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Seed dormancy and germination niches of Mediterranean species along an altitudinal gradient in Sardinia

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...A mia madre,
la donna più forte che conosco,
che mi ama più di se stessa e a cui devo tutto

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General Introduction

Seed biology

Seeds are the dispersal and propagation units of the Spermatophyta (seed plants): Gymnosperms (conifers and related clades) and Angiosperms (flowering plants). Spermatophytes have been an important evolutionary success respect to more primitive plants. This evolutionary success is, at least in part, due to the seed habit, being the most complex and evolutionary method of sexual reproduction found in vascular plants. Indeed, the seed plants present an important adaptive advantage, occur in a wide variety of habitats and dominate biological niches both in hot and cold climates (Strasburger, 1992; Leubner, 2008; Baskin and Baskin, 2014).

The seed, containing the embryo as the new plant in miniature, is structurally and physiologically equipped for its role as a dispersal unit and is well provided with food reserves to sustain the growing seedling until it establishes itself as a self-sufficient, autotrophic organism (Bewley, 1997). The embryo is constituted by one cotyledon in monocotyledons and by two cotyledons in almost all dicotyledons and two or more in gymnosperms (Salisbury and Ross, 1994). In typical angiosperm seeds the embryo is derived from the parent plant via double fertilization and is surrounded by two covering layers: 1) the endosperm, a food storage tissue which is rich in oils and proteins (the micropylar endosperm is the part of the endosperm that covers the radicle tip); and 2) testa (seed coat), an outer protective layer of the seed developed from the integuments of the ovule that protect the embryo from mechanical injury and from drying out (Fig. 1); (Leubner, 2008; Müller *et al*., 2006; Baskin and Baskin, 2014).

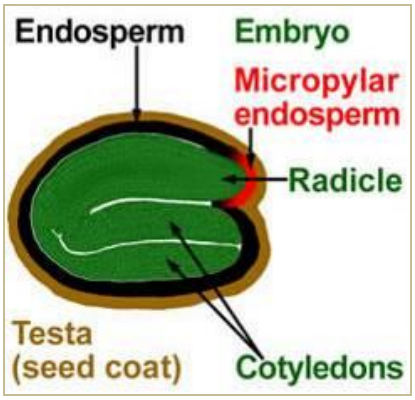

Figure 1 - Drawing of a mature *Arabidopsis thaliana* seed; from Müller *et al*. (2006).

Seed germination

Germination commences with the uptake of water by imbibition of the dry seed, followed by embryo expansion. This usually culminates in rupture of the covering layers and emergence of the radicle, generally considered as the completion of the germination process. Radicle protrusion at the completion of seed germination depends on embryo growth driven by water uptake. Uptake of water by a seed is triphasic, with a rapid initial uptake (phase I, imbibition) followed by a plateau phase (phase II). A further increase in water uptake (phase III) occurs only when germination is completed, as the embryo axis elongates and breaks through its covering structures (Bewley, 1997; Kucera *et al*., 2005; Müller *et al*., 2006; Finch-Savage and Leubner-Metzger, 2006) (see Fig. 2). The emergence of the seedling above the soil surface is the next phase of the plant's growth and it is called seedling establishment (Black *et al*., 2006).

Three fundamental conditions must exist before germination can occur: 1) the embryo must be alive; 2) any dormancy requirements that prevent germination must be overcome; 3)

the proper environmental conditions must exist for germination. Furthermore, seed germination is affected by several external factors. Broadly, there are of two kinds: 1) factors that are found in the field situation; and 2) factors imposed experimentally, generally in the laboratory. Some factors, however, fall within both groups, as for example temperature, light and water availability (Black *et al*., 2006).

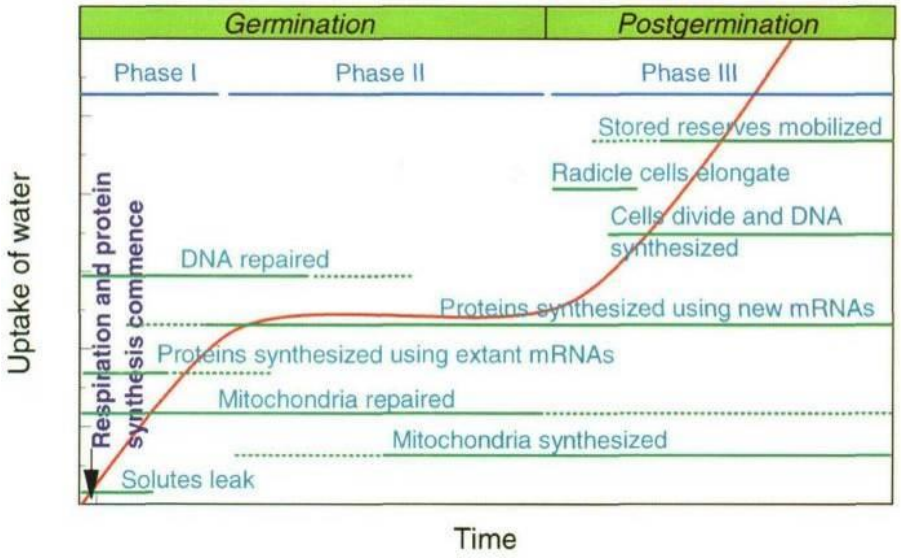

Figure 2 - Time course of major events associated with germination and subsequent postgerminative growth. The time for events to be completed varies from several hours to many weeks, depending on the plant species and the germination conditions; from Bewley (1997).

Germination – Influence of temperature

The most important physical factor that influences seed germination is the temperature, which affects either directly, through action on germination itself, or indirectly, by affecting dormancy and viability (Black *et al*., 2006; Baskin and Baskin, 2014).

Temperatures for germination are typical of each species, but they can vary e.g. according to the variety, the geographical origin of the seeds, the duration of storage, the extent of any dormancy or treatments (e.g. hormones such as gibberellins) applied to the seeds. Therefore, each *taxon* is characterized by a temperature range over which germination is possible, that is related to the climatic and ecological conditions to which species is adapted (Black *et al*., 2006). For example in a series of studies on geographical variation in germination temperature in Europe, P.A. Thompson (cited in Probert, 2000) reported that both minimum and maximum temperatures for germination varied consistently along a north-south gradient; both were lower in Mediterranean species compared with those from northern Europe. Indeed, in the northern Europe, the priority is to avoid germinating during or immediately before the severe winter, thus these species need relatively high temperatures for germination (Fenner and Thompson, 2005).

Constant and alternating temperature regimes often affect seed germination differently (Probert, 2000). In particular, different studies reported an increasing of germination responses of species under the fluctuating temperature regime (e.g. Thompson and Grime, 1983; Schütz and Rave, 1999; Liu *et al*., 2013). The interaction between a requirement for light and for temperature alternation varies between species. Sometimes light can substitute entirely for alternating temperatures, while in other cases the effect of light is merely to reduce the amplitude of alternation necessary to stimulate germination (Fenner and Thompson, 2005).

Germination – Influence of light

Light prevents or delays germination in seeds of many species, acting at different stages of germination process and on incipient radicle emergence (Black *et al*., 2006). In particular, the responses of seeds to light are important for preventing the occurrence of germination in places and at times that are unfavourable to seedling establishment. The ability to detect different aspects of the light environment enables the seed to have at least some control over where and when germination takes place (Fenner and Thompson, 2005). The chances of successful establishment may be determined by whether the germinating seed is buried in the soil or is on the surface. In particular, if it is buried, the precise depth is crucial for emergence; while, if it is on the surface, then the degree of shade (especially from surrounding vegetation) can be decisive. Therefore, if a seed that is lying in darkness below the soil surface germinates, then its shoot may not be able to reach the surface. This hazard is greatest for small seeds, so the ability to detect light (or its absence) is of great survival value Fenner and Thompson, 2005). In addition, seeds of some species are sensitive to the photoperiod, i.e. the relative lengths of the light and dark periods corresponding to day and night (Cumming, 1963), and in some cases, day length plays a part in determining the timing of germination (Densmore, 1997). In all these situations, the ability to detect the intensity, quality or periodicity of the light provides the seed with information it requires about its environment (Fenner and Thompson, 2005).

Germination – Influence of water

The external factor on which germination of most seed types entirely depends is water, necessary for the assumption of the physiology, metabolism and molecular process that drive germination. According to Evans and Etherington (1990), the sensitivity of germination depends on the soil water content of species provenience. Therefore, the availability of water in the soil is critically important and the water status of seeds is central to their survival and their germination (Black *et al*., 2006). A seed may become fully imbibed but remain ungerminated indefinitely if its dormancy-breaking or germination-inducing requirements are not met. Indeed, seeds of many species may persist in the soil for years or decades in a non-dormant state, forming a persistent soil seed bank (Thompson, 2000).

Seeds, depending on their tolerance to drying and low temperatures are commonly classified as orthodox, intermediate and recalcitrant seeds (Roberts, 1973). Specifically, orthodox seeds are desiccation tolerant and are usually highly dehydrated at maturity. Their moisture content is about 10-15% dry weight basis. Owing to their low moisture content, they also tolerate very low temperatures. Most of them tolerate ultra-drying and even freeze-drying. In contrast, recalcitrant seeds remain rich in water at shedding and are intolerant of dehydration. They rapidly die if they lose too much water. The moisture content under which they do not survive varies from 20-50% dry weight basis depending on the species. They must germinate as soon as they fall to the ground or remain on moist soil until they germinate, otherwise they do not survive. They usually do not require external water for germination since their natural water content is sufficient for them to complete germination (Black *et al*., 2006). For all these traits, it is difficult to store recalcitrant seeds due to their tissue complexity and desiccation sensitive (Chandel *et al*., 1995). Finally, the category identified as "intermediate seeds", include seeds that tolerate dehydration better than recalcitrant but worse than orthodox seeds, and seeds that have the same tolerance to dehydration as orthodox seeds but age very fast. Additionally, seeds that tolerate dehydration, but not temperatures
below zero, are considered intermediate. Conservation of orthodox seeds is routinely done by seed desiccation and storage at low temperatures. Long-term conservation procedures of recalcitrant and, often, intermediate seeds envisage cryopreservation (Bacchetta *et al*., 2006; Ballesteros *et al*., 2015).

Seed dormancy

A dormant seed is one that does not have the capacity to germinate in a specified period of time under any combination of normal physical environmental factors (temperature, light/dark, etc.) that otherwise is favourable for its germination (Baskin and Baskin, 2004). Thus, the crucial function of dormancy is to prevent germination when conditions are suitable for germination but the probability to survival and growth of the seedling is low (Fenner and Thompson, 2005). By contrast, a non-dormant seed is one that has the capacity to germinate over the widest range of normal physical environmental factors (temperature, light/dark, etc.) possible for the genotype (Baskin and Baskin, 2004, 2014). The non-dormant seed that does not germinate because of the absence of one or more of these factors is said to be in a state of quiescence. The seed will germinate when the appropriate set of environmental conditions is within its range of requirements for radicle emergence, providing it has not entered secondary dormancy (Baskin and Baskin, 2004).

Nikolaeva (1977) devised a dormancy classification system reflecting the fact that dormancy is determined by both morphological and physiological properties of the seed. Based on this scheme, Baskin and Baskin (2004) have proposed a comprehensive classification system which includes different classes of seed dormancy: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) and combinational (PY + PD). The system of dormancy classification is hierarchical, and consists of five classes, further divided into levels and types.

Physiological dormancy (PD)

Physiological dormancy (PD) is the most prevalent form of seed dormancy and can be triggered by divergent environmental cues (Finch-Savage *et al.*, 2007; Baskin and Baskin 2014). Temperatures are known to be instrumental in the induction of dormancy in seeds that have permeable seed coats and fully developed embryos - i.e., those that potentially have physiological dormancy (Baskin and Baskin, 2014). PD can be divided into three levels: deep, intermediate and non-deep. In addition, Baskin and Baskin (2004) recognized five types of non-deep PD based on patterns of change in physiological responses to temperature. In particular, in PD non-deep Type 1, the temperature range for germination increases during dormancy release as a continuum from low to high, and conversely, from high to low for Type 2 (Fig. 3). Baskin and Baskin (2004) reported that most seeds belong to these two Types of non-deep PD. In Type 3, the temperature for germination increases during dormancy release as a continuum from medium to both high and low temperatures.

Whereas, in PD non-deep Type 4 and Type 5, there is no temperature and dormancy continuum, thus, non-dormant seeds germinate only at high temperature (Type 4) or low temperature (Type 5), (see Fig. 3; Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006).

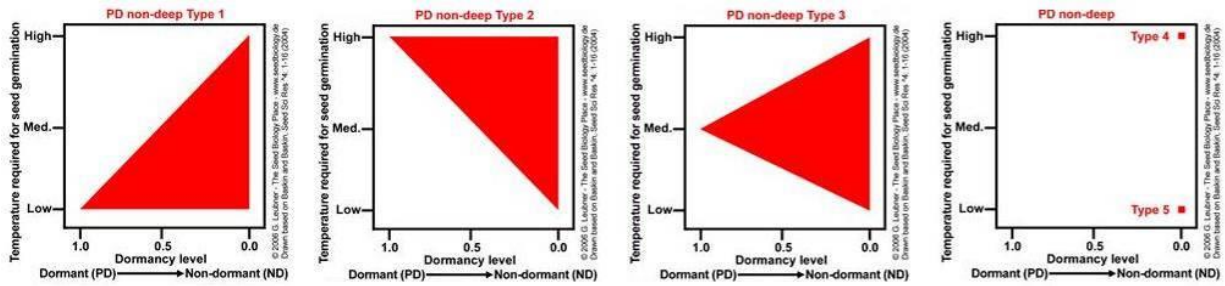

Figure 3 - Types of non-deep physiological dormancy in seeds according to Baskin and Baskin (2004), modified from Leubner, 2008.

Morphological dormancy (MD)

MD is evident in seeds with embryos that are underdeveloped (in terms of size), but differentiated (e.g. into cotyledons and hypocotyl-radical). These embryos are not (physiologically) dormant, but simply need time to grow and then germinate (radical protrusion). This group does not include seeds with undifferentiated embryos. The dormancy period is the time elapsed between incubation of fresh seeds and radical emerges (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006).

Morphophysiological dormancy (MPD)

MPD is present in seeds with underdeveloped (in terms of size) embryos, but in addition they have a physiological component to their dormancy. These seeds therefore require a dormancy-breaking treatment, e.g. a defined combination of warm and/or cold stratification which in some cases can be replaced by GA application. In MPD-seeds embryo growth/emergence requires a considerably longer period of time than in MD-seeds. There are eight known levels of MPD, based on the protocol for seed dormancy break and germination (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006; Baskin and Baskin, 2014).

