# Biological and ecological trade-offs of seed oil content in alpine species

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## Summary

## Introduction

Seed importance for plant regeneration, seed traits are understudied.

### Seeds reservoirs

Seeds act as reservoirs of energy basically accumulating 3 macromolecules: protein, fatty acids (FA) or starch. The quantity and quality of these reserves influence dormancy, viability and germination potential, thus directly related to plant fitness (Levin, 1974; Westoby et al., 1992). Oil (FA) and carbohydrates are predominant for angiosperms (Bretagnolle et al 2016), but most plants rely on oil content, as has already been shown positively correlated with the earliness of germination (Gardarin et al., 2011)). Seed oil content and fatty acid composition thus determine plant fitness and validate the adaptive hypothesis, i.e. these traits are crucial for plant adaptation Sanyal 2016, germination success [Linder 2000], emergence and establishment of a plant [Bewley 1994]. Carbons in fatty acids are highly reduced and the oxidation of oils in germinating seeds releases more than twice as much energy as the oxidation of storage carbohydrates or proteins on a per g basis (Baud & Lepiniec, 2010; Luttge, 2012; Theodoulou & Eastmond, 2012). Lipid composition determines oil quality and membrane structure and has profound effects on seed viability in the dry state (Hoekstra, 2005).

### Seed oil composition (importance)

Although oil content variation is found within and among genera of the same family (Bretagnolle 2016) it is also highly constrained by phylogeny and subject to evolutionary change (Levin 1974). Both oil content and relative FA frequency vary as response to environmental, geographical location and maternal genotypes (Linder, 2000; Ghebretinsae et al., 2008). Most FA found in seeds are the saturated palmitic (16:0, PA) and stearic (18:0, SA) acids and the unsaturated oleic acid (18:1n-9, OLA), linoleic acid (18:2n-6, LA) and a-linolenic acid (18:3n3, ALA). FA can be divided in saturated (SFA) and unsaturated (UFA) which have differential properties that may influence those responses. On a per-carbon basis, UFA cost more to produce and yield less energy when oxidized than SFAs [41, Linder 2000). Moreover, the storage of unsaturated FAs and particularly PUFAs necessitates the storage of antioxidant molecules to prevent damage to FAs. Hence, the oily seeds should maximise SFA storage instead of UFA (Linder, 2000). Hence, a maximal storage strategy would be oriented towards the synthesis of oily seeds with only saturated FAs. However, the relative abundance of unsaturated to saturated FAs highly varies in angiosperms and many species synthesise a very low amount of saturated FAs (Linder, 2000; Voelker & Kinney, 2001).

### Global seed oil content patterns

Levin 1974 found that seed oil content increased with woodiness and shade tolerance. Sanyal 2016 found that seed oil content has been seen to be significantly higher in tropical plants, probably because need higher energy reserved in seeds to survive first life stages with high competition and/or low illumination (Salisbury 1942 ), compared to temperate plants. Also, higher proportions of UFA (oleic and eicosenoic) with increased latitude (Sanyal 2016). Concordantly previous studies have shown that the proportions of saturated and unsaturated FAs and subsequently their melting points vary with latitude [2, 40]. At lower latitudes, seeds with higher proportions of saturated oils would be favoured because they would have more energy for growth without delaying or slowing germination. At higher latitude and cooler germination temperatures, seeds that have a higher proportion of unsaturated oils (with lower melting points) may germinate earlier and/or more rapidly than seeds that are higher in saturated FAs (with higher melting points). Rich UFA seeds could then germinate faster and earlier than rich SFA seeds in cold conditions, providing a competitive advantage where cold temperature regulates seed germination (Linder, 2000).

### Biological tradeoffs (seed longevity, seed mass, other germination traits?)

Sanyal 2016 suggests that selection could be acting simultaneously on multiple seed traits: seed size, oil content and seed oil composition to facilitate faster growth and reproduction and higher latitudes or lower temperatures.

