than not specialist
* Snowbed have higher variability and faster decrease, fellfield have intermediate response while neutral show the better response (i.e less viability loss)

These results seem to be driven by 3 particular families; Cyperaceae (Carex sempervirens); Gentianaceae (Gentiana verna and Gentianella campestris) and Scrophulariaceae (Pedicualris pyrenaica) all 3 specialist snowbed calcareous have low p50, high negative slope (faster decrease) and low Ki (initial viability). But Solidago virgaurea from compositae family (siliceous, fellfield and generalist) and Phyteuma hemisphaerica from campanulacea family (siliceous, snowbed, specialist) also have low p50 values, high negative slope but medium/high Ki values.

All species show viability decrease across 30 days in ageing solution but for 7 species estimated p50 value exceed 30 days. Suggesting that some alpine species might not be short-lived (Dianthus langeanus, Neochischikinia truncatula, Helianthemum canum, Spergula morisoni and Silene acaulis (only one of the populations))

Preliminary conclusions

Calcareous + snowbed + specialist were the less long-lived accessions (Correlation vs causation)

Precisely there communitites (i.e. snowbeds) have been previously identified as the most detrimentaly affected by climate change (responses in growth, richness, cover changes REF) but there was no information aobut seed longevity as underlying factor for the shrinking of these communities (habitat loss).

Climate change is predicted to keep raising of temperatures but also an increase of the variability in extreme precipitation events (both drought and floods). For the same vegetation plot to suffer co-ocurrent extreme stresses (REF)

However, in the current warming climate, snowbed communities will probably be negatively affected by a shorter soil seed bank persistence due to an increased deterioration rate elicited by higher soil temperature and not last sufficiently to buffer lengthy detrimental periods for community regeneration.

EXTRA options:

Use Andrea data set from the alps with longevity responses to obtain a wider point of view and more general pattern across geographic areas. However not 100% comparable because seeds were kepts at -20ºC and 15% RH. My study area might not be as wet and cold as the Alps. (very warm-dry summer)

Possibility to add iButtons data or even WP data from dataloggers in the field?

Use Landolt indicator values (available for Italian flora, database from Francesco Porro)

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

USEFUL NOTES/REFERENCES FROM SEEDALP PROPOSAL

Surprisingly, regeneration traits, which are largely responsible for species replacement and community diversity (Grubb 1977), are scarcely investigated in the context of trait-based ecology. As a response to this gap, recent studies highlighted the role of the regeneration niche for understanding the diversity and relative abundance of plants. Such studies relied on the independent functionality of seeds (Hoyle et al. 2015), the necessity of integrating seed traits in plant community ecology (Jiménez-Alfaro et al. 2016),

the performance of seeds is primarily regulated by environmental factors, thus defining a *seed ecological spectrum* (Saatkamp et al. 2018). However, the complexity of the processes regulating seed dispersal, persistence, germination timing and seedling establishment in response to environmental factors makes fundamental questions regarding seed ecology difficult to answer, especially because these factors have rarely been addressed in concert.

the myriad of morphological, physiological and biochemical processes regulating seed functions necessitates new research to demonstrate the role of seed traits across species and habitats (Jiménez-Alfaro et al. 2019).

The research gap on seed-trait ecology is especially evident in alpine vegetation. In a review about the influence of climate change in seed regeneration of alpine plants, Briceño et al. (2015) demonstrate how the role of seeds has been largely neglected in alpine plant biology.

Recently, it has been noted that the abundance and distribution of alpine plants strongly depends on micro-topographic variation, which accounts for drastic changes in temperature and related factors (Scherrer & Körner 2010; Kulonen et al. 2018). An increasing number of studies have reported relationships between micro-habitat variation and the compositional and functional diversity of alpine plant communities (e.g. Choler 2005; Opedal et al. 2014; Stark et al. 2017; Blonder et al. 2018) but functional approaches have mostly focused on adult-plant traits.