Physical dormancy (PY)

PY is caused by one or more water-impermeable layers of palisade cells in the seed or fruit coat (Baskin *et al*., 2000, Baskin, 2003, Baskin and Baskin, 2004; Baskin and Baskin, 2014). Seeds will remain dormant until some factor(s) render the covering layer(s) permeable to water. In nature, these factors include high temperatures, widely fluctuating temperatures, fire, drying, freezing/thawing and passage through the digestive tracts of animals. In seed technology, mechanical or chemical scarification can break PY dormancy. Once PY is broken, i.e. the seed or fruit coat becomes permeable to water, the seeds can germinate over a wide range of ambient conditions (Baskin and Baskin, 2014).

Combinational dormancy (PY+PD)

Combinational dormancy, PY+PD is evident in seeds with water-impermeable coats (PY) combined with physiological embryo dormancy (PD non-deep) (Baskin and Baskin, 2004).

Inter-population variation in seed germination along an altitudinal gradient

Altitude is an important factor in germination variation in many species (Holm, 1994; Vera, 1997). In particular, positive correlations between higher altitude sites and higher dormancy levels have usually been reported at broader geographical scales (Holm, 1994; Meyer *et al*., 1995, Cavieres and Arroyo, 2000; Wang *et al*., 2010). At any given time, spatial variation in seed dormancy can be observed also among individuals and populations of the same species (Black *et al*., 2006). For example, Fernández-Pascual *et al*. (2013) reported that *Centaurium somedanum* plants from higher altitudes produce seeds with stronger dormancy, where the increased dormancy with elevation may be explained as an adaptation strategy against the long period of snow cover for high-elevation populations (Meyer *et al*. 1995). These finding are also in agreement with Zhou and Bao (2015) which found that the level of dormancy of the rose achenes increased with increasing elevation, which is an adaptation strategy to the colder and longer winter and prevents premature germination. However, exceptions to this pattern have also been found (Farhadi *et al*., 2013). Therefore, the inter-population variability in seed germination behaviour could reflect local adaptation to particular environments (Andersson and Milberg, 1998; Pérez-García *et al*., 2003; Pérez-García *et al*., 2006).

Seed germination of Mediterranean species

The Mediterranean climate it is characterised by a considerable unpredictability of temperature and precipitation, with hot dry summers and cold wet winters (Joffre *et al*., 1999; Doussi and Thanos, 2002; Valladares and Sáchez-Gómez, 2006; Galmés *et al*., 2007). Under a Mediterranean climate, the plant growth and reproduction must occur in a window of favourable conditions that may vary in length and in which environmental cues and constraints play a central role (Thanos *et al*., 1995; Doussi and Thanos, 2002; Debussche *et al*., 2004; Gresta *et al*., 2010). In particular, the long periods of drought during summer impose severe abiotic stresses that limit plant growth and subsequently compromise their survival (Medrano *et al*., 2009). Therefore, the arid summer in Mediterranean environments represents the most dangerous season for seedling germination and growth (Fenner and Thompson, 2005).

Previous studies reported that in typical Mediterranean coastal species germination occurs a rather narrow range of cool temperatures (typically 5-15°C; Thanos *et al*., 1989, 1995), and a remarkably slow rate (Doussi and Thanos, 2002). Both germination characteristics seem to be associated with autumn/winter seed germination and seedling establishment. This pattern, generally known as "Mediterranean germination syndrome", is considered ecologically advantageous within an unpredictable rainfall regime, during the start of the rainy period under the Mediterranean climate (Thanos *et al*., 1995; Doussi and Thanos, 2002).

On the contrary, in temperate, boreal and arctic climates, seed germination occurs immediately after snowmelt in spring, in order to avoid the low temperatures during seedling establishment in the autumn (Thompson, 2000; Baskin and Baskin, 2003; Fenner and Thompson, 2005; Cochrane *et al*., 2011; Rosbakh and Poschlod, 2014). Exposure to cold temperatures is regarded as the most important mechanism for breaking dormancy in the seeds of most temperate perennials (Probert, 2000; Baskin and Baskin, 2014). While, chilling is not supposed to enhance seed germination in Mediterranean species, and in some cases, cold exposure would have a negative effect on germination (Luna *et al*., 2008). However, the extrapolation of the germination requirements of high mountain Mediterranean plants should be conducted with caution, because it is reported that are generally positively affected by cold-wet stratification treatment but need to germinate quickly after snow melt in the early spring, before the arrival of the dry summer season (Giménez-Benavides *et al*., 2005, 2007; Mattana *et al*., 2012; Porceddu *et al*., 2013).

Thermal time model

Three cardinal temperatures (base, optimum and ceiling) describe the range of temperature over which seeds of a particular species can germinate (Bewley and Black, 1994). The minimum or base temperature (T_b) is the lowest of temperature at which germination can occur, while the optimum temperature (T_o) is the temperature at which germination is most rapid, and the maximum or ceiling temperature (T_c) is the highest temperature at which seeds can germinate.

Many mathematical models have been developed to describe germination patterns in response to temperature (e.g, Garcia-Huidobro *et al*., 1982; Covell *et al*., 1986; Ellis *et al*., 1986; Pritchard and Manger, 1990; Hardegree, 2006). In these models, seeds accumulate units of thermal time (°Cd) to germinate for a percentile g of the population. When seeds are *subjected* to temperatures (T) above a base temperature for germination (T_b), germination rate increases linearly with temperature to an optimum temperature (T_o), above which germination rate starts to decrease (Garcia-Huidobro *et al*., 1982). In the sub-optimal range ($T_o - T_b$), germination occurs in the time t_g, when the thermal time accumulated has reached the critical value (θ_g) for a percentile g of the population.

The germination response of the seeds to constant temperature in the suboptimal range has been modelled using the equation (Garcia-Huidobro *et al*., 1982):

$$1/t_g \text{ (days}^{-1}) = (T_g - T_b) / \theta$$

where $1/t_g$ is the rate of germination of different percentiles of a seed population at sub-optimal constant temperatures, t_g is the time taken for cumulative germination to reach percentile g, T_g is the temperature (°C), T_b is the base temperature for subset g of the population, and θ is the thermal time (°Cd above T_b) required for cumulative germination to achieve percentile g. The use of thermal time to describe the temperature dependence of germination can be extended to supra-optimal temperatures where $1/t_g$ decreases linearly with an increase in temperature above the optimum, T_o, reaching zero at a maximum temperature, T_c (Garcia-Huidobro *et al.*, 1982).

Linear regression has been used to express probit(g) as a function of thermal time (θ_g) and the form of cumulative germination response of seeds has been described by the equation (Covell *et al.*, 1986):

$$\text{probit}(g) = K + \theta_g / \sigma,$$

where K is an intercept constant when θ_g is zero, θ_g may be normal or log-normal distributed (and the best model evaluated on the basis of the r^2 values; Hardegree, 2006), and σ is the standard deviation of the response to θ_g (i.e. the reciprocal of the slope), and represents the sensitivity of the population to θ_g (Covell *et al.*, 1986).

The models have been used to describe and compare thermal responses of species and local populations from varying climates and disparate locations (Garcia-Huidobro *et al.*, 1982) as well as to predict seed germination of non-dormant seeds (and subsequent seedling emergence) in the field describing the seed response to ambient soil conditions (e.g. Steadman *et al.*, 2003; Porceddu *et al*., 2013), and also to assess the impact of different simulated climate change scenarios on seed dormancy release and germination timing (Orrù *et al.*, 2012).

Sardinia

Sardinia (Italy) and its ca. 399 satellite islands and islets is located in the Western part of the Mediterranean Basin and, covering a surface-area of c. 24,090 km^2, is the second largest island after Sicily (Fenu *et al*., 2015) (see Fig. 4). Its geographical isolation and high geological and geomorphological diversity contributed to develop a wide range of habitats and a consequent high rate of endemics (Thompson, 2005), especially on its mountain massifs, where conditions of ecological insularity occur (Médail and Quézel, 1997; Bacchetta *et al*., 2012b). The checklist of the Italian vascular flora (Conti *et al*., 2005; 2007) ascribes 2,494 *taxa* to the Sardinian territory, even if after the latest floristic researches, the number of *taxa* has risen approximately up to 3000 (Bacchetta *et al*., unpublished data). Among them, about the 11.6% are considered as Sardinian endemic vascular plants (Bacchetta *et al*., 2012b; Fenu *et al*., 2014), which include the exclusive Sardinian plant species, the Sardo-Corsican endemics, as well as the endemics in common with the Tuscan Archipelago (Bacchetta *et al*., 2012a; Fenu *et al*. 2014). However, several *taxa* of the Sardinian vascular flora are included in the regional Red Lists of Italy (Conti *et al*., 1997; Rossi *et al*., 2015) and many natural habitats that characterize the Sardinian territory and its important plant areas (IPAs) are still not under protection.

Figure 4 - Mediterranean Basin. The red square shows the Sardinia island.

Species selection

For the species selection, priority was given to endemic species, considering that they are better adapted to the habitats where they grow and thus characterize them; as well as to the *taxa* with a high phytogeographical interest.

Seed collection was carried out from the sea level to the main mountain regions of the Island. Specifically, 26 seed lots of 18 species were collected directly from mother plants and randomly chosen during the time of the natural dispersal. For the species listed in the annexes of the Habitat Directive, as required by the European and national laws (articles 9 and 10 of DPR 357/97 modified by DPR 120/03), seeds were collected after obtaining permits from the "Ministero dell'Ambiente e della Tutela del Territorio e del Mare". For each seed collection, a part of seeds has been used to perform the germination tests, while the remaining seeds were stored at the Sardinian Germplasm Bank (BG-SAR).

BG-SAR is part of the service centre and research Hortus Botanicus Karalitanus (HBK), which main objective is the conservation, study and management of germplasm of Sardinia and the Mediterranean area. To date, BG-SAR preserved approximately 2,500 seed

lots of which ca. 210 belonging to exclusive endemics of Sardinia and ca. 60 listed in the Habitat Directive 92/43/EEC (Porceddu *et al*., 2015).

PhD thesis structure

The PhD thesis is composed of four chapters, each of them it is organised as an indipendent paper.

The first chapter investigates thermal thresholds and the progress of seed germination of Mediterranean species along an altitudinal gradient in Sardinia, with a focus on seed germination responses to the applied pre-treatments (cold and warm stratification and dry after ripening) and hormones (gibberellins). Furthermore, evaluates the possible differences in germination strategies between Mediterranean and temperate species.

In the second chapter thermal requirements for seed dormancy loss and germination of three Mediterranean *Rhamnus* species, which grow on different habitats and climatic conditions, were investigated.

The third chapter evaluates intra-specific differences on thermal thresholds for seed dormancy loss and germination of four Tyrrhenian endemic species along an altitudinal gradient.

Finally, in the fourth chapter, lipid thermal profile and water activity of target *taxa* was studied by Differential Scanner Calorimetry analysis to determine their biophysical and physiological characteristics.

The main objectives of this work are to:
(1) evaluate the effect of temperature variation with altitude on the thermal requirements for dormancy loss and germination of different Mediterranean species, as well as among species belonging to the same genus and also between different populations of the same species;
(2) assess the effect of different pre-treatments (cold and warm stratification and dry after ripening) and hormones (gibberellins) on the thermal requirements for seed dormancy release and germination of the target *taxa*;
(3) evaluate lipid thermal profile of the selected dry seeds and find out if they are correlated with the altitudinal gradient, as well as with the actual climate data of soil temperature and also with the base temperatures for germination;
(4) study the biophysical adaptation of two target species at two different extreme altitudes through their freezing behaviour and water activity.

References

Andersson L., Milberg P., 1998. Variation in seed dormancy among mother plants, populations and year of seed collection. Seed Science Research, 8: 29–38.

Bacchetta G., Fenu G., Mattana E., Piotto B., Virevaire M (Eds), 2006. Manuale per la raccolta, studio, conservazione e gestione ex situ del germoplasma. Agenzia per la Protezione dell'Ambiente e per i servizi Tecnici (APAT), Rome.

Bacchetta G., Farris E., Pontecorvo C., 2012a. A new method to set conservation priorities in biodiversity hot-spots. Plant Biosystems, 146: 638–648.

Bacchetta G., Fenu G., Mattana E., 2012b. A checklist of the exclusive vascular flora of Sardinia with priority rankings for conservation. Anales del Jardín Botánico de Madrid, 69: 81–89.

Ballesteros D., Meloni F., Bacchetta G. (Eds.), 2015. Manual for the propagation of selected Mediterranean native plant species. Ecoplantmed, ENPI, CBC-MED.

Baskin J.M., Baskin C.C., Li X., 2000. Taxonomy, anatomy and evolution of physical dormancy in seeds. Plant Species Biology, 15: 139–152.

Baskin C.C., 2003. Breaking physical dormancy in seeds – focussing on the lens. New Phytologist, 158: 229–232.

Baskin C.C., Baskin J.M., 2003. When breaking seed dormancy is a problem. Try a move-along experiment. Native Plants|Spring, 4: 17–21.

Baskin J.M., Baskin C.C., 2004. A classification system for seed dormancy. Seed Science Research, 14: 1–16.

Baskin C.C., Baskin J.M., 2014. Seeds: ecology, biogeography, and evolution of dormancy and germination. San Diego: Academic Press

Bewley J.D., Black M., 1994. Seeds: Physiology of Development and Germination (New York: Plenum Press).

Bewley J.D., 1997. Seed germination and dormancy. Plant Cell, 9: 1055–1066.

Black M., Bewley J.D., Halmer P., 2006. The encyclopedia of seeds. Science, technology and uses. Wallingford, UK: CABI Publishing.

Cavieres L.A., Arroyo M.T.K. 2000. Seed germination response to cold stratification period and thermal regime in *Phacelia secunda* (Hydrophyllaceae). Plant Ecology, 149: 1–8.

Chandel K.P.S., Chaudhury R., Radhamani J., Malik S.K., 1995. Desiccation and freezing sensitivity in recalcitrant seeds of tea, cocoa and jackfruit. Annals of Botany, 76: 443–450.

Cochrane A., Daws M.I., Hay F.R., 2011. Seed-based approach for identifying flora at risk from climate warming. Austral Ecology, 36: 923–935.

Conti F., Manzi A., Pedrotti F., 1997. Liste Rosse Regionali delle Piante d'Italia. WWF Italia: Società Botanica Italiana, CIAS, Università di Camerino.

Conti F., Abbate G., Alessandrini A., Blasi C. (Eds.), 2005. An annotated checklist of the Italian Vascular Flora. Palombi Editori, Roma, Italia. 420 pp.

Conti F., Alessandrini A., Bacchetta G., Banfi E., Barberis G., Bartolucci F., Bernardo L., Bonacquisti S., Bouvet D., Bovio M., Brusa G., Del Guacchio E., Foggi B., Frattini S., Galasso G., Gallo L., Gangale C., Gottschlich G., Grünanger P., Gubellini L., Iiriti G., Lucarini D., Marchetti D., Moraldo B., Peruzzi L., Poldini L., Prosser F., Raffaelli M., Santangelo A., Scassellati E., Scortegagna S., Selvi F., Soldano A., Tinti D., Ubaldi D., Uzunov D., Vidali M., 2007. Integrazioni alla checklist della flora vascolare italiana. Natura Vicentina, 10: 5–74.