### Seed mass and oil content

Bretagnolle found a negative correlation between seed mass and oil content and a strong positive correlation between the energy stored in the seed and oil content. Large seeds generally store less oil than small seeds, whereas small seeds which have higher oil content are rich in PUFAs. This relation make sense because the carbons in fatty acids are highly reduced and the oxidation of oils in germinating seeds releases more than twice as much energy as the oxidation of storage carbohydrates or proteins on a per g basis (Baud & Lepiniec, 2010; Luttge, 2012; Theodoulou & Eastmond, 2012). Hence, a small oily seed can release as much as energy as a starchy seed that is twice as heavy. A huge variation of oil content exists in small seeds, although such variation strongly decreases towards low oil values in large seeds (corroborated in our preliminar results). Such a relationship suggests that as oil synthesis is energetically costly relative to carbohydrates, oil synthesis could be an advantage only for small seeds which can store energy in a smaller volume (Bretagnolle 2016).

### Seed size and persistence

Small-seeded species often produce numerous seeds with higher persistence capacities, whereas large-seeded species often produce less but bigger seeds that generate seedlings with higher competitive ability and better buffering capacities of environmental stresses (Thompson et al., 1993; Coomes et al., 2002; Moles & Westoby, 2006; Muller-Landau, 2010).

### Seed longevity and oil content.

Seed longevity, i.e. the ability of seeds to remain viable over certain storage periods, is determined by an intricate network of genetic and environmental factors. The genetic factors are associated with seed morphology and composition, whereas the environment affects by a combination of conditions prevailing during seed development, ripening, at harvest and during storage. Walters et al. (2005) suggested taxonomic and climatic effects on interspecific differences in longevity. According to these authors, seeds of species from cold and temperate climates have shorter longevity than seeds of species from hot and arid climates (McDonald, 1999; Kranner et al., 2010; Walters et al., 2010). According to Mondoni et al. (2014) a relationship between seed longevity and the environment was considerably strong and greatly affected by maternal genetics. But according to Merritt et al. (2014b) environmental conditions of seed origin or production are weakly associated with seed longevity. On the other hand, according Probert et al. (2009) seeds from more stressing environments, as hot and dry, are more tolerant to desiccation and from moist and colder are more susceptible.

It is believed that lipid peroxidation is the main cause of seed deterioration in dry seeds during storage, which in turn influences longevity (Bewley et al., 2013). Stored lipids within the seeds deteriorate mainly due to (i) oxidation caused by high temperature and moisture content, (ii) hydrolysis, wherein fat is degraded into fatty acids and (iii) contamination (Abdellah and Ishag, 2012). To prevent the deleterious effect of lipid oxidation, many lipophylic antioxidants such as tocopherols and carotenoids are also stored in oily seeds, and a positive correlation has been shown between the tocopherol level and the degree of unsaturation in FAs (Kamal-Eldin & Andersson, 1997; Sattler et al., 2004; Falk & Munn\_e-Bosch, 2010). Seeds that contain a high concentration of lipids are susceptible to generate reactive oxygen species (ROS) during ageing that are responsible for cellular damage (Yao et al., 2012). . Supporting evidence was originally based on the finding that auto-oxidation of polyunsaturated fatty acids produces free radicals, thereby compromising membrane integrity (Priestley and Leopold, 1979).Thus, oily seeds being more sensitive to ageing (Nagel & Borner, 2010).Concordingly, Neto 2019 found a significant negative correlation between seed longevity and oil content. However, in general, the correlation between seed oil content and longevity has been described as weak (Nagel and Börner, 2010; Priestley et al., 1985; Walters et al., 2005)., in other cases no significant effect of oil in seed longevity has been reported (Probert 2009, Mederios et al 1998, Pritchard and Dickie lab not published)

In particular, the oxidative resistance of the oil decreases with increasing proportion of Polyunsaturated fatty acids (PUFA). Thus, the concentrations of UFAs and SFAs affect the storage behaviour of seeds (Walters et al. 2004; Volk et al. 2006; Walters et al. 2015). Oxidations of unsaturated fatty acids are considered to be primary reactions in ageing, contributing to free radicals production and subsequent attacks on other macromolecules (Benson 1990). Higher values of linolenic acid (UFA) associated with shorter longevity (Ponquett et al 1992). Further investigations on the effects of seed oils (content and composition) on longevity are desirable (Pritchard and Dickie 2004).