Seed persistence and germination of alpine species is regulated by seasonal cycles in temperature and moisture conditions (Baskin & Baskin 2014), but these responses may not be the same for all species living in the same habitat, determining spatial and temporal heterogeneity in this key selective process. Studies suggested that the seed germination niche is influenced by micro-site temperature and snow cover (Fernández-Pascual et al. 2016) and soil bedrock (Tudela-Isanta et al. 2018). Relatively higher temperatures at the microsite also may determine shifts in germination timing and increasing recruitment of alpine populations in both temperate (Mondoni et al. 2015) and Mediterranean (Giménez-Benavides et al. 2018) systems.

However, apart from seed mass, regeneration traits are hardly ever considered, despite existing evidence that life-history stages determine different plant responses across micro-habitats (Hülber et al. 2018). So far, we do not know whether regeneration stages may follow similar or different strategies in alpine plants, and if regeneration traits can provide additional insights into assembly processes, in particular at the level of microsite variation.

To this end, studies on seed traits collected from multiple co-occurring species, dealing with micro-site environmental variation, and combined with community compositional data, may provide a new view about the role of the regeneration niche in alpine plant diversity, along with the understanding of unexplored patterns and processes in plant communities.

We will focus on communities above the treeline (between 1750 and 2560 m a.s.l) dominated by grass-like species (mostly *Poaceae* and *Cyperaceae*) but also rich in Hemicryptophytes and Chamaephytes. We will place four experimental sites along a topographical and compositional gradient in one calcareous and one siliceous mountain. For each site, we will establish a macroplot of 50 m radius, in which we will establish 20 plots of 1 m2 (Figure 2). Then we will characterize seed traits of co-occurring species to be linked with compositional, environmental and soil information collected in the plots.

**Objective 2: to evaluate seed persistence and longevity of co-occurring species**

*Background.* The ability of seeds to persist alive in the soil for months or years mostly depends on species-specific responses to the environmental conditions in the micro-site and how seed physiology matches local climatic seasonality (Jaganathan 2015). Either seed dormancy or the lack of high temperatures may prevent seed germination after seed shedding, as a natural adaptation of alpine populations to avoid seedling emergence in winter (Baskin & Baskin 2014). Seeds that do not germinate (and are not lost by e.g. predation) begin a metabolic ageing process that will determine in-situ survival, mostly depending on moisture and temperature. Soil seed banks have been consistently reported to exist in alpine habitats (Körner 2003; Jaganathan 2015) and the very few studies conducting burial experiments showed that most alpine species can persist at least 5 years (Schwienbacher et al. 2010). However, ex-situ laboratory experiments of longevity have shown that alpine seeds are more sensitive to artificial ageing (inferred with increased moisture) than lowland species (Mondoni et al. 2011; Santyanti et al. 2018). Although there is evidence that ex-situ longevity and desiccation tolerance correlate with other seed traits, plant traits and habitat conditions (Probert et al. 2009; Merritt et al. 2014; Wyse et al. 2017), these relationships have not been explored yet in alpine communities (nor in many other habitats).

*Hypotheses.* We expect that microsite conditions - moisture, temperature and snow cover - influence the survival of alpine seeds in a predictable way for each species or population. If this hypothesis is true, there should be a physiologically-based relationship between the persistence of buried seeds in the field and seed longevity and/or desiccation tolerance estimated ex situ. For example, we expect that those species/populations with seeds that persist worse under long snow accumulation (high in-situ humidity) will also be more sensitive to artificial ageing (high ex-situ humidity). Such relationships should relate to the ecology of the species in the field and other plant or seed traits. If species responses are relatively consistent among populations, then persistence traits may be also used to infer responses at the community level.