Covell S., Ellis R.H., Roberts E.H., Summerfield R.J., 1986. The influence of temperature on seed germination rate in grain legumes. Journal of Experimental Botany, 37: 705–715.

Cruz A., Pérez B., Velasco A., Moreno J.M., 2003. Variability in seed germination at the interpopulation and intraindividual levels of shrub *Erica australis* in response to fire-related cues. Plant Ecology, 169: 93–103.

Cumming B.G., 1963. The dependence of germination on photoperiod, light quality, and temperature, in *Chenopodium* spp. Canadian Journal of Botany, 41(8): 1211–1233.

Debussche M., Garnier E., Thompson J.D., 2004. Exploring the causes of variation in phenology and morphology in Mediterranean geophytes: A genus-wide study of Cyclamen. Botanical Journal of the Linnean Society, 145: 469–484.

Densmore R.V., 1997. Effect of day length on germination of seeds collected in Alaska. American Journal of Botany, 84: 274–278.

Doussi M.A., Thanos C.A., 2002. Ecophysiology of seed germination in Mediterranean geophytes. 1. *Muscari* spp. Seed Science Research, 12: 193–201.

Ellis R.H., Covell S., Roberts E.H., Summerfield R.J., 1986. The influence of temperature on seed germination rate in grain legumes. II. Intraspecific variation in chickpea (*Cicer arietinum* L.) at constant temperatures. Journal of Experimental Botany, 37: 1503–1515.

Evans C.E., Etherington J.R., 1990. The effect of soil water potential on seed germination of some British plants. New Phytologist, 115: 539–548.

Farhadi M., Tigabu M., Arian A.G., Sharifani M.M., Daneshvar A., Oden P.C., 2013. Pre-sowing treatment for breaking dormancy in *Acer velutinum* Boiss. seed lots. Journal of Forest Research, 24: 273–278.

Fenner M., Thompson K., 2005. The Ecology of Seeds. Cambridge University Press, Cambridge.

Fenu G., Fois M., Cañadas E.M., Bacchetta G., 2014. Using endemic-plant distribution, geology and geomorphology in biogeography: the case of Sardinia (Mediterranean Basin). Systematics and Biodiversity, 12: 181–193.

Fenu G., Fois M., Cogoni D., Porceddu M., Pinna M.S., Cuena Lombraña A., Nebot A., Sulis E., Picciau R., Santo A., Murru V., Orrù M., Bacchetta G., 2015. The Aichi Biodiversity Target 12 at regional level: an achievable goal?. Biodiversity, 16(2-3): 120–135.

Fernández-Pascual E., Jiménez-Alfaro B., Caujapé-Castells J., Jaén-Molina R., Díaz T.E., 2013. A local dormancy cline is related to the seed maturation environment, population genetic composition and climate. Annals of Botany, 112: 937–945.

Finch-Savage W.E., Leubner-Metzger G., 2006. Seed dormancy and the control of germination. New Phytologist, 171: 501–523.

Galmés J., Flexas J., Savé R., Medrano H., 2007. Water relations and stomatal characteristics of Mediterranean plants with different growth forms and leaf habits: responses to water stress and recovery. Plant Soil, 290: 139–155.

Garcia-Huidobro J,, Monteith J.L., Squire G.R., 1982. Time, temperature and germination of pearl millet (*Pennisetum typhoides* S. & H.). Journal of Experimental Botany, 33: 288–296.

Giménez-Benavides L., Escudero A., Pérez-García F., 2005. Seed germination of high mountain Mediterranean species: altitudinal, interpopulation and interannual variability. Ecological Research, 20: 433–444.

Giménez-Benavides L., Escudero A., Iriondo J.M., 2007. Local adaptation enhances seedling recruitment along an altitudinal gradient in a high mountain Mediterranean plant. Annals of Botany, 99: 723–734.

Gresta F., Cristaudo A., Onofri A., Restuccia A., Avola G., 2010. Germination response of four pasture species to temperature, light, and post-harvest period. Plant Biosystems, 144(4): 849–856.

Hardegree S.P., 2006. Predicting germination response to temperature. I. Cardinal-temperature models and subpopulation-specific regression. Annals of Botany, 97: 1115–1125.

Holm S.O., 1994. Reproductive patterns of *Betula pendula* and *B. pubescens* coll. along a regional altitudinal gradient in northern Sweden. Ecography, 17: 60–72.

Joffre R., Rambal S., Damesin C., 1999. Functional attributes in Mediterranean-type ecosystems. In: Pugnaire FI, Valladares F, eds. Handbook of functional plant ecology. NewYork: Marcel Dekker, 347–380.

Kigel J., 1995. Seed germination in arid and semiarid regions. In: Kigel, J., Galili, G., eds. Seed development and germination. Marcel Dekker, New York, pp. 645–699.

Leubner G., 2008. The Seed Biology Place - http://www.seedbiology.de

Liu K., Baskin J.M., Baskin C.C., Bu H., Du G., Ma M., 2013. Effect of diurnal fluctuating versus constant temperatures on germination of 445 species from the Eastern Tibet Plateau. PLoS ONE 8(7): e69364. doi:10.1371/journal.pone.0069364.

Luna B., Pérez B., Céspedes B., Moreno J.M., 2008. Effect of cold exposure on seed germination of 58 plant species comprising several functional groups from a mid-mountain Mediterranean area. Ecoscience, 15(4): 478–484.

Mattana E., Fenu G., Bacchetta G., 2012. Seed production and in situ germination of Lamyropsis microcephala (Asteraceae), a threatened mediterranean mountain species. Arctic, Antarctic, and Alpine Research, 44(3): 343–349.

Médail F., Quézel P., 1997. Hot-spots analysis for conservation of plant biodiversity in the Mediterranean Basin. Annals of the Missouri Botanical Garden, 84: 112–127.

Medrano H., Flexas J., Galmés J., 2009. Variability in water use efficiency at the leaf level among Mediterranean plants with different growth forms. Plant Soil, 317: 17–29.

Meyer S., Kitchen S., Beckstead J., 1995. Seed germination timing patterns in intermountain *Penstemon* (Scrophulariaceae). American Journal of Botany, 82: 377–389.

Müller K., Tintelnot S., Leubner-Metzger G., 2006. Endosperm-limited Brassicaceae Seed Germination: Abscisic Acid Inhibits Embryo-induced Endosperm Weakening of *Lepidium sativum* (cress) and Endosperm Rupture of Cress and *Arabidopsis thaliana*. Plant Cell Physiology, 47(7): 864–877.

Nikolaeva M.G., 1977. Factors controlling the seed dormancy pattern. In: Khan AA, ed. The physiology and biochemistry of seed dormancy and germination. Amsterdam: North-Holland, 51–74.

Orrù M., Mattana E., Pritchard H.W., Bacchetta G., 2012. Thermal thresholds as predictors of seed dormancy release and germination timing: altitude-related risks from climate warming for the wild grapevine *Vitis vinifera* subsp. *sylvestris*. Annals of Botany, 110: 1651–1660.

Pérez-García F., Hornero J., González-Benito M.E., 2003. Interpopulation variation in seed germination of five Mediterranean Labiatae shrubby species. Israel Journal of Plant Sciences, 51: 117–124.

Pérez-García F., Huertas M., Mora E., Peña B., Varela F.; González-Benito M.E., 2006. *Hypericum perforatum* L. seed germination: interpopulation variation and effect of light, temperature, presowing treatments and seed desiccation. Genetic Resources and Crop Evolution, 53: 1187–1198.

Porceddu M., Mattana E., Pritchard H.W., Bacchetta G., 2013. Thermal niche for in situ seed germination in Mediterranean mountain streams: model prediction and validation for *Rhamnus persicifolia* seeds. Annals of Botany, 112(9): 1887–1897.

Porceddu M., Santo A., Picciau R., Murru V., Bacchetta G., 2015. *Ex situ* conservation of plant diversity in the Mediterranean islands: the role of the Sardinian Germplasm Bank (BG-SAR). In: Mariotti M. & Magrini S. (eds.), Conservation of threatened

species: activities and collaborations within the network, Edition: 1, Chapter: 6, Publisher RIBES, Editors: Mauro Mariotti, Sara Magrini, pp. 27–30.
Pritchard H.W., Manger K.R., 1990. Quantal response of fruit and seed germination rate in *Quercus robur* L. and *Castanea sativa* Mill., to constant temperatures and photon dose. Journal of Experimental Botany, 41: 1549–1557.
Probert R.J., 2000. The role of temperature in the regulation of seed dormancy and germination. In: Fenner M., ed. Seeds: the ecology of regeneration in plant communities. Wallingford, UK: CAB International, pp. 261–292.
Roberts E.H., 1973. Predicting the storage life of seeds. Seed Science Technology 1: 499–514.
Rosbakh S., Poschlod P., 2014. Initial temperature of seed germination as related to species occurrence along a temperature gradient. Functional Ecology, doi: 10.1111/1365-2435.12304.
Rossi G., Orsenigo S., Montagnani C., Fenu G., Gargano D., Peruzzi L., Wagensommer R.P., Foggi B., Bacchetta G., Domina G., Conti F., Bartolucci F., Gennai M., Ravera S., Cogoni A., Magrini S., Gentili R., Castello M., Blasi C., Abeli T., 2015. Is legal protection sufficient to ensure plant conservation? The Italian Red List of policy species as a case study. Oryx, doi: 10.1017/S003060531500006X.
Salisbury F.B., Ross C.W., 1994. Fisiologia vegetale. Zanichelli, Bologna.
Schütz W., Rave G., 1999. The effect of cold stratification and light on the seed germination of temperate sedges (Carex) from various habitats and implications for regenerative strategies. Plant Ecology, 144: 215–230.
Steadman K.J., Bignell G.P., Ellery A.J., 2003. Field assessment of thermal after-ripening time for dormancy release prediction in *Lolium rigidum* seeds. Weed Research, 43: 458–465.
Strasburger E., 1992. Trattato di botanica: Sistematica e Geobotanica. Vol. 2. Antonio Delfino Editore, Roma.
Thanos C.A., Georghiou K., Skarou F., 1989. Glaucium flavum seed germination – an ecophysiological approach. Annals of Botany, 63: 121–130.
Thanos C.A., Georghiou K., Dimitra J.D., Marangaki C.J., 1991. Photoinhibition of seed germination in Mediterranean maritime plants. Annals of Botany, 68: 469–475.
Thanos C.A., Costas C.K., Skarou F., 1995. Ecophysiology of germination in the aromatic plants thyme, savory and oregano (Labiatae). Seed Science Research, 5: 161–170.
Thompson J.D., 2005. Plant evolution in the Mediterranean. Oxford, UK: Oxford University Press.
Thompson K., Grime J.P., 1983. A comparative study of germination responses to diurnally fluctuating temperatures. Journal of Applied Ecology, 20: 141–156.
Thompson K., 2000. The functional ecology of soil seed banks. In: Fenner M., ed. Seeds: The ecology of regeneration in plant communities. Wallingford, UK: CAB International, pp. 215–235.
Valladares F., Sánchez-Gómez D., 2006. Ecophysiological traits associated with drought in Mediterranean tree seedlings: individual responses versus interspecific trends in eleven species. Plant Biology, 8: 688–697.
Vera M.L., 1997. Effects of altitude and seed size on germination and seedling survival of heathland plants in north Spain. Plant Ecology, 133: 101–106.
Wang J.H., Baskin C.C., Du Chen W.G.Z., 2010. Variation in seed germination between populations of five sub-alpine woody species from eastern Qinghai-Tibet Plateau following dry storage at low temperatures. Ecological Research, 25: 195–203.
Zhou Z., Bao W., 2014. Changes in seed dormancy of *Rosa multibracteata* Hemsl. & E. H. Wilson with increasing elevation in an arid valley in the eastern Tibetan Plateau. Ecological Research, 29: 693–700.

Chapter 1: Thermal thresholds for seed germination of Mediterranean species along an altitudinal gradient

Introduction

Germination is the process that commences with the uptake of water by the quiescent dry seed, continues with the elongation of the embryonic axis and is completed when a part of the embryo, usually the radicle, extends to penetrate the structures that surround it (Bewley and Black, 1994; Bewley, 1997). With the progress of seed germination, the process becomes irreversible such that a seedling must either establish or die. Therefore, in order to increase survival chances of the seedling, time of seed germination should match environmental conditions favourable for further plant establishment (Grubb, 1977; Fenner and Thompson 2005; Poschlod *et al*., 2013).

Dormancy is defined as a physiological state in which a seed disposed to germinate does not, even in the presence of favorable environmental conditions (Bonner, 1984). Dormancy breaking and germination requirements are specific for each species and depend on phylogeny, distribution range and habitat (Baskin and Baskin, 2004, 2014; Finch-Savage and Leubner Metzger, 2006). Even closely related species, either growing in a variety of habitats (e.g. Vandelook *et al.*, 2008) or co-occurring in a given habitat, may differ in germination response to environmental signals (e.g. Daws *et al*., 2002; Karlsson *et al*., 2008). Baskin and Baskin (2004) have proposed a comprehensive classification system which includes five classes of seed dormancy: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) and combinational (PY + PD). PD is the most prevalent form of seed dormancy and can be triggered by divergent environmental cues (Finch-Savage *et al.*, 2007; Baskin and Baskin, 2014). In many species cold stratification or pre-chilling (C), followed by favourable temperatures, represents a natural mechanism to release PD (Baker, 1989; Probert, 1990; Vera, 1997). Also the application of warm stratification or pre-warming (W), the plant hormone gibberellic acid (GA_3) as well as dry after-ripening treatment (DAR) are a common treatments used to relieve this type of dormancy (Baskin and Baskin, 2004, 2014; Finch-Savage and Leubner-Metzger, 2006; Finch-Savage *et al.*, 2007). Cold / warm stratification or their combination (which in some cases can be replaced by GA) may be required to break MPD. Dormancy-break in seeds with PY may occur following different methods, i.e., mechanical or chemical scarification (Baskin and Baskin, 2004, 2014).

The most crucial environmental factor for the failure of seedling emergence / establishment is temperature stress; consequently, seed germination should be triggered by favourable temperature conditions for the further development of the seedling (Rosbakh and Poschlod, 2014). Furthermore, temperature is the major environmental factor responsible for changes in dormancy states of seeds (Baskin and Baskin, 2014).

Three cardinal temperatures (base, optimum and ceiling) describe the range of temperature over which seeds of a particular species can germinate (Bewley and Black, 1994). The minimum or base temperature (T_b) is the lowest of temperature at which germination can occur, the optimum temperature (T_o) is the temperature at which germination is most rapid, while the maximum or ceiling temperature (T_c) is the highest temperature at which seeds can germinate. Therefore, each *taxon* is characterized by a temperature range over which germination is possible, which is related to the climatic and ecological conditions to which species is adapted (Black *et al*., 2006).