Although its importance very few studies on native species address and analyze seed oil content and composition. A lot of specific studies in commercial species (cotton, brassica, soybean) gene expression, irrigation effects (focused on plant breeding programs).

### Ecological significance (species optimal microclimatic conditions)

### Aims, questions and hypotheses.

The goal of the study is to explore and understand the seed oil content and composition (proportion of saturated fatty acids, SFA, and unsaturated fatty acids, UFA) in alpine plants. This kind of data is barely available for most plants and especially for wild alpine species. Although oil content is known to influence a wide range of biological processes, few studies have investigated biological and ecological correlates. The trade-offs identified in the literature have not been consistent across showing divergent patterns. Our study is structured in 3 main sections/questions:

1. Exploration and description of seed oil content in alpine species. Does oil content and composition in alpine species follow global patterns?
2. Seed oil content biological trade-offs. How do oil content and oil composition (SFA vs UFA) correlate with other seed traits? Explore seed mass, seed longevity (p50) or other traits from previous experiments like t50?
   1. H2.1. Higher seed mass will correlate with less oil content and less UFA/SFA ratio
   2. H2.2. Higher oil content and higher UFA/SFA ratio will correlate with less longevity
   3. H2.3. Higher UFA/SFA ratio will correlate with faster germination (lower t50) (still to be tested)
3. Ecological trade-offs. How does oil content and oil composition (SFA vs UFA) correlate with the ecological optimal of the species/community?
   1. H3.1. Strict alpine species will have less oil content and higher UFA/SFA ratio
   2. H3.2. Species living in exposed sites (+ warm in summer, + cold in winter) will have higher oil content and higher UFA/SFA ratio

We will use a combination of techniques from laboratory based and physiological traits o other ecological traits. Seed traits like oil content could be a potential predictors for many biological responses.

Notes seed oil content vs longevity seminar 21/2/24

* Beta distribution para ratios
* Gamma distribution para datos sin transformar
* Añadir community como factor fijo?
* Ecological tradeoffs (GDD/FDD) divider Mediterranean vs Temperate?
* Árboles de regression (Adri)

## Methods

### Study system

We focused on alpine species from grassland communities in both acidic and basic bedrocks in the Cantabrian mountains (Northwestern Spain). These grassland communities are continuously distributed along the mountain range, occupying reduced areas above the treeline and around mountain tops, between 1750 and 2500 meters (figure 1). Grassland vegetation is mostly dominated by *Poaceae* and *Cyperaceae*, and the main lifeforms are Hemicryptophytes and Chamaephytes and local richness ranges from 4 to 28 species, basic grasslands being more species-rich. Grazing impact is restricted to wild populations of Cantabrian chamois (*Rupricapra pyrenaica parva*).

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.

### Species data

Within each bedrock type, we established four sampling sites separated at least 500 m. To cover spatial community composition variation, we established 20 additional five plots (1m2) in each cardinal direction with a 10 m separation per sampling site (cross design, fig x). In total, we established 160 vegetation plots (4x20 in acid and 4x20 in basic bedrock) and collected floristic and community composition data for 128 alpine grassland species (46 sp in acidic and 82 sp in basic). To calculate species ecological optimums (following the methodology of picos paper JVA add doi), we measure microenvironmental gradients using an iButton datalogger, buried at 5 cm deep, in each vegetation plot (Thermochron, iButton, Newbury, UK; accuracy: +/- 0.5 °C from -10 °C to +65 °C, resolution: 0.5 °C, records every four hours) which recorded temperatures across 11 months. In basic plots, the iButtons recording period went from 1st October 2018 to 31st August 2019 (330 days) while in acidic vegetation plots, the recording period for the iButtons went from 12th July 2021 to 29th May 2022 (321 days, all raw data available in GitHub repository).

All species were classified between specialist (strict alpine) and generalist distribution. We identified plant specialists as those that are significantly associated with the target vegetation type, using the Indicator Values (IndVal) in the indicspecies R package (REF). The calculations were based on 12,000 vegetation plots of grasslands stored in the SIVIM database for the Cantabrian Mixed Forests ecoregion. From the preliminary list of indicator species for the studied vegetation, we removed species with median values of elevation below 1800 m, most of them characteristic of subalpine or nitrophilous habitats. We finally identified 40 plant specialists which are widely recognized as characteristic species of alpine and subalpine grasslands.