*Methods.* We will establish four experimental sites in one calcareous and one siliceous mountain of the study area (M1 and M2 in Figure 1). In each mountain, the four sites will cover contrasting soil conditions, from “fellfields” (open areas subjected to freeze-thaw cycles without snow protection on winter, and dry cycles) to “snowbeds” (here, referred to areas with dense plant cover, long snow cover and high moisture). In M1 (Picos de Europa National Park) we will use part of the sites included in a local monitoring program of climate change coordinated by the PI during the last 10 years. In M2 (Sierra de Villabandín) the sites will be selected by using the data collected in 2019 by the PI to fill gaps in the vegetation database. In both M1 and M2, the sites will be selected by maximizing plant diversity and their representativeness in the study area. We will select a homogeneous and relatively flat area to place a reference centre for the site, where we will bury one logger of water potential and temperature )MicroLog SP3, EMS, Brno) and will extract a soil sample of 10 cm3 for physic-chemical analysis. Then we will define two areas based on circular plots (*Figure 2*): (1) a “target community” defined around the logger using a radius of 3 m (circular plot of 28.3 m2) and (2) a “site community” defined by a radius of 50 m (7853 m2). In year 1, we will collect seeds from all species observed in the target community but collected from the whole site community. We will mix the seeds of each species and will imitate short-distance dispersal from the whole population burying the seeds in the target community during the same day they will be collected.

Species will be ranked by the time it takes for 50% of the seeds within an accession to lose viability (p50, Ellis & Roberts 1980) and by the standard deviation of the frequency distribution of seed deaths in time (r) (Newton et al. 2009). These metrics will be calculated for in-situ and ex-situ longevity. Then we will test the best predictors explaining the variation between p50 across species using GLMs and ANOVAs. Predictors will be: site, species, species group based on dominant/subordinate and specialists/generalists, seed traits, plant traits, and environmental variables extracted from the soil and the datalogger (days of snow cover, growing-degree days, and freezing degree days, see Choler 2018).

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)

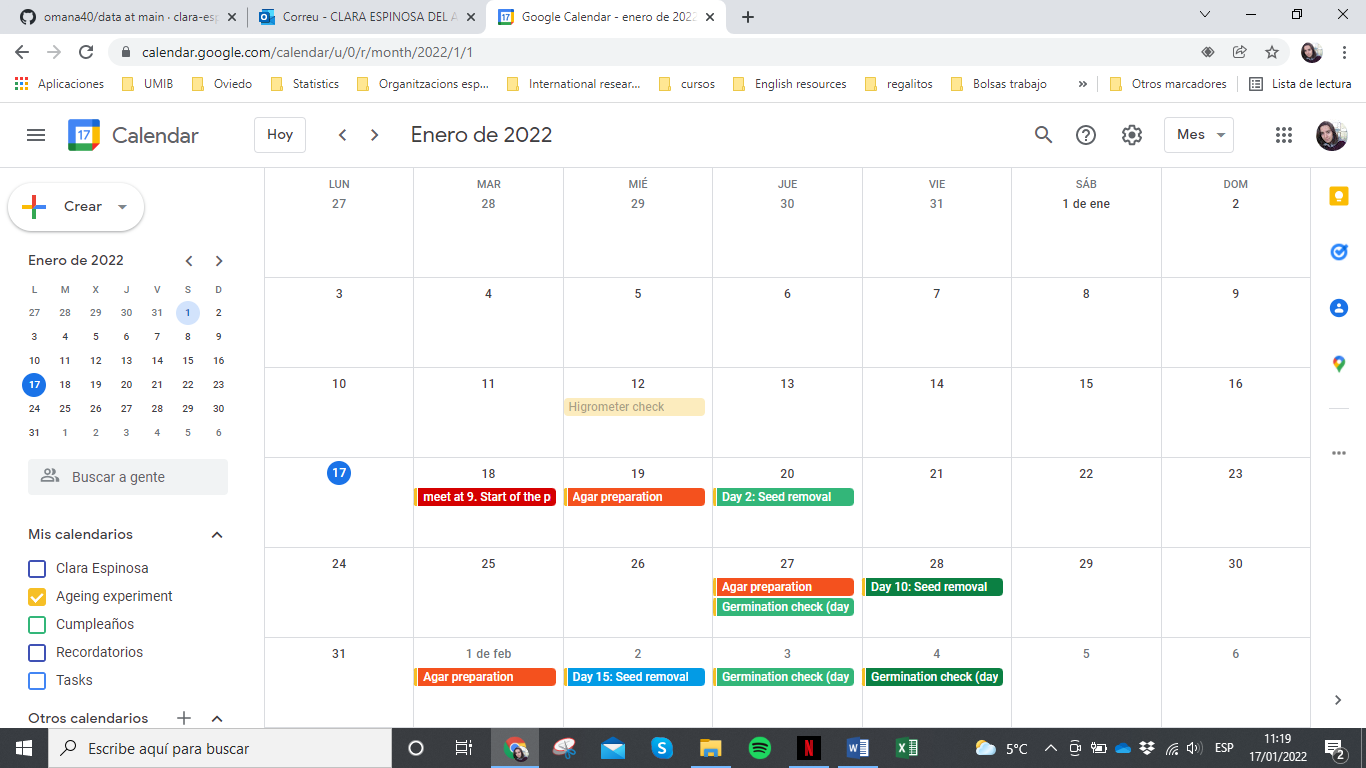
Box stands: 2 per sealable box (can be stacked)