In the temperate climate, the priority is to avoid germination during or immediately before a severe winter, and therefore seeds are 'programmed' to germinate at relatively high temperatures immediately after snowmelt in spring (Baskin and Baskin, 2003; Fenner and Thompson, 2005). However, in the Mediterranean regions, by far the least dangerous season for seedlings is the damp, cool but mostly frost-free winter. For that reason, chilling is not

supposed to enhance seed germination in Mediterranean species, even may have a detrimental effect on seed germination as reported by Skordilis and Thanos (1995) for seeds of *Pinus halepensis*, for which long term chilling led to gradual loss of viability. Therefore, under a Mediterranean climate, characterised by a high seasonality with hot dry summers and cold wet winters (Joffre *et al*., 1999; Valladares and Sáchez-Gómez, 2006; Galmés *et al*., 2007), plant growth and reproduction must occur in a window of favourable conditions that may vary in length and in which environmental cues and constraints play a central role (Thanos *et al*., 1995; Doussi and Thanos, 2002; Debussche *et al*., 2004; Gresta *et al*., 2010). A "Mediterranean germination syndrome" has been identified for typical Mediterranean coastal species (Thanos *et al*., 1991; Skordilis and Thanos, 1995; Thanos *et al*., 1995; Doussi and Thanos, 2002). This seed germination pattern is characterized by low optimal germination temperatures (5-15°C, according to Thanos *et al*., 1989) and slow germination rate (Doussi and Thanos, 2002). This device is considered an advantageous ecological adaptation of species under the Mediterranean climate, as it ensures that germination occurs well into the wet season, in late autumn, and maximises the length of the growing season before the onset of summer drought (Doussi and Thanos, 2002). Therefore, in this way seed germination and subsequent seedling establishment are secured against the dry spells frequently encountered during the early phase of the rainy period in the Mediterranean climate (Thanos *et al*., 1989, 1995).

Water shortage is a key factor controlling plant performance in high Mediterranean mountains (Cavieres *et al*., 2006; Michalet, 2006). Mediterranean mountain specialists cope with both very short growth season, typical of all mountain environments, and a severe water shortage in summer. Therefore mountain habitats are considered to be extremely stressful for plant life (Giménez-Benavides *et al.*, 2007). Giménez-Benavides *et al*. (2005) detected that most of the 20 species from high altitude Mediterranean climates, readily germinate without treatment, reaching an optimum at relatively high temperatures in contrast to lowland Mediterranean species. Furthermore, cold-wet stratification increased germination capacity in several dormant species, as widely reported for many arctic, boreal and alpine species (Baskin and Baskin, 2014). Thus, high mountain Mediterranean species do not differ from alpine species except that a relatively high number of species are ready to germinate without any treatment (Giménez-Benavides *et al.*, 2005). As for many temperate and alpine species, a spring germination was detected also for two endemic mountain species of Sardinia, *Lamyropsis microcephala* (Mattana *et al*., 2012) and *Rhamnus persicifolia* (Porceddu *et al*., 2013).

Recently, Rosbakh and Poschlod (2014) highlighted to what extent germination requirements of species with different distributional ranges are related to macroclimatic habitat characteristics in a temperate climate zone. Similarly, Fernández-Pascual *et al*. (2013) have shown that dormancy variations are influenced by both a climatic gradient and the seed maturation environment. However, there is no data available to date on thermal threshold for seed germination that grow along an altitudinal gradient in the Mediterranean Basin.

The aims of this work were to (1) evaluate the effect of temperature variation with altitude on the thermal requirements for dormancy loss and germination of Mediterranean species; (2) assess the effect of different treatments (GA_3, C, W, DAR) on the thermal requirements for dormancy loss and germination of Mediterranean species along an altitudinal gradient; and finally (3) characterize quantitatively and model the "Mediterranean germination syndrome".

Materials and methods

Study areas and species

This study was carried out in Sardinia, the second largest island in the Mediterranean Sea (ca. 24,090 km^2), along an altitudinal gradient, from sea level up to 1834 m a.s.l. in the highest mountain region (Gennargentu Massif, CE-Sardinia) (Table 1). The experimental sites have been chosen in order to have the entire representativeness in geological substrata (Carmignani *et al*., 2001), bioclimatic conditions (Bacchetta *et al*., 2009) and a coherence with the biogeographic sectors (Fenu *et al*., 2014). Taking in consideration this premise, species were chosen that belong to the most representative families in the Mediterranean area. In addition, priority was given to: (1) Sardinian endemics (Sardinian biogeographic subprovince and meso-hotspot); (2) the Sardinian, the Corsican and the Tuscan Archipelago endemics (Italo-Tyrrhenian province); and (3) Tyrrhenian insular endemics (Italo-Tyrrhenian superprovince and macro-hotspot) (Rivas-Martínez *et al*., 2004, Rivas-Martínez, 2007; Cañadas *et al*., 2014).

Seed collecting and soil temperature recording

Seeds of 26 provenances of 18 species were collected (Table 1). Several collecting trips were carried out each year from late spring (May) to the autumn (October), during the period 2012-2013 and seeds were collected directly from the plants at the time of natural dispersal (see Table 1). For the species listed in the annexes of the Habitat Directive, as required by the European and national laws (articles 9 and 10 of DPR 357/97 modified by DPR 120/03), seeds were collected after obtaining permits from the "Ministero dell'Ambiente e della Tutela del Territorio e del Mare".

To study and monitor the annual trend of soil temperature, 24 data-loggers (TidbiT® v2 Temp logger, Onset Computer Corporation, Cape Cod, MA, U.S.A.) were buried at a depth of 2–3 cm, at different altitudes (from ca. 25 to ca. 1800 m a.s.l., see Table 1 and 2) and times over two years (Table 2). The loggers recorded the soil temperature at 90-min intervals and most of them covered at least two winter and summer seasons (Table 2).

Table 1 - Seed lots details and information on the species collected. *Sources: Bacchetta, 2006; Bacchetta *et al*., 2012; Fenu *et al*., 2014. Abbreviations on the endemic species distribution: SA = Sardinia; CO = Corsica; BL = Balearic Islands; GA = France; H = Hyères Islands; AT = Tuscan Archipelago. Species were collected close to the data loggers (see Table 2).

N°	*Taxon*	Family	Distribution*	Locality	Coordinates (WGS84)	Altitude (m a.s.l.)	Collection date	mc (% ± S.D.)
1	*Brassica tournefortii* Gouan	Brassicaceae	S-Medit	Poetto - Cagliari (CA)	N 39°12' E 9°10'	5	07/05/2012	11.0 ± 2.7
2	*Clematis vitalba* L.	Ranunculaceae	Europ-Caucas.	Monte Padenteddu - Pula (CA)	N 39°02' E 8°54'	760	12/10/2012	9.0 ± 0.7
3	*Dianthus morisianus* Vals.	Caryophyllaceae	Endem. SA	Portixeddu - Buggerru (CI)	N 39°26' E 8°26'	65	24/07/2012	10.5 ± 0.3
4	*Digitalis purpurea* L. var. *gyspergerae* (Rouy) Fiori	Plantaginaceae	Endem. SA-CO	Is Cioffus - Capoterra (CA)	N 39°06' E 8°57'	360	26/06/2012	9.8 ± 3.5
5	*Digitalis purpurea* L. var. *gyspergerae* (Rouy) Fiori	Scrophulariaceae	Endem. SA-CO	Monte Lattias - Uta (CA)	N 39°08' E 8°50'	904	23/07/2012	ND
6	*Digitalis purpurea* L. var. *gyspergerae* (Rouy) Fiori	Plantaginaceae	Endem. SA-CO	Bruncu Spina - Desulo (NU)	N 40°00' E 9°18'	1800	30/08/2012	ND
7	*Helianthemum caput-felis* Boiss.	Cistaceae	SW-Medit.	Sa Mesa Longa - S. V. Milis (OR)	N 40°02' E 8°23'	38	29/07/2012	9.3 ± 1.6
8	*Helichrysum microphyllum* ssp. *tyrrhenicum* Bacch., Brullo *et* Giusso	Asteraceae	Endem. SA-CO-BL	Monte Albo - Lula (NU)	N 40°28' E 9°30'	610	16/07/2013	ND
9	*Lamyropsis microcephala* (Moris) Dittrich *et* Greuter	Asteraceae	Endem. SA	Bruncu Spina - Desulo (NU)	N 40°01' E 9°17'	1757	28/08/2012	7.1 ± 0.6
10	*Lupinus luteus* L.	Fabaceae	W-Medit.	Buggerru (CI)	N 39°26' E 8°23'	103	29/05/2012	8.1 ± 0.2
11	*Nepeta foliosa* Moris	Lamiaceae	Endem. SA	Prados - Oliena (NU)	N 40°15' E 9°25'	1146	07/08/2012	8.1 ± 1.1
12	*Ptilostemon casabonae* (L.) Greuter	Asteraceae	Endem. SA-CO-H-AT	Miniera Luigi - Buggerru (CI)	N 39°22' E 8°25'	135	11/07/2013	5.5 ± 0.1
13	*Ptilostemon casabonae* (L.) Greuter	Asteraceae	Endem. SA-CO-H-AT	Mitzaorxia - Laconi (OR)	N 39°52' E 9°04'	686	22/07/2013	5.5 ± 0.1
14	*Ptilostemon casabonae* (L.) Greuter	Asteraceae	Endem. SA-CO-H-AT	Is Terr'e Molentes - Fonni (NU)	N 40°03' E 9°19'	1300	23/08/2013	5.9 ± 0.0
15	*Rhamnus alaternus* L. ssp. *alaternus*	Rhamnaceae	Circum-Medit.	S. Barbara - Capoterra (CA)	N 39°08' E 8°56'	505	12/07/2013	ND
16	*Rhamnus lycioides* L. ssp. *oleoides* (L.) J. & Maire	Rhamnaceae	S-Medit.	Perdu Collu - Pula (CA)	N 39°00' E 8°56'	60	10/08/2012	6.2 ± 0.0
17	*Ruta lamarmorae* Bacch., Brullo *et* Giusso	Rutaceae	Endem. SA	Bruncu Spina - Desulo (NU)	N 40°01' E 9°17'	1675	19/09/2012	9.6 ± 0.1
18	*Santolina insularis* (Gennari *ex* Fiori) Arrigoni	Asteraceae	Endem. SA	Miniera Luigi - Buggerru (CI)	N 39°22' E 8°25'	147	11/07/2013	7.6 ± 0.7
19	*Santolina insularis* (Gennari *ex* Fiori) Arrigoni	Asteraceae	Endem. SA	Sugalaffricu - Laconi (OR)	N 39°52' E 9°00'	500	21/06/2013	6.1 ± 1.7
20	*Santolina insularis* (Gennari *ex* Fiori) Arrigoni	Asteraceae	Endem. SA	Separadorgiu - Fonni (NU)	N 40°02' E 9°17'	1531	12/09/2013	8.4 ± 0.2
21	*Scrophularia ramosissima* Loisel.	Scrophulariaceae	Endem. SA-CO-BL-GA	Is Arenas - Arbus (VS)	N 39°31' E 8°25'	25	19/07/2012	9.0 ± 5.1
22	*Scrophularia trifoliata* L.	Scrophulariaceae	Endem. SA-CO-AT	Miniera Luigi - Buggerru (CI)	N 39°22' E 8°25'	217	11/07/2012	8.8 ± 1.2
23	*Scrophularia trifoliata* L.	Scrophulariaceae	Endem. SA-CO-AT	Laconi (OR)	N 39°51' E 9°03'	510	24/07/2013	ND
24	*Scrophularia trifoliata* L.	Scrophulariaceae	Endem. SA-CO-AT	Su Thuttureli - Oliena (NU)	N 40°14' E 9°25'	1238	17/07/2013	8.4 ± 4.1
25	*Silene succulenta* Forssk. ssp. *corsica* (DC.) Nyman	Caryophyllaceae	Endem. SA-CO	Foce Coghinas - Badesi (OT)	N 40°56' E 8°48'	5	02/06/2012	10.4 ± 1.3
26	*Verbascum plantagineum* Moris	Scrophulariaceae	Endem. SA	Monte Nieddu - Pula (CA)	N 39°09' E 8°54'	200	20/06/2012	15.8 ± 2.2

Table 2 - Site information and data-loggers for soil temperature measurement details. Abbreviations on the province: VS = Medio Campidano; OR = Oristano; CI = Carbonia-Iglesias; NU = Nuoro; OG= Ogliastra.

Locality	Altitude (m a.s.l.)	Coordinates (WGS84)	Start of the measurement	End of the measurement	Days of the measurement
Is Arenas - Arbus (VS)	25	N 39°31' E 8°25'	14/02/2011	09/06/2013	846
Sa Mesa Longa – S. V. Milis (OR)	38	N 40°02' E 8°23'	18/06/2012	27/12/2012	192
Buggerru - Portixeddu (CI)	63	N 39°26' E 8°26'	23/01/2011	24/07/2012	548
Rio Siddo - Ghilarza (OR)	128	N 40°08' E 8°50'	17/06/2012	24/09/2014	828
Domusnovas Canales - Norbello (OR)	227	N 40°08' E 8°52'	17/06/2012	22/03/2013	278
Su Costarbu - Abbasanta (OR)	357	N 40°09' E 8°46'	17/06/2012	23/09/2014	828
Su Monte 'e su Cavalleri - Abbasanta (OR)	430	N 40°08' E 8°43'	17/06/2012	23/09/2014	828
Genna Ferracesus - Gonnosfanadiga (VS)	569	N 39°27' E 8°39'	17/05/2012	29/04/2013	346
Perda Pibera - Gonnosfanadiga (VS)	700	N 39°26' E 8°39'	20/08/2012	30/09/2014	771
Iscala 'e Prados - Oliena (NU)	700	N 40°15' E 9°24'	08/08/2012	11/10/2014	794
Perda Pibera - Gonnosfanadiga (VS)	818	N 39°26' E 8°39'	17/05/2012	30/09/2014	866
Rio Olai - Orgosolo (NU)	945	N 40°08' E 9°21'	22/04/2011	12/09/2013	874
Iscala 'e Prados - Oliena (NU)	1040	N 40°15' E 9°25'	08/08/2012	11/10/2014	794
Dolina di Prados - Oliena (NU)	1146	N 40°15' E 9°25'	09/04/2009	11/10/2014	2011*
Rio Correboi - Villagrande (OG)	1200	N 40°04' E 9°20'	22/04/2011	04/07/2013	804
M. Novo S. Giovanni - Orgosolo (NU)	1255	N 40°07' E 9°24'	15/05/2011	30/08/2012	473
Rio Correboi - Villagrande (OG)	1267	N 40°03' E 9°20'	22/04/2011	04/07/2013	804
Monte Spada - Fonni (NU)	1340	N 40°04' E 9°16'	22/04/2011	30/08/2012	496
Rio Correboi - Villagrande (OG)	1344	N 40°03' E 9°20'	22/04/2011	04/07/2013	804
Palumbrosa - Oliena (NU)	1361	N 40°14' E 9°25'	04/10/2010	17/07/2013	1017
Punta Corrasi - Oliena (NU)	1412	N 40°14' E 9°25'	05/08/2011	11/10/2014	1163
Bae e Laccos - Fonni (NU)	1520	N 40°00' E 9°19'	26/08/2010	25/08/2011	364
Rio Aratu - Desulo (NU)	1665	N 40°01' E 9°17'	26/08/2010	20/08/2013	1090
Punta Bruncu Spina - Desulo (NU)	1810	N 40°00' E 9°18'	02/09/2012	11/10/2014	769

*Data deficient from 19/08/2011 to 08/08/2012

Germination experiments

According to seed availability, four replicates of 30 seeds each or three replicates of 20 seeds each, were sown on the surface of 1% agar water in 60 or 90 mm diameter plastic Petri dishes for small or large seeds, respectively.