### Oil content and composition

We preliminary explored within-accession variation in 5 species analysing 3 subsamples per species, however, we could not detect any statistical differences. Consequently, a single sample for each species seed accession was investigated, so that within-species variation was not explored. Fatty acids analysis (oil content and composition) requires a sample of 200mg of dry seeds. We have oil content and composition data from 36 species (18 from acid and 18 from basic) many of the data obtained about FAs content and composition of these species were not known before. We sent the samples to an external analytical laboratory (USTA-CSIC) where they used a gas chromatographer with a Flame Ionization Detector (Agilent 7820A, EZChrom Elite software). Samples were manually ground with liquid nitrogen, then fatty acids were transformed to fatty acid methyl esters (FAMEs) using a mix of sodium methoxide 0.5M in methanol and acetyl chloride in methanol (1:10 v/v) (table xx in appendix with complete list of Fame’s possible to identify).

### Other seed traits data

Seed mass data was calculated for all species weighting 5 replicates of 50 dry seeds per species.

Germination traits are available for almost all species. T50 calculated as the time to reach 50% germination from a phenology germination experiment (Espinosa del Alba et al. 2024).

Longevity data is available for 20 of those 36 species. We applied a standard comparative longevity protocol (Newton et al., 2009, Probert et al., 2009) optimised for short-lived species (Davies et al., 2016) like alpine species (Mondoni 2012). The artificial accelerated ageing protocol allows the measurement of p50 value (amount of time for seed viability to drip to 50%). The initial RH value was 30-35% (Hygropalm 3 display unit; Rotronic Instrument UK Ltd, Crawley,UK). Before the start of the ageing protocol species from the Cistaceae family were physically scarified with sandpaper. Seed samples (200 seeds per species) were first rehydrated to 47% RH at 20ºC for 5 days in a non-saturated LiCl solution (Hay et al., 2008) in crystal petri dishes and kept in in a 300 x 300 x 130 mm sealed electric enclosure box (Ensto UK Ltd, Southampton, UK) before moving the vials into the ageing conditions. The ageing conditions consisted in a temperature of 45°C, a RH of 60% and darkness. A subsample of S 42 seeds was withdrawn after 2, 10, 15 and 30 days and sowed in petri dishes 1% agar with 250 ml/L of GA3 (Kew Royal Botanic Garden Technical Information sheet\_13a). In some species, the number of seeds for subsamples was reduced due to the lack of seeds. Germination conditions for most species were set at 22-12ºC alternating temperatures with a 12/12 h photoperiod, for those whose germination required colder temperatures a second germination chamber was set at 15/5ºC alternating temperatures with a 12/12h photoperiod (see table xx for details). After the sowing, the seeds were checked once a week for one month, in which the germination was scored by removing the seedlings with a visible root (>1.5 mm); after the end of each germination test the ungerminated seeds were cut-tested under the binocular loupe to visually assess the state of the embryo. We considered seeds with white and firm embryos viable, i.e. potentially germinable (Baskin and Baskin, 2014) and we removed empty or infected seeds from further analysis.

Longevity analysis have been widely used by seed bank managers based on the following viability equation: v = Ki – (p/σ), where v is viability in NED (Normal equivalent deviates), p are the days of ageing, Ki is the initial viability, σ is the standard deviation of the distribution of deaths over time.

### Statistical analysis

We use GENSTAT software to apply a Probit analysis (Hay et al., 2014) and calculate p50, Ki and slope values.

The phylogeny tree was created using V.PhyloMaker R package (Ref), see fig xx)