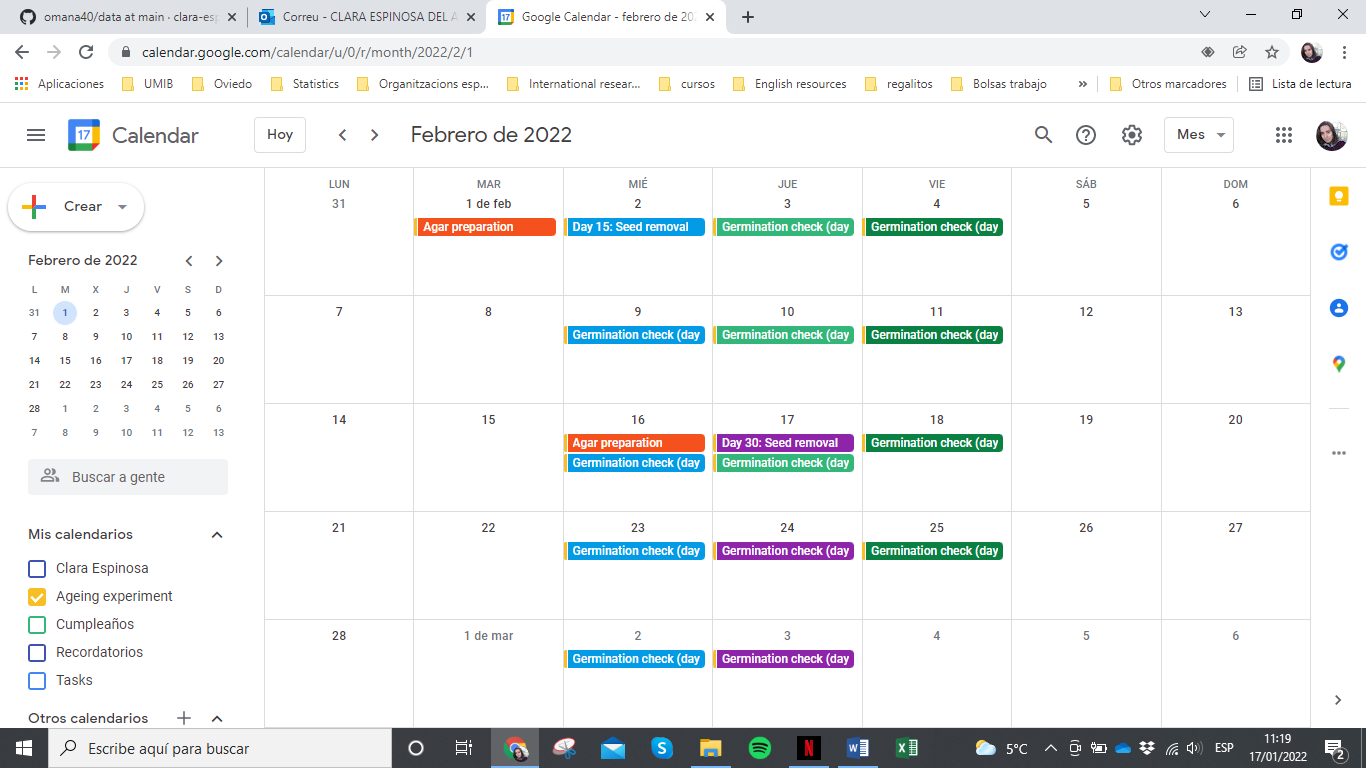
Lab-based hygrometer: Andrea uses Rotronic to assess the HR of the seeds (same we were considering) and to check TºC and HR inside sealable boxes uses: TINYTAG TV-4500 VIEW 2 (color azul) protección IP65 (185 € sin IVA) <https://e-berman.info/producto/tv-4500/>