Seeds were incubated in the light (12 h light ⁄ 12 h dark) at a range of constant germination temperatures (5, 10, 15, 20 and 25°C) and under an alternating temperature regime (25/10°C) for a maximum of four months. In addition, different pre-treatments were also applied:
1) pre-chilling ("C", seeds were incubated for three months at 5°C in 1% agar water in the darkness) ; 2) pre-warming ("W", seeds were stratified for three months at 25°C in 1% agar water); and 3) dry after ripening ("DAR", seeds were stored for three months at 25°C inside a sealed glass container with silica gel in a ratio seed / silica gel 1:1). At the end of each pre-treatment, seeds were incubated at the above listed temperatures (Table 3). During stratifications (i.e., C and W) seeds were incubated in continuous dark achieved by wrapping dishes in aluminium foil and then incubated to the light condition at the germination temperatures specified above. Extra replicates of 30 or 20 seeds each were sown on the surface of 1% agar water with 250 mg l^{-1} of GA_3 and incubated in the light (12 h light ⁄ 12 h dark), at the previously cited temperatures (Table 3).

Furthermore, seeds of species for which a physical dormancy (PY) was reported, i.e., *Helianthemum caput-felis* and *Lupinus luteus* (Royal Botanic Gardens Kew, 2014), were scarified by chipping with a scalpel, before starting all germination experiments.

Table 3 - Information on the tested species. 0 = Control, C = cold stratification (three months at 5°C in agar), W = warm stratification (three months at 25°C in agar); DAR = dry after ripening (three months at 25°C on silica gel); GA_3 = 250 mg L^{-1} of gibberellic acid on agar.

Taxon	**Germination experiments**				
	0	C	W	DAR	GA_3
Brassica tournefortii Gouan	X	X	X	X	X
Clematis vitalba L.	X	X	X	X	X
Dianthus morisianus Vals.	X	X	X	X	
Digitalis purpurea L. var. *gyspergerae* (Rouy) Fiori (3 seedlots)	X	X	X	X	X
Helianthemum caput-felis Boiss.	X			X	
Helichrysum microphyllum ssp. *tyrrhenicum* Bacch., Brullo *et* Giusso	X	X	X	X	X
Lamyropsis microcephala (Moris) Dittrich *et* Greuter	X	X		X	
Lupinus luteus L.	X			X	
Nepeta foliosa Moris	X	X	X	X	X
Ptilostemon casabonae (L.) Greuter (3 seedlots)	X	X	X	X	X
Rhamnus alaternus L. ssp. *alaternus*	X	X			
Rhamnus lycioides L. ssp. *oleoides* (L.) J. *et* Maire	X	X	X	X	X
Ruta lamarmorae Bacch., Brullo *et* Giusso	X	X	X	X	X
Santolina insularis (Gennari *ex* Fiori) Arrigoni (3 seedlots)	X	X	X	X	X
Scrophularia ramosissima Loisel.	X	X	X	X	X
Scrophularia trifoliata L. (3 seedlots)	X	X	X	X	X
Silene succulenta Forssk. ssp. *corsica* (DC.) Nyman	X	X	X	X	
Verbascum plantagineum Moris	X	X	X	X	X

Germination, defined as visible radicle emergence (> 1 mm), was recorded three times a week, except for dark-incubated seeds that were only scored once, at the end of the stratifications, to avoid any exposure to light. At the end of the germination tests, when no additional germination had occurred for two weeks, after a minimum of one month from sowing, a cut-test was carried out to determine the viability of the remaining seeds and the number of empty seeds in particular. Firm seeds were considered to be viable.

Data analysis

Soil temperatures were analysed for winter and summer seasons. Winter period was considered from the 21st of December to the 20th of March while summer ran from the 21st of June to the 21st of September. The minimum daily temperature, the mean daily temperature and the duration of the winter period (i.e., number of days with mean daily temperatures ≤ 5°C) were calculated for the winter season. The maximum daily temperature, the mean daily temperature and the duration of the summer period (i.e., days with mean daily temperatures ≥ 25°C) were calculated for the summer season.

Final germination percentages were calculated on the basis of the total number of filled seeds as the mean of the four (or three) replicates ± standard deviation (S.D.) for each tested condition. Seed germination during pre-treatments (i.e., cold and warm stratifications) was also recorded and when seeds germinated during pre-treatments before moving to the incubation temperatures, these where not considered in the final germination percentages which were calculated on the basis of the remaining filled seeds after pre-treatments.

Theoretical base temperature for germination (T_b) at which the germination rate is equal to zero (Ellis *et al*., 1986) was evaluated for each seed lot. T_b was calculated by determining the seed germination rate, defined as the reciprocal of time to reach 50% of actual germination for the tests carried out at constant temperatures (5–25°C). Subsequently, data were regressed using a linear model, by averaging the *x*-intercept for the suboptimal temperature range. Furthermore, when 50% of final germination was not reached, the T_b value was estimated *sensu* Trudgill *et al*. (2000), reporting the lowest incubation temperature at which seed germination was recorded.

The thermal constant (*S*) expressed in degree days (ºCd), i.e. the thermal requirement to achieve 50% of germination, given by the reciprocal of the slope of the linear regression (Garcia-Huidobro *et al*., 1982; Trudgill *et al*., 2000), was also calculated for each seed lot after each pre-treatment. Regression analyses were carried out using SigmaPlot Version 11.0 (Systat Software, Inc., San Jose California USA).

In order to improve the dataset, data on T_b and *S* of other species were also taken from the literature, i.e. *Centranthus amazonum* Fridl. & A. Raynal, *Centranthus ruber* (L.) DC. subsp. *ruber* and *Clinopodium sandalioticum* (Bacch. & Brullo) Bacch. & Brullo *ex* Peruzzi & Conti. In addition, the dataset of Trudgill *et al*. (2000) was employed in order to compare the results of this study for Mediterranean species with those achieved for some temperate species.

Statistical analysis

Effect of pre-treatments, temperatures and their combinations on germination percentages were determined by fitting Generalized Linear Models (GLMs), with a logit link function and a quasi-binomial error structure, using *F*-tests with an empirical scale parameter to overcome residual overdispersion (Crawley, 2007). GLMs were also used for analysing the effect of base temperatures and the thermal constant for 50% of germination (as above detailed), the three groups identified (i.e., Mediterranean lowland, Mediterranean high mountain and temperate species), followed by a *post-hoc* pairwise comparisons *t*-test (with Bonferroni adjustment). A two-sample Student's *t*-tests were used to test whether the means of two

normally distributed populations were equal (McDonald, 2008). Furthermore, the Wilcoxon rank-sum test which is based solely on the order in which the observations from the two samples fall was applied. All the analyses were carried out using R v. 2.14.1 (R Development Core Team, 2011).

Results

Climatic data

Soil temperatures data highlighted that in winter the minimum daily temperatures decreased with altitudes from approx. 4°C (ca. 200 m a.s.l.) to approx. -1°C (ca. 1800 m a.s.l.) and this pattern could be modelled by a linear correlation (Fig. 1a). The mean daily temperatures followed the same pattern decreasing from approximately 12°C (ca. 200 m a.s.l.) to about 0°C (ca. 1800 m a.s.l.; Fig. 1b). The winter period increased linearly with altitude from 0 to around 25 days at ca. 800 m a.s.l. (Fig. 1c). At altitudes > 800 m a.s.l., the period increased faster as highlighted by a three times higher slope of the regression line than at lower altitudes, reaching a duration of 147 days at ca. 1800 m a.s.l. (Fig. 1c), and this model explained a higher proportion of the variance (higher r^2 values) than when the correlation was modelled by a continous line. A winter period of 90 days, corresponding to the duration of the cold stratification at 5°C (C) in the lab, was reached at altitudes ≥ 1146 m a.s.l. (Fig. 1c). According to this threshold, it was possibile to distinguish between "Mediterranean Lowland" (ML) up to altitudes of 1146 m a.s.l., and "Mediterranean Mountain" (MM) at altitudes ≥ 1146 m a.s.l. (Fig. 1c).

The maximum daily temperatures in summer ranged from 19°C (ca. 1350 m a.s.l.) to 60°C (ca. 200 m a.s.l.; Fig. 2a), without a significant correlation with altitude ($P > 0.05$; Fig. 2a). The mean daily temperatures decreased with altitude from 31°C (0 m a.s.l) to 16°C (ca. 1350 m a.s.l.; Fig. 2b) as well as the duration of the summer period from 114 (0 m a.s.l.) to 0 days (ca. 1800 m a.s.l.; Fig. 2c). A summer period of 90 days, corresponding to the duration of the warm stratification (W) and dry after ripening (DAR) at 25°C in the lab, was reached at altitudes ≤ 228 m a.s.l (Fig. 2c). Almost all the data-loggers were at altitudes higher than these thresholds with only five recordings at lower altitudes (Fig. 2c).

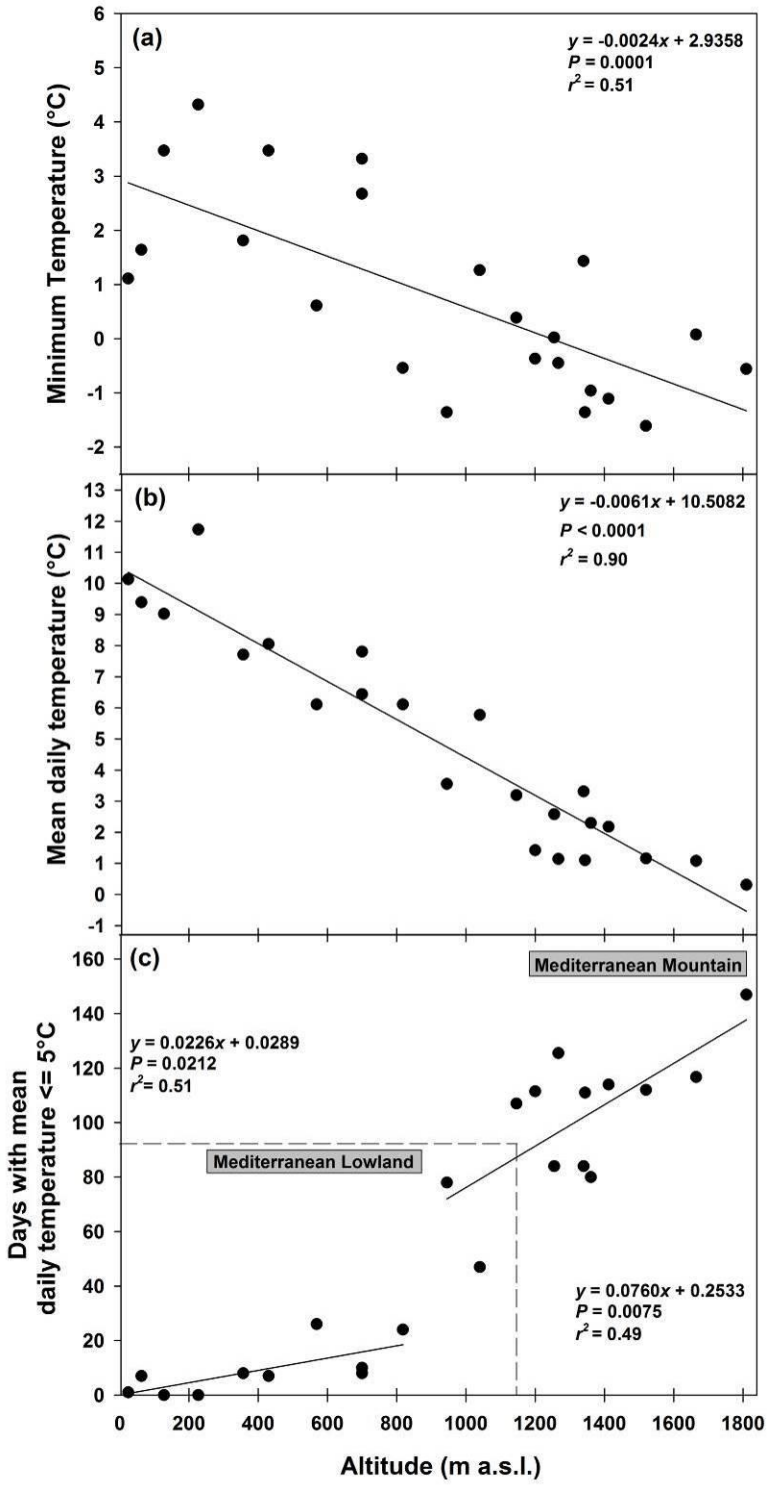

Figure 1 - Soil temperatures recorded in winter by 24 data-loggers at different altitudes. Fitted lines parameters are shown in each plot. In (c) the dashed lines highlight the value of 90 days which correspond to the cold stratification (C; 5°C for three months) applied in the lab (see Table 3). According to this threshold, that was reached at altitudes ≥ 1146 m a.s.l., it was possibile distinguish between Mediterranean Lowland and Mediterranean Mountain.