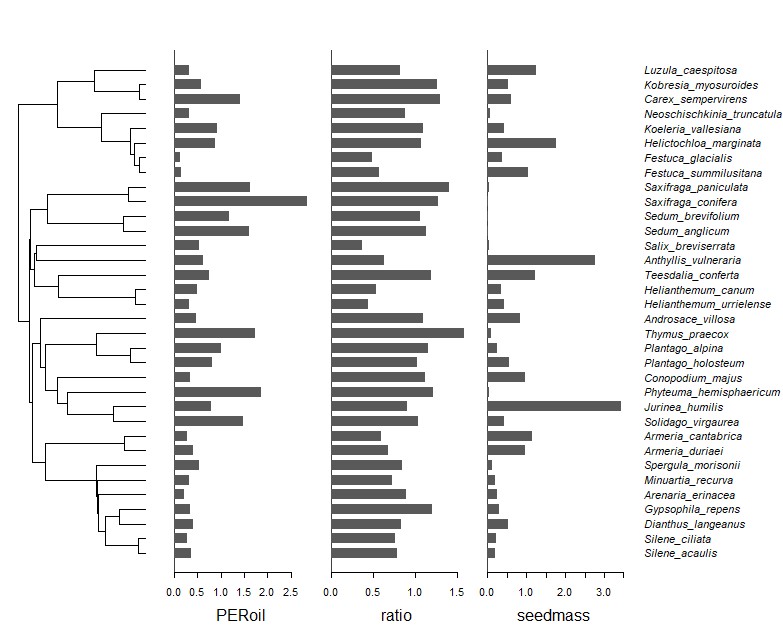
Exploratory PCA for FAME’s composition and total oil content (in percentage)

How two response variables are total oil content (in percentage) and UFA/SFA ratio.

Species phylogenetic tree (so far)

## Results

Congeneric species has very similar levels and proportions of FA and oil content (as stated in literature, and see figure 2)



### Seed oil content patterns in alpine species

The exploratory PCA showed relatively low explained variation within the first two axes (21.7 and 16.2 5 respectively). FAMEs of C22:1n9 and C22:2n6 are the ones contributing the most in PC1 and PC2 (around 11%) along with the percentage of oil content (11%) also contributing in PC2. Additionally, PCA revealed that oil content percentage is not highly correlated with any specific FAME type (table in appendix). It is important to note that erucic acid (C22:1n9) is highly abundant but only in Brassicaceae family. Most species are distributed along oil content variable.

Within our species, oil content varies from 1.3% in *Festuca glacialis* (Poaceae) to 30.1% in *Saxifraga conifera* (Saxifragaceae). Most abundant FAME in alpine seeds were the unsaturated linoleic acid (18:2n-6, LA, 43.1 ± 16.1%), oleic acid (18:1n-9, OLA, 22.9±13.3%) and alpha-linolenic acid (18:3n3, ALA, 16.2 ± 19.3%); and saturated palmitic (16:0, PA, 10.6 ± 4%). These 4 represent a mean of 92.7% (SD = 52.75). The next with higher % were stearic acid (C18:0, 2 ± 0.9%, saturated) and c-linolenic acid (C18:3n6, 1.1±2.9%, unsaturated). Additionally, in our data set Erucic acid (C22:1n9) had also high values due to Brassiacea species. The mean frequency of saturated fatty acids (SFA is 14.7% with sd of 5.3%) and the mean ratio between unsaturated fatty acids (UFA) and SFA is 6.8 (SD= 2.1), ranging from 2.7 in Salicaceae up to 11.4 Lamiaceae. In general, the Salicaceae and Cistaceae had low values of the ratio, indicating higher synthesis SFAs, while other families such as the Lamiaceae or the Saxifragaceae were characterised by a higher ratios showing a tendency to synthesise more UFAs.