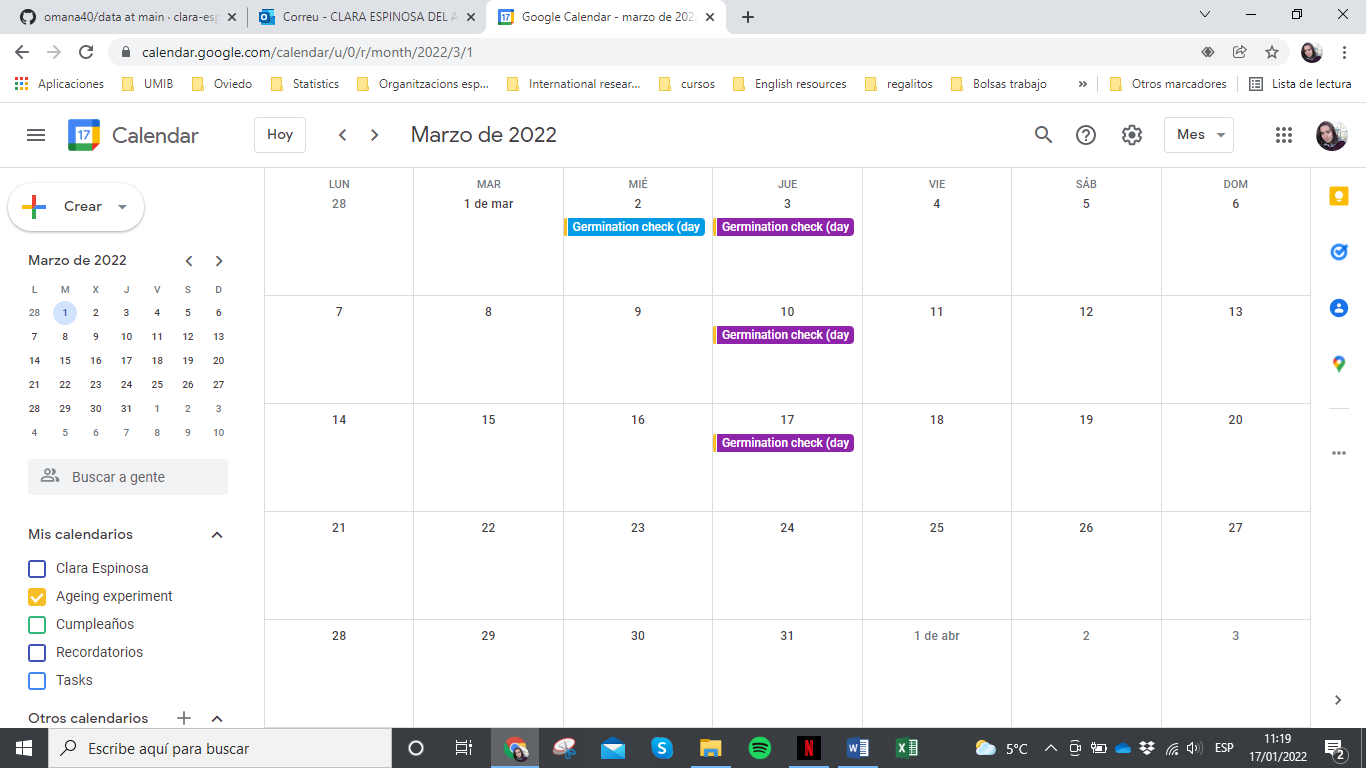
Statistical analysis software: Genstat es de pago, su doctorante había intentado hacer las seed survival curve mediante R pero no había encontrado una manera “sencilla”

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 number 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.
* Standars 14 days in rehydration solution before ageing solution

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 the 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\*