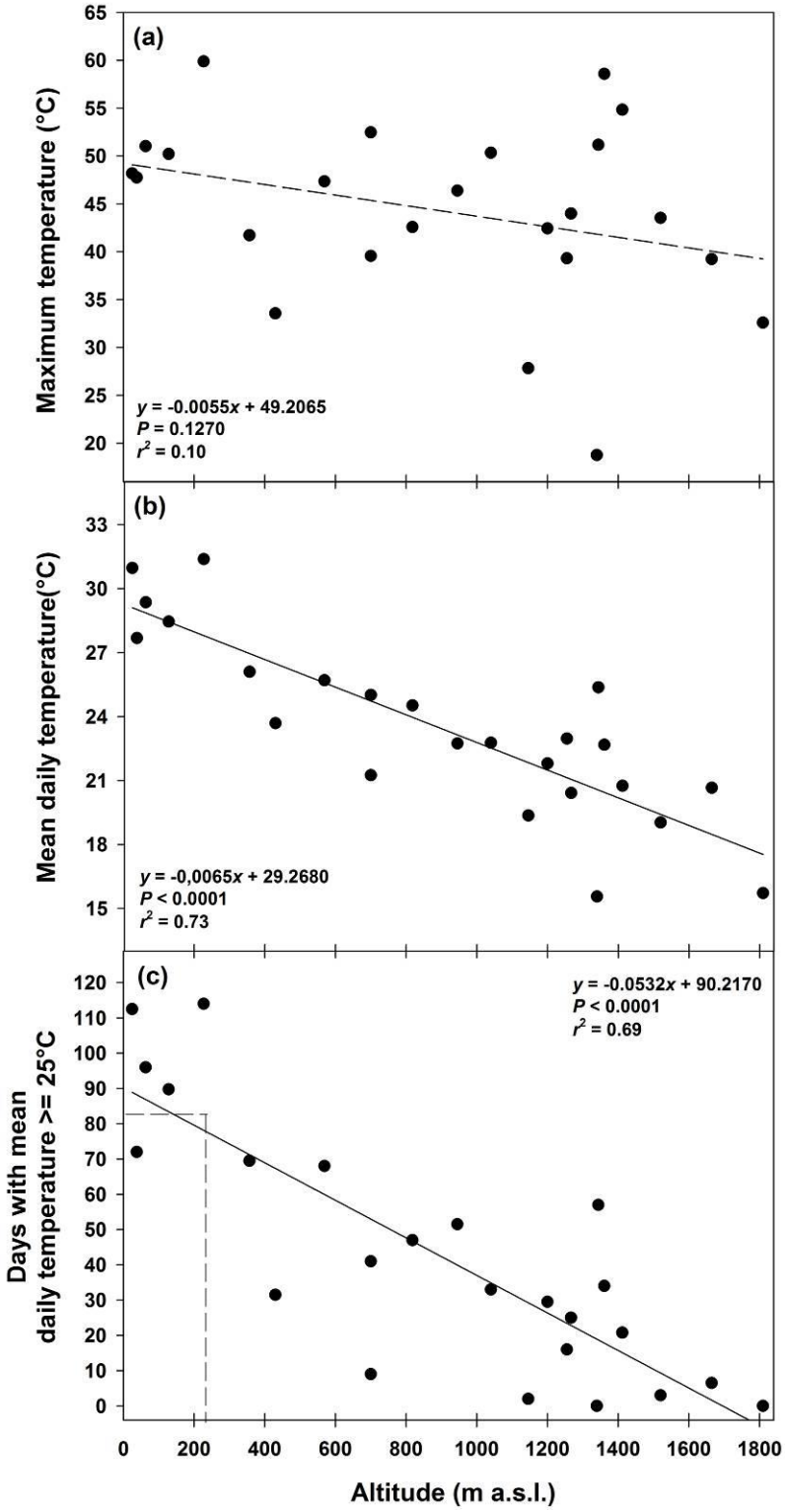

Figure 2 - Soil temperatures recorded in summer by 24 data-loggers at different altitudes. Fitted lines parameters are shown in each plot. In (c) the dashed lines highlight the value of 90 days which correspond to the warm stratification (W; 25°C for three months) and the dry after ripening (DAR; 25°C for three months on silica gel) applied in the lab (see Table 3).

Seed germination

The highest germination percentage for untreated seeds was reached by *Lupinus luteus* (100% at all tested temperatures, see Appendix 1). Similar germination percentages close to 100% as a maximum value, and averaging between 75-100%, were observed for other five species: *Digitalis purpurea* var. *gyspergerae* (the lowest altitude population), *Helianthemum caput-felis*, *Helichrysum microphyllum* ssp. *tyrrhenicum*, *Ptilostemon casabonae* (all the three populations) and *Santolina insularis* (populations at middle and high elevation). However, very high germination percentages (> 75%) were also recorded for *Dianthus morisianus*, *Digitalis purpurea* var. *gyspergerae* (the middle altitude population), *Rhamnus lycioides* ssp. *oleoides*, *Scrophularia ramosissima*, *Scrophularia trifoliata* (all the three populations) and *Verbascum plantagineum*. Seeds of *Digitalis purpurea* var. *gyspergerae* (the highest altitude population), *Lamyropsis microcephala*, *Nepeta foliosa*, *Rhamnus alaternus* ssp. *alaternus*, *Santolina insularis* (population at low elevation) and *Silene succulenta* ssp. *corsica*, germinated with maximum values included between 50-75%. Finally, two species (*Brassica tournefortii* and *Ruta lamarmorae*) failed to achieve 25% of seeds germinated (Appendix 1).

A fully positive germination response was achieved with GA_3 treatment by seeds of all species, which germinated with percentages > 50%. In particular, the highest germination was recorded by *Brassica tournefortii* (ca. 100% at all tested temperatures; Appendix 1). In relation to the control, GA_3 increased germination significantly in *Clematis vitalba*, *Digitalis purpurea* var. *gyspergerae* (the highest altitude population), *Nepeta foliosa*, *Ruta lamarmorae*, *Scrophularia trifoliata* (both low and high altitude population) and *Verbascum plantagineum* seeds (Appendix 1). For all these species, generalized linear models highlighted a statistically significant ($P < 0.001$) effect for all the factors (treatment and temperature) as well as for their interactions (Appendix 2).

Seeds of *Clematis vitalba*, *Dianthus morisianus*, *Helichrysum microphyllum* ssp. *tyrrhenicum*, *Ptilostemon casabonae* (the lowest altitude population), *Rhamnus alaternus* ssp. *alaternus*, *Rhamnus lycioides* ssp. *oleoides*, *Ruta lamarmorae* and *Santolina insularis* (the lowest altitude population) germinated during C stratification before moving to the range of incubation temperatures, to values between ca. 15 and 32% (Fig. 3a, 3b). To a lesser extent, seeds of *Lamyropsis microcephala*, *Nepeta foliosa*, *Ptilostemon casabonae* (the highest altitude population), *Scrophularia ramosissima* and *Scrophularia trifoliata* (the middle altitude population) also germinated, with percentages varying from 5 to 10% (Fig. 3a, 3b). Although the linear regression did not highlighted a statistical trend ($P > 0.05$), the graph shows that only the low altitude seedlots germinated to high percentages; in particular, seeds of *Clematis vitalba*, *Dianthus morisianus*, *Helichrysum microphyllum* ssp. *tyrrhenicum* and *Rhamnus lycioides* ssp. *oleoides* reached > 25% of germination (Fig. 3a).

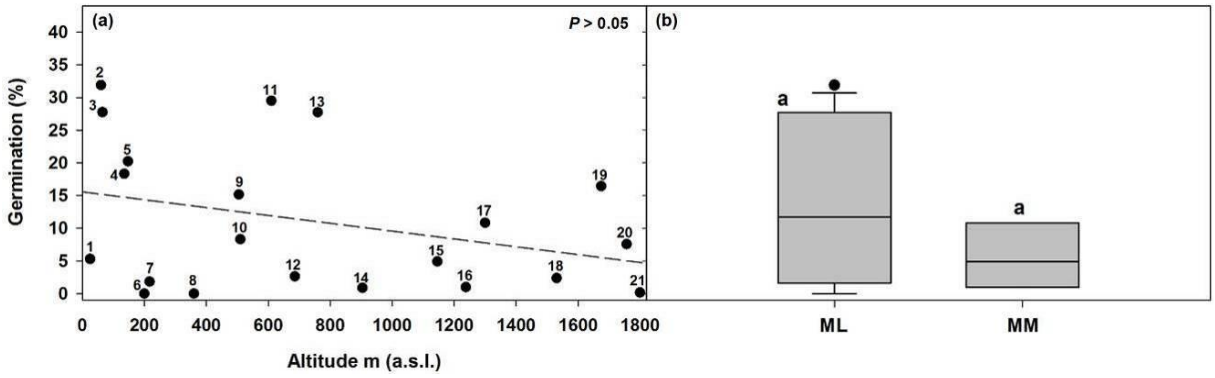

Figure 3 - (a) Germination percentages of seeds treated with pre-chilling during the three months at 5°C. (Species are listed according to the altitude: 1 = *Scrophularia ramosissima*, 2 = *Rhamnus lycioides* ssp. *oleoides*, 3 = *Dianthus morisianus*, 4 = *Ptilostemon casabonae* (low altitude), 5 = *Santolina insularis* (low altitude), 6 = *Verbascum plantaginenum*, 7 = *Scrophularia trifoliata* (low altitude), 8 = *Digitalis purpurea* var. *gyspergerae* (low altitude), 9 = *Rhamnus alaternus* ssp. *alaternus*, 10 = *Scrophularia trifoliata* (middle altitude), 11 = *Helichrysum microphyllum* ssp. *tyrrhenicum*, 12 = *Ptilostemon casabonae* (middle altitude), 13 = *Clematis vitalba*, 14 = *Digitalis purpurea* var. *gyspergerae* (middle altitude), 15 = *Nepeta foliosa*, 16 = *Scrophularia trifoliata* (high altitude), 17 = *Ptilostemon casabonae* (high altitude), 18 = *Santolina insularis* (high altitude), 19 = *Ruta lamarmorae*, 20 = *Lamyropsis microcephala*, 21 = *Digitalis purpurea* var. *gyspergerae* (high altitude)).
(b) Referring to climatic data achieved by data-loggers (Fig. 1c), the study species were divided in two different boxplots depending on altitude: Mediterranean Lowland "ML" species (0 - 1146 m a.s.l.) and Mediterranean Mountain "MM" species (1146 - 1810 m a.s.l.). *Post hoc* pairwise *t*-test comparisons were carried out and boxplots with the same letter signify that are not statistically different ($P > 0.05$).

With C treatment the highest germination percentage was observed for *Clematis viltaba* seeds (ca. 95%). Nevertheless, very high germination percentages (> 75%) were also recorded for other seven species: *Digitalis purpurea* var. *gyspergerae* (population at low and middle elevation), *Helichrysum microphyllum* ssp. *tyrrhenicum*, *Nepeta foliosa*, *Ptilostemon casabonae* (both populations at middle and high elevation), *Santolina insularis* (the highest altitude population), *Scrophularia trifoliata* (the middle altitude population) and *Silene succulenta* ssp. *corsica*. Seeds of other six species reached maximum values between 50-75%: *Dianthus morisianus*, *Digitalis purpurea* var. *gyspergerae* (the highest altitude population), *Lamyropsis microcephala*, *Santolina insularis* (the middle altitude population), *Scrophularia ramosissima* and *Verbascum plantagineum*. While seeds of *Rhamnus alaternus* ssp. *alaternus*, *Rhamnus lycioides* ssp. *oleoides*, *Santolina insularis* (the lowest altitude population) and *Scrophularia trifoliata* (the population at high altitude) germinated with maximum percentages between 25-50%. In contrast, seed germination of *Brassica tournefortii*, *Ruta lamarmorae* and *Scrophularia trifoliata* (the lowest altitude population) was very low (< 25%; see details in Appendix 1). C increased germination significantly of only two species in comparision to the control, i.e. *Clematis vitalba* and *Nepeta foliosa* (Appendix 1). On the contrary, C decreased significantly the mean germination of nine species: *Dianthus morisianus*, *Digitalis purpurea* var. *gyspergerae* (population at low and middle elevation), *Helichrysum microphyllum* ssp. *tyrrhenicum*, *Ptilostemon casabonae* (all the three populations), *Rhamnus lycioides* ssp. *oleoides*, *Santolina insularis* (population at low and middle altitude), *Scrophularia trifoliata* (all the three populations), *Scrophularia ramosissima* and *Verbascum plantagineum* (Appendix 1). For all these species, generalized linear models highlighted a statistically significant ($P < 0.001$) effect for all the factors (treatment and temperature) as well as for their interactions ($P < 0.001$, $P < 0.01$ and $P < 0.05$), except for *Helichrysum microphyllum* ssp. *tyrrhenicum* for which $P > 0.05$ for the interaction between treatment and temperature (Appendix 2).

Seeds of seven species treated with W had highest values for germination > 75%; specifically, *Dianthus morisianus*, *Digitalis purpurea* var. *gyspergerae* (at the lowest and middle altitude populations), *Helichrysum microphyllum* ssp. *tyrrhenicum*, *Nepeta foliosa*, *Ptilostemon casabonae* (all the three populations), *Santolina insularis* (the highest altitude population) and *Scrophularia trifoliata* (all the three populations). Seeds of *Santolina insularis* (the middle altitude population) and *Scrophularia ramosissima* germinated to maxima of 50-75%, while only one species *Santolina insularis* (the lowest altitude population) reached percentages between 25-50%. Whilst five species, specifically *Brassica tournefortii*, *Digitalis purpurea* var. *gyspergerae* (the highest altitude population), *Ruta lamarmorae*, *Rhamnus lycioides* ssp. *oleoides*, *Silene succulenta* ssp. *corsica*, failed to achieve 25% seed germination (Appendix 1). W treatment did not significantly increase germination of all the tested species in comparison to the control (Appendix 1). In contrast, W decreased significantly the mean seed germination of five species: *Digitalis purpurea* var. *gyspergerae* (the highest altitude population), *Rhamnus lycioides* ssp. *oleoides*, *Santolina insularis* (the middle altitude population), *Scrophularia ramosissima* and *Verbascum plantagineum* (Appendix 1). For all these five species, generalized linear models highlighted a statistically significant ($P < 0.001$) effect for all the factors (treatment and temperature) as well as for their interactions (Appendix 2).

The highest seed germination percentages following DAR were observed in two species: *Helianthemum caput-felis* and *Lupinus luteus*; indeed, both species achieved 100% at all tested temperatures, except *L. luteus* at 5°C that achieved 98% germination. However, high germination > 75% were also recorded for *Brassica tournefortii*, *Dianthus morisianus*, *Digitalis purpurea* var. *gyspergerae* (population at low and middle altitude), *Helichrysum microphyllum* ssp. *tyrrhenicum*, *Lamyropsis microcephala*, *Ptilostemon casabonae* (all the three populations), *Rhamnus lycioides* ssp. *oleoides*, *Santolina insularis* (population at middle and high elevation), *Scrophularia ramosissima*, *Scrophularia trifoliata* (all the three populations) and *Verbascum plantagineum*. Seeds of *Digitalis purpurea* var. *gyspergerae* (the highest altitude population), *Nepeta foliosa*, *Ruta lamarmorae*, *Santolina insularis* (population at low elevation) and *Silene succulenta* ssp. *corsica* reached, as maximum values, percentages between 50-75%. This response range also included the lowest germination percentage achieved by *Ruta lamarmorae*, with 56% (Appendix 1). DAR did not increase germination significantly for the study species compared to the Control, except for *Brassica tournefortii*, *Helianthemum caput-felis* and *Ruta lamarmorae* (Appendix 1). Moreover, DAR decreased germination significantly in only two species, i.e., *Digitalis purpurea* var. *gyspergerae* and *Scrophularia trifoliata*, both concerning the lowest altitude population (Appendix 1). For all these species, generalized linear models highlighted a statistically significant ($P < 0.001$) effect for all the factors (treatment and temperature) as well as for their interactions, with the exception of *Helianthemum caput-felis* for which $P > 0.05$ for the temperature factor (Appendix 2).