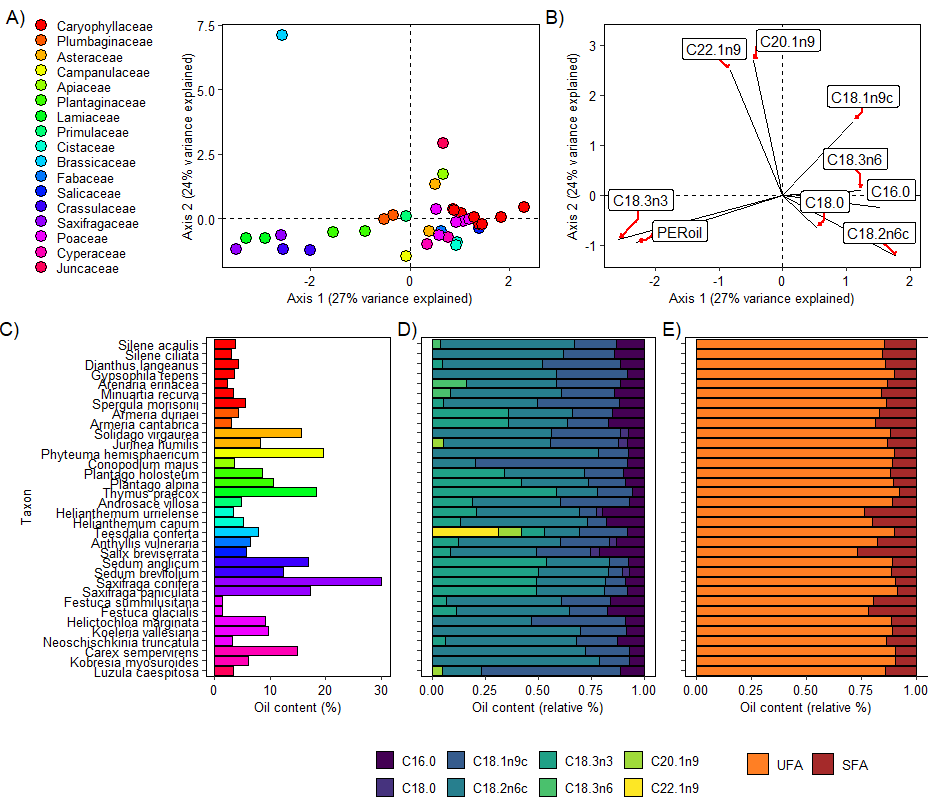


Fig 3: Alpine species seed oil content and composition exploration (n=34). A) Exploratory PCA species points and B) variables directions and contributions. C) Oil content per species in percentage, colors representing families as panel A. D) Seed oil composition of those FAME’s with more than 3% of relative abundance. E) Seed oil composition per species with FAME’s divided between Unsaturated Fatty Acids (UFA) and Saturated Fatty Acids (SFA).

### Biological trade-offs

#### Seed mass

We observe a notable variation of oil content in small seeds, but it diminish in large seeds which at the same time have lower oil content.

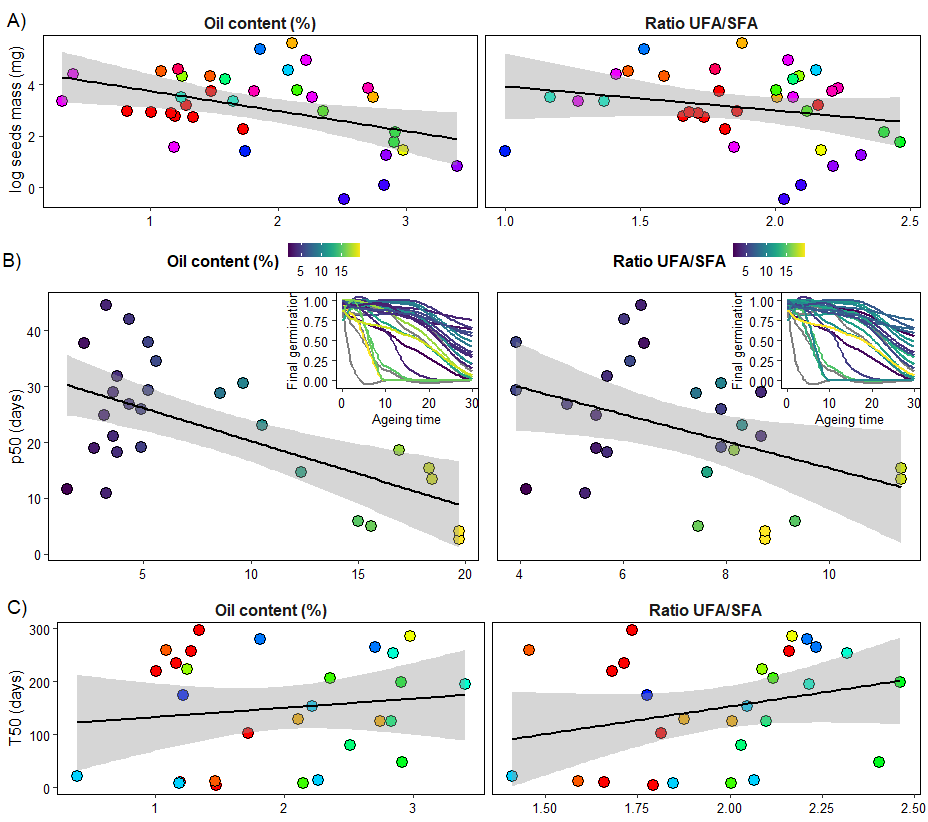
H2.1a Higher seed mass correlate with lower oil content (variables log transform to met assumptions H2.1a Higher seed mass correlate less UFA/SFA ratio (seed mass log transform to met assumptions) optimize space for more energy reserves

Longevity (analysed as raw germination data with MCMC-GLMM and using P50 from probit analysis)

H2.2a Higher oil content less longevity. Ageing was significantly modified by oil content (<0.001 in MCMC-GLMM and 0.05 in normal GLM for p50)

H2.2b A higher UFA/SFA ratio will correlate with less longevity. The ratio was not found significant with the data available. To be tested again with more data.

H2.3. A higher UFA/SFA ratio will correlate with faster germination (lower t50) (still to be tested)

Fig 4. Seed oil content and Ratio UFA/SFA biological trade-offs. A) correlation between seed mass (log transformed) and oil content (% log transformed) and ratio Unsaturated Fatty Acids (UFA)/ Saturated Fatty Acids (SFA), also log transformed. Colours represent families as in figure 3. B) P50: time for viability to drop to 50% , based on probit analysis (GENSTAT software) and raw germination curves across artificial accelerated ageing protocol in the lab, colour represent, oil content percentage (left panel) and UFA/SFA ratio (right panel). C)T50: time to reach 50% germination, data from germination phenology which uses the same species from the same area (Espinosa del Alba et al., 2024) depending on oil content (%log transformed) and ratio UFA/SFA (log transformed). Colours represent families as in figure 3.

EXTRA:: will ecological preferences modify longevity? AS preliminary results from Giovanni tesis and IAVS congress. Significant only when using raw germination data.

Gráfico, Diagrama

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### Ecological trade-offs

#### Species distributions

H3.1 Specialist species will have less oil content and higher UFA/SFA ratio

#### Optimal ecological conditions

H3.2a Species living in exposed sites (+ warm in summer (GDD), + cold in winter (FDD)) will have higher oil content

H3.2b Species living in exposed sites (+ warm in summer (GDD), + cold in winter (FDD)) will have higher UFA/SFA ratio