Seeds of *Clematis vitalba* showed a preference for alternating temperatures, with a higher proportion of germinating seeds compared with constant temperatures. Specifically, untreated seeds, as well as W and DAR seeds germinated only under the alternating temperature regime (25/10°C) with values of 87, 84 and 72%, respectively (Appendix 1). A positive effect of fluctuating temperatures on germination was also observed for *Verbascum plantagineum* seeds treated with W (88% germination at 25/10°C compared with a maximum of 18% at a constant 25°C; see Appendix 1). For both these species, generalized linear models highlighted a statistically significant ($P < 0.001$) effect for all the factors (treatment and temperature) as well as for their interactions (Appendix 2).

Thermal thresholds for seed germination

Estimated T_b of untreated (0, Control) seeds varied by 14ºC, from -9 (*Lupinus luteus*) to ca. 5°C (*Verbascum plantagineum* and *Clinopodium sandalioticum*); while GA_3 treated seeds had estimated T_b varying by 11ºC, from -5 (*Santolina insularis*, seeds collected at the middle altitude population) to 6°C (*Rhamnus lycioides* ssp. *oleoides*). C treatment of seeds reduced the range of values to 6°C, from approx. 1 (*Digitalis purpurea* var. *gyspergerae*, the lowest altitude population) to approx. 7°C (*Santolina insularis*, concerning seeds collected at the highest altitude population). DAR treated seed T_b varied by 13ºC, similar to the range of values for the Control; however, T_b was shifted to higher temperatures, by 3 - 4°C, varying from -5 (the lowest altitude population of *Digitalis purpurea* var. *gyspergerae* and *Lupinus luteus*) to 8°C (*D. purpurea* var. *gyspergerae*, for seeds collected at the middle altitude population). Finally, T_b of seeds treated with W varied from 0°C (for seeds of *Scrophularia trifoliata* collected at the middle altitude population and for the highest population of *Santolina insularis*) to 9°C (for the middle altitude population of *Digitalis purpurea* var. *gyspergerae*; Appendix 3).

Untreated seeds had *S* values that varied more than 10-fold, between 22 (*Helianthemum caput-felis*) to 357°Cd (*Rhamnus lycioides* ssp. *oleoides*). The *S* value range changed little with GA_3, from 52 to 196°Cd (*Santolina insularis*, seeds collected at high and low elevation, respectively). A similar range was recorded for C treated seeds with *S* values from 33 (*S. insularis*, seeds collected at the highest altitude population) to 204°Cd (*Digitalis purpurea* var. *gyspergerae*, seeds collected at low elevation). In contrast, DAR treatment yielded a range of *S* values similar to that of the Control, from 41 (*Helianthemum caput-felis*) to 313°Cd (*Rhamnus lycioides* ssp. *oleoides*). Finally, for seeds treated with W, *S* varied from 56 to 238°Cd (*Digitalis purpurea* var. *gyspergerae* and *Scrophularia trifoliata*, respectively; in both cases concerning populations at low altitude; Appendix 3).

The analysis was performed only for the species for which was possible to calculate both T_b and *S* in all pre-treatments. With the exception of W, a negative correlation between these thresholds was detected in all treatments, although only for untreated seeds was it statistically significant (Fig. 4a).

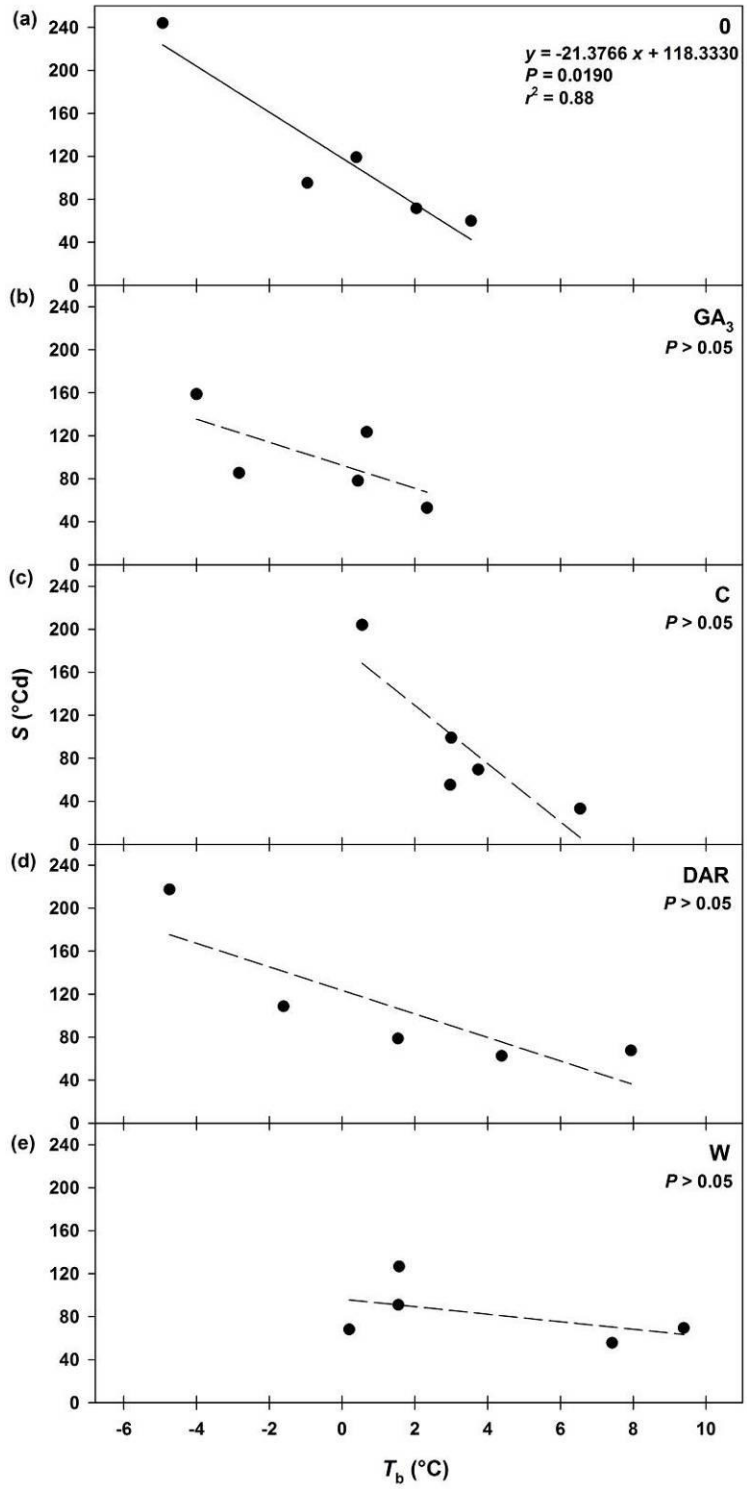

Figure 4 - Relationships between base temperatures for germination (T_b) of the investigated species and thermal constant *S* (°Cd) without any pre-treatment "0; Control" (a), for gibberellic acid "GA_3; 250 mg L^{-1} in the germination substrate" treated seeds (b), after pre-chilling "C; 5°C for three months" (c), after "DAR; 25°C for three months on silica gel" (d), and after pre-warming "W; 25°C for three months" (e). For each treatment are reported the same data for the same species for which was possible to calculate T_b and *S*. Only species for which both T_b and *S* could be calculated in all pre-treatments are included in these analysis.

Correlation between base temperature (T_b) and altitude

A positive correlation between T_b and altitude was highlighted for Control (0) and for seeds treated with DAR. In both cases, seedlots collected at low altitudes had a T_b ranging from -5 to 5°C but with increasing altitude, T_b was estimated to be around 10°C (Fig. 5a and Fig. 5d, respectively). On the contrary, altitude did not seem to have an effect on seeds treated with GA_3, showing a T_b around 2°C for all species (Fig. 5b) as well as for seeds treated with C and W, that showed a T_b around 5°C, regardless of altitude (Fig. 5c and 5e).

To explore further these apparent differences of T_b in response to altitude, species were divided between Mediterranean lowland ("ML") and Mediterranean mountain species ("MM") based on the climate data from the data-loggers (Fig. 1c). For Control seeds *post hoc* pairwise *t*-test comparison highlighted significant differences among ML and MM. Both GA_3 and C treatment caused a reduction in T_b values of MM species, nullifying the effect of altitude (Fig. 6).

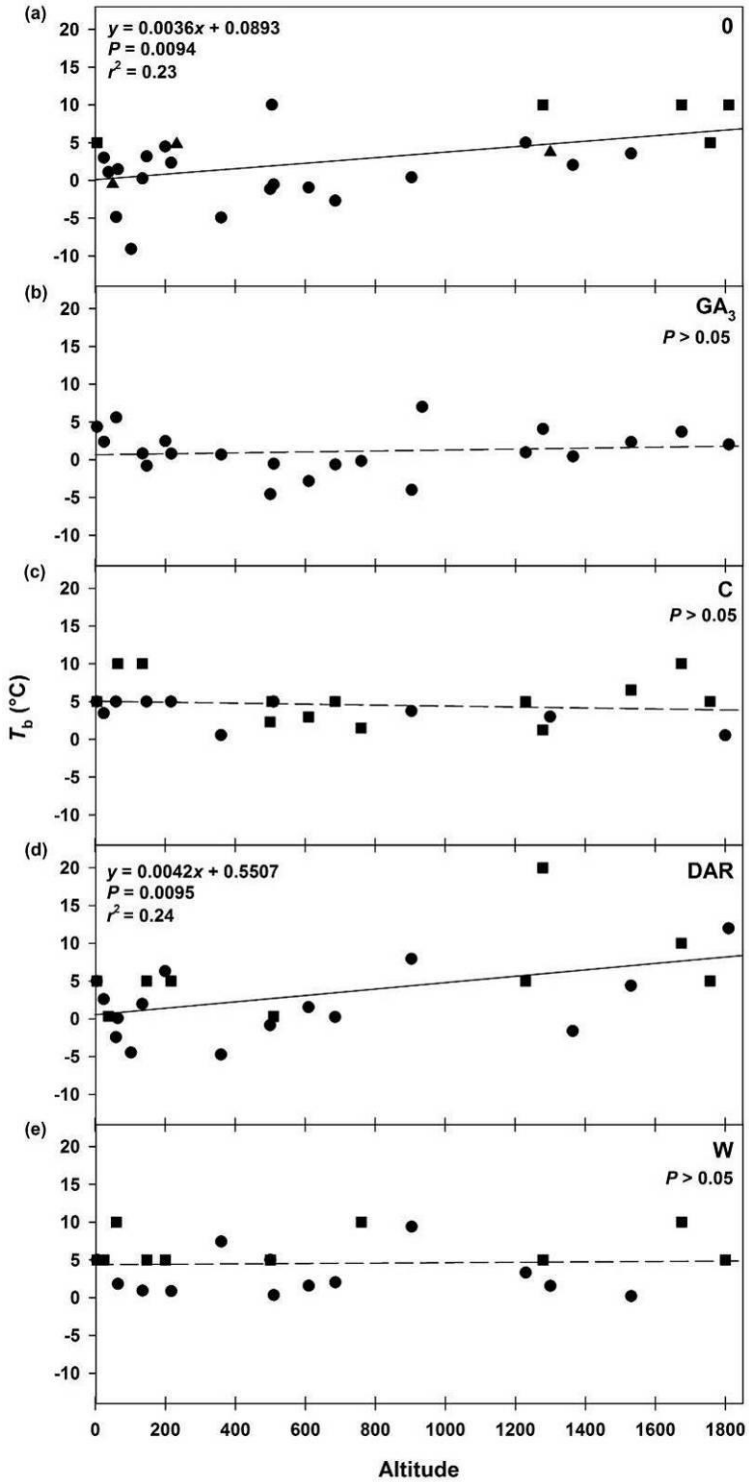

Figure 5 - Relationships between base temperatures for germination (T_b) of the investigated species and altitude without any pre-treatment "0; Control" (a), for gibberellic acid "GA_3; 250 mg L^{-1} in the germination substrate" treated seeds (b), after pre-chilling "C; 5°C for three months" (c), after "DAR; 25°C for three months on silica gel" (d), and after pre-warming "W; 25°C for three months" (e). Circles represent calculated values, squares estimated (i.e. the lowest temperature at which seeds germinated) and triangles signify data taken from the literature (*Centranthus amazonum*, *C. ruber* and *Clinopodium sandalioticum*).

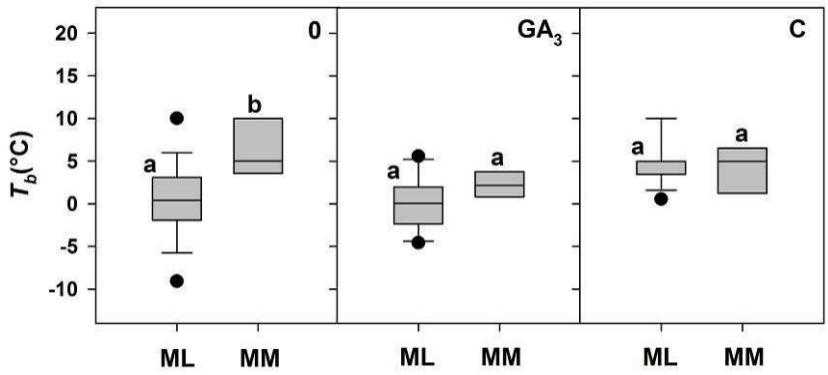

Figure 6 – Based on climate data recorded by the data-loggers (Fig. 1c), for 0 (Control), GA_3 (250 mg L^{-1} in the germination substrate) and C (5°C for three months) treatments the species were divided in two different boxplots depending on altitude: Mediterranean Lowland "ML" species (0 - 1146 m a.s.l.) and Mediterranean Mountain "MM" species (1146 - 1810 m a.s.l.). *Post hoc* pairwise *t*-test comparisons were carried out for each treatment, and boxplots with different letters indicate significant ($P < 0.05$) variation.

GLMs highlighted a statistically significant effect of the treatment and altitude factors ($P < 0.001$ and $P < 0.01$) on T_b of the study species. Also their interactions were statistically significant ($P < 0.01$; Table 4).

Table 4 - GLMs results for base temperature of germination (T_b) of the following factors: "Treatment" (0, Control; C, 5°C for 3 months; W, 25°C for three months; GA_3, 250 mg L^{-1} in the germination substrate; DAR, 25°C for three months on silica gel) and "Altitude" (Mediterranean 0 - 1146 m a.s.l.; temperate 1146 - 1810 m a.s.l.), and their interaction.