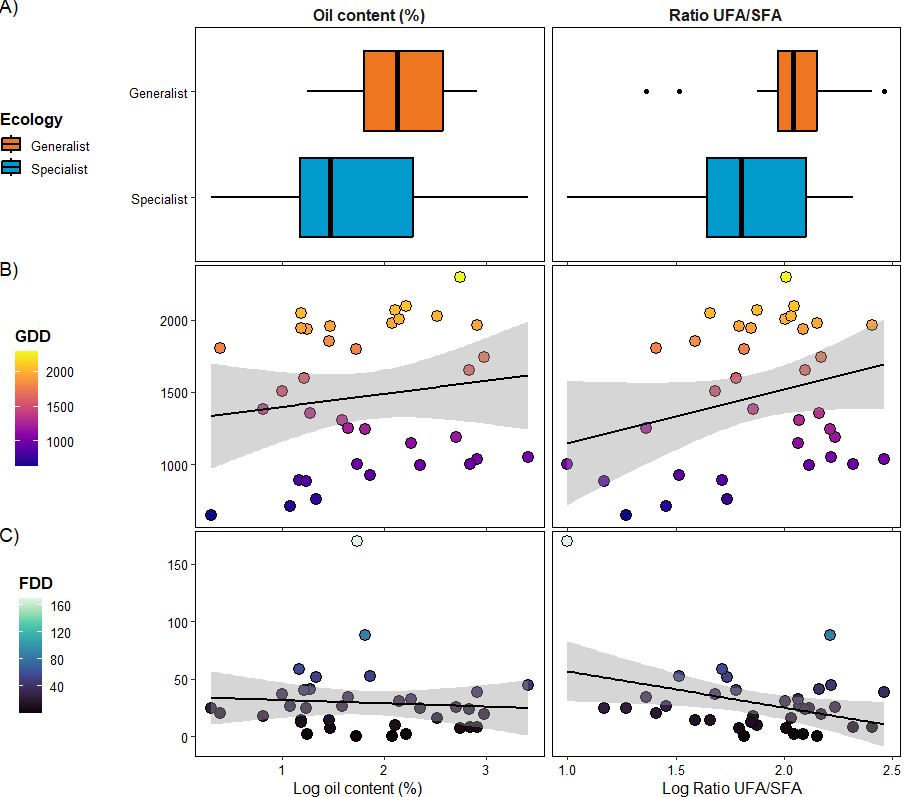


Fig 5. Seed oil content ecological trade-offs.

## Discussion

Our study is xxx

### Comparison with global patterns

Sanyal 2016 found significant negative correlation between seed oil content and palmitic (C16:0) and linoleic acids (C18:2n6) and positive correlation with oleic (C18:1n9), arachidic (C20:0) and eicosenoic (C20:1n9). In our preliminary analysis no significant high correlations between total oil content and other components in either direction.

The oil content can vary from 1 % in Musa paradisiaca to 76 % in Chrysobalanus icaco [9]. Bretagnolle found a huge variation in seed oil content ranging from seeds with virtually no oil (Trifolium pratense L., 0%) to very rich seeds (Papaver rhoeas L., 54%) (Bretagnolle 2016). In our preliminary results oil content varies from 1.3 % (F. glacialis) to 30.1% (S. conifera).

According to Bretagnolle 2016 most FA found in seeds are the saturated palmitic (16:0, PA) and stearic (18:0, SA) acids and the unsaturated oleic acid (18:1n-9, OLA), linoleic acid (18:2n-6, LA) and a-linolenic acid (18:3n3, ALA). Corroborated in our results + 2 extra FAs erucic (C22 1n9, mainly from Brassicaceae) and eicosenoic (c20. 1n9, mainly from Juncaceae and Brassicaceae).

Bretagnolle 2016. the three most common fatty acids were palmitic acid (C16:0, PA), oleic acid (18:1n- 9, OLA) and linoleic acid (18:2n-6, LA). These three FAs represented a mean of 76.5% (SD = 20.5%) of the total FAs in the seeds of the species analysed and can represent more than 90% of the FA synthesized by the seed. In our case also linoleic (18:2n-6, 43.1 ± 16.1%), oleic (C18:1n9, 22.9±13.3%), alpha-linolenic (C18:3n3, 16.2 ± 19.3%) and palmitic (C16:0, 10.6 ± 4%). These 4 represent a mean of 92.7% (SD = 52.75)

Two other FAs were found highly represented: the a-linolenic acid (18:3n3, ALA) and the c-linolenic acid (18:3n6, GLA). These five FAs (PA, OLA, LA, ALA and GLA) represented 85.7% of the FAs (SD = 13.1%) among all the species analysed, and for the majority of the species, these five FA’s represented more than 70% of the seed oil content. In our case, the next with higher % were stearic acid (C18:0, 2 ± 0.9%), c-linolenic acid (C18:3n6, 1.1±2.9%).

According to Bretagnolle 2016 the mean frequency of saturated fatty acids (SFA) was 15.6% (SD = 8.8%) and the ratio between unsaturated fatty acids (UFA) and SFA was 7.1 (SD = 4.3). In general, the Asteraceae and Poaceae had low values of the ratio, indicating the synthesis of a high proportion of SFAs, while other families such as the Apiaceae or the Brassicaceae were characterised by a high ratio indicating a tendency to synthesise mostly UFAs. In our case the mean frequency of SFA is 14.7% with SD 5.3%, and the mean ratio between UFA and SFA is 6.8% with SD 2.1%. Ranging from 2.7 in salicacea up to 11.4 in lamiaceae.

### Biological trade-offs

Bretagnolle 2016 found a huge variation of oil content exists in small seeds, although such variation strongly decreases towards low oil values in large seeds (Corroborated in our preliminary results).

## Acknowledgements

We are grateful to the Analysis Service Unit facilities of ICTAN for the analysis of Chromatography.