Base temperature (T_b)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
NULL			106	1915.8		
Altitude	1	198.16	105	1717.6	146.178	< 0.001
Treatment	4	201.85	101	1515.8	37.224	< 0.01
Altitude:Treatment	4	200.79	97	1315.0	37.028	< 0.01

Thermal thresholds of Mediterranean vs. temperate species

Differences in the relationships between T_b and S among Mediterranean species and temperate species are shown in Fig. 7. Specifically, Mediterranean species showed a range of T_b varying from -9 to 5°C, while the T_b values of temperate species were never below -2°C (Fig. 7). Differences between Mediterranean and temperate species were evident also for the S values. In particular, Mediterranean species ranged from 22 to 357°Cd, highlighting a slower germination than temperate species, whose values varied from 15 to 136°Cd (Fig. 7).

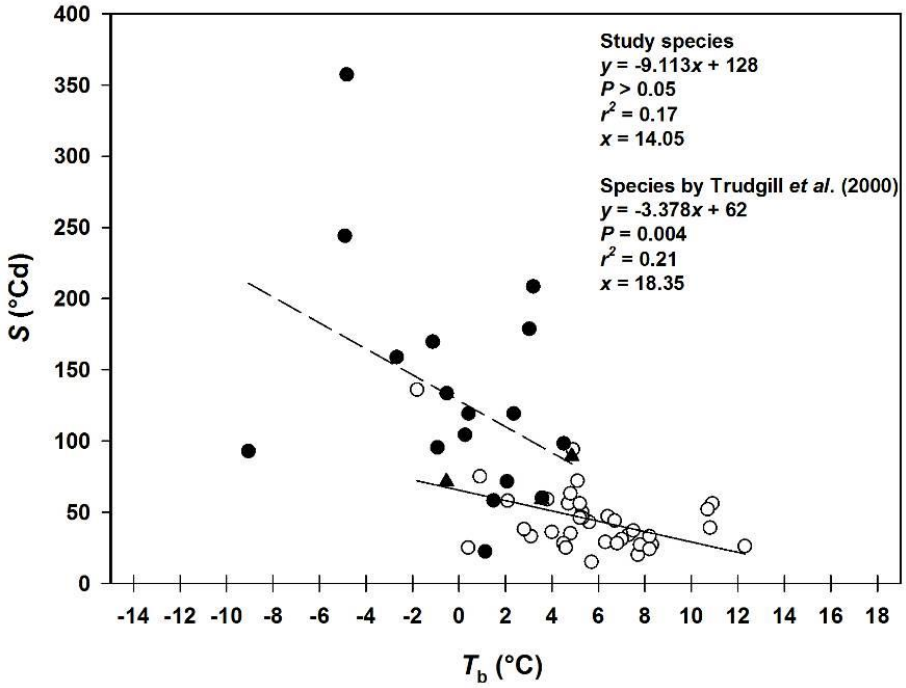

Figure 7 - Relationships between base temperatures for germination (T_b) and thermal constant S (°Cd) without any pre-treatment for 50% germination. Black circles represent calculated values of the study species, white circles represent estimated values of temperate species studied by Trudgill *et al*. (2000), finally triangles signify data taken from the literature (*Centranthus amazonum*, *C. ruber* and *Clinopodium sandalioticum*).

More detailed analyses were carried out to evaluate possible differences among the Mediterranean species, in particular between the Mediterranean lowland "ML" and the Mediterranean mountain "ML" and between them and the temperate species "T" (as above detailed) in relation to T_b and S (Fig. 8a, 8b). Significant differences were highlighted between T and ML species in relation with T_b but also the MM differed from ML species (Fig. 8a). Concerning S, T species were different from the Mediterranean (broad sense), and unlike T_b, in this case the analysis highlighted that among MM and ML there was no difference (Fig. 8b).

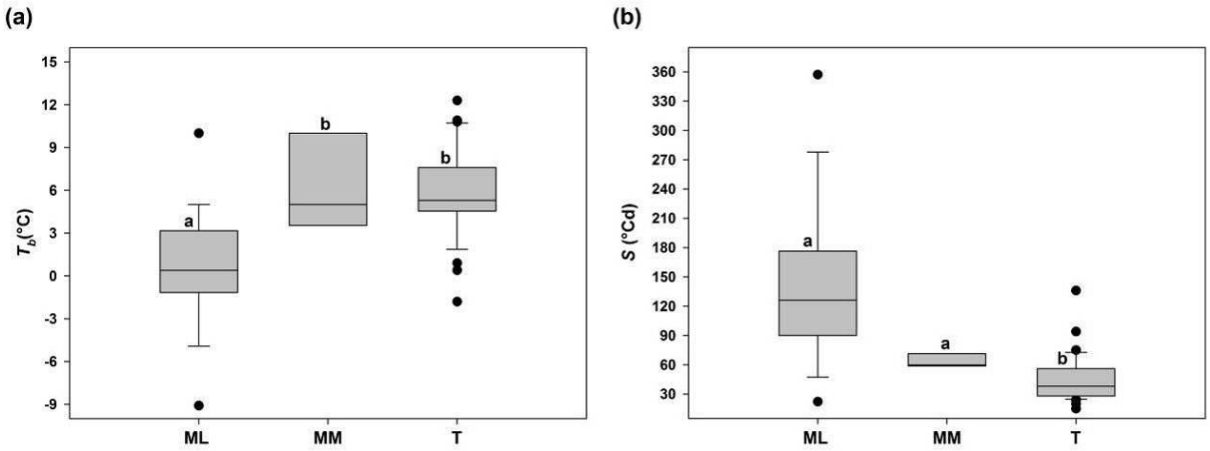

Figure 8 - Relationships between base temperatures for germination (T_b) and thermal constant *S* (°Cd) without any pre-treatment for 50% germination between the study species that were divided two different boxplots depending on altitude: Mediterranean Lowland "ML" species (0 - 1146 m a.s.l.) and Mediterranean Mountain "MM" species (1146 - 1810 m a.s.l.), and the temperate species, studied by Trudgill *et al*. (2000). *Post hoc* pairwise *t*-test comparisons were carried out and boxplots with different letters indicate significant ($P < 0.05$) variation.

Discussion

The data recorded for the soil temperature reflect the Mediterranean climate characterized by high temperatures in summer and cool, wet winters (Joffre *et al*., 1999; Medrano *et al*., 2009; Kadis and Georghiou, 2010). In particular, a colder and longer winter, that extends approximately from November to March, was noted at high altitudes unlike the lowlands (until 800 m a.s.l.), for which a milder winter season was detected. Conversely, a longer dry period over the summer, that extend approximately from June to September, was observed at low elevation (up to 200 m a.s.l.). However, the high temperatures recorded in summer along the whole altitudinal gradient, highlight as also the high Mediterranean mountains encounter a water stress, which can have important effects on the timing of seed germination and on plant growth (Mooney *et al*., 1965; Rundel *et al*., 2003; Giménez-Benavides *et al*., 2005).

Clearly, there is considerable inter-specific variation in the sensitivity of seed germination to the applied treatments (Appendix 1, 2). All species respond positively to GA_3 treatment. Furthermore, GA_3 widened the temperature range for germination of eight species (*Brassica tournefortii*, *Verbascum plantagineum*, *Clematis vitalba*, *Nepeta foliosa*, the highest altitude population of *Scrophularia trifoliata*, *Ruta lamarmorae* and *Digitalis purpurea* for both populations at middle and high elevation altitude) which seeds germinated with very low values without any treatment at 5°C (Appendix 1). This indicates that certain degree of dormancy could be found in some of the study species, especially at low temperatures. Indeed, it is well known that GA plays a key role in dormancy release and in the promotion of seed germination in species exhibiting physiological or morphophysiological dormancy (Bewley and Black, 1994; Finch-Savage and Leubner- Metzger, 2006; Baskin and Baskin, 2014). With the exception of four species (*Clematis vitalba*, *Rhamnus alaternus*, *Nepeta foliosa* and *Digitalis purpurea* var. *gyspergerae*), cold stratification either inhibits or has a neutral effect on seed germination. These results agree with Valbuena and Vera (2002) who found that the germination for species of Mediterranean heathland was not enhanced by cold pre-treatment of seed; similar results were obtained by Luna *et al*. (2008) where sprouters and endemic species from Iberian Peninsula were negatively affected by cold stratification treatment. However, a modified germination strategy is apparent in high mountain Mediterranean plants which are generally positively affected by cold-wet stratification treatment but need to germinate quickly after snow melt in the early spring, before the arrival of the dry summer season (Giménez-Benavides *et al*., 2005, 2007; Mattana *et al*., 2012; Porceddu *et al*., 2013). The seeds are also insensitive to constant W temperature (Appendix 1). By contrast, in literature it is reported that in many species warm stratification can improve germination. Furthermore, it is well known that such treatment, followed by cold periods of stratification, is necessary to overcome morphophysiological dormancy (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzge, 2006; Baskin and Baskin, 2014). For example, a similar germination requirement has been found in *Arum maculatum* L. seeds (Pritchard *et al*., 1993), *Fraxinus excelsior* L. (Villiers and Wareing, 1964) and in *Jeffersonia diphylla* (L.) Pers. (Baskin and Baskin, 1989). While, DAR only has a positive effect on germination in a limited number of species (*Brassica tournefortii*, *Helianthemum caput-felis* and *Ruta lamarmorae*). The lack of a significant effect of dry after-ripening pre-treatment was also reported for *Brassica insularis* Moris, a Mediterranean species growing on both coastal and inland cliffs (Santo *et al*., 2014a). Along this line, Santo *et al*. (2014b) found that in *Phleum sardoum* (Hackel) Hackel, an endemic psammophilous species of Sardinia, growing exclusively on coastal sandy dunes, dry after-ripening increased germination rate at low temperatures, but did not affect final germination percentages. By contrast, Peishi *et al*. (1999) reported that short-term storage (< 18 months) at high temperatures improved germination in seeds of three Australian Asteraceae. In agreement, Finch-Savage *et al*. (2007) found that dormancy was released by dry after-ripening in *Arabidopsis thaliana* Cape Verde

Island accession. Indeed, dry after-ripening is an important treatment to break dormancy (Bewley, 1997; Kucera *et al*., 2005; Probert, 2000), and may represent a natural mechanism that can control dormancy in dry climates (Probert, 2000).

In many species, constant and alternating temperature regimes often affect seed germination differently (Probert, 2000). Different studies reported an increasing of germination responses of species under the fluctuating temperature regime (e.g. Thompson and Grime, 1983; Schütz and Rave, 1999; Liu *et al*., 2013). In agreement, alternating temperature regimes improve the germination percentages of some of the Sardinian species studied here, e.g. for untreated seeds of *Clematis vitalba*, as well for those treated with W and DAR (Appendix 1).

A relative lack of responsiveness to pre-treatment does not infer a lack of temperature sensitivity for germination. Indeed, seed germination responses to temperature, reflect a climate-adapted strategy on the timing of seedling emergence (Skordilis and Thanos, 1995) and the identification of thermal thresholds for seed germination could help to explain the cumulative differences detected among species along the altitudinal gradient. T_b and S were evaluated for each species and the results highlight that thermal thresholds do change among species as well as populations of the same species (Ellis *et al*., 1987; Daws *et al*., 2004), and they are affected also by the application of the treatments.

The relationship between T_b and altitude highlight a positive correlation for Control (0) and DAR, while, seeds treated with GA_3, C and W have no showed any correlation. Different responses of T_b according to altitude (i.e. separating ML and MM species) were also highlighted for untreated seeds, whereas GA_3 and C reduced T_b values in MM species. In particular, the diverse behaviour of species located at high altitudes could be explained by different environmental cues which seeds are subjected to during late autumn, through the winter and into springtime. Specifically, seeds of high altitude may have higher T_b values so as to prevent premature germination under cool conditions when the risk of freezing may still persist. But GA_3 and C seem to be effective at lowering T_b, indicating the effective widening of the temperature range over which germination can occur (Vera, 1997; Cavieres and Arroyo, 2000; Baskin and Baskin, 2014). Interestingly, the application of C pre-treatment is not necessary to improve germination of the study species collected at the low altitude, with these seeds being able to germinate during cold stratification before moving to the range of incubation temperatures. This behaviour is totally in agreement with the Mediterranean character of the species, where seeds are non-dormant (*sensu* Baskin and Baskin, 2004) in autumn at the time of dispersal and the maximal response of germination is achieved under relatively cool conditions of 5-15°C (Thanos *et al*., 1989; Skordilis and Thanos, 1995). This device provides a considerable ecological advantage for seeds by ensuring that germination is completed at the most appropriate period (mid to late autumn), and thus allowing seedlings to avoid arid conditions during summer (Kadis and Georghiou, 2010; Luna *et al*., 2008).

From the comparison performed between the T_b and S of Mediterranean species of Sardinia with those of temperate species (Trudgill *et al*., 2000), it is clear that the thermal thresholds vary between the two groups of species. Specifically, Mediterranean species have lower T_b values than those of temperate species, while, S of Mediterranean species highlights a slower germination than temperate species. These results are in agreement with the findings of studies (Thanos *et al*., 1991; Skordilis and Thanos, 1995; Thanos *et al*., 1995; Doussi and Thanos, 2002) on the Mediterranean germination syndrome, suggesting for Mediterranean species (especially for those of Mediterranen lowland) a low base temperature for germination and a slow germination rate. However, Mediterranean mountain species showed a thermal character more similar to temperate species compared to the typical Mediterranean coastal species. That is also in agreement with previous work (e.g. Mattana *et al.*, 2012; Porceddu *et al*., 2013) on Mediterranean mountain species in Sardinia which reported a seed thermal behaviour typical of temperate and alpine regions, where early spring germination prevails

due to a requirement for cold stratification over winter. Consequently, it is established that subtle differences in germination strategy between species are modulated by both T_b and S.

Conclusion

This study characterizes the Mediterranean climate along an altitudinal gradient in Sardinia. Furthermore, seed germination responses of Mediterranean species along an altitudinal gradient are identified both in relation with the altitude and different treatments. The thermal thresholds for seed germination of the study species highlight the Mediterranean germination syndrome for lowland species and a thermal temperate behaviour, with a spring germination, for the high Mediterranean mountain species.

References

Bacchetta G., 2006. La flora del Sulcis (Sardegna sud-occidentale). Guineana, 12: 1–369.

Bacchetta G., Bagella S., Biondi E., Farris E., Filigheddu R., Mossa L., 2009. Vegetazione forestale e serie di vegetazione della Sardegna (con rappresentazione cartografica alla scala 1:350.000). Fitosociologia, Vol. 46 (1) suppl. 1: 3–82.

Bacchetta G., Fenu G., Mattana E., 2012. A checklist of the exclusive vascular flora of Sardinia with priority rankings for conservation. Anales del Jardín Botánico de Madrid, 69(1): 81-89.

Baker G.H., 1989. Some aspects of the natural history of seed banks. In: Leck, M.A., Parker, V.T. & Simpson, R.L. (eds), Ecology of soil seed banks, Academic Press, San Diego.

Baskin C.C., Baskin J.M., 1989. Seed germination ecophysiology of *Jeffersonia diphylla*, a perennial herb of mesic deciduous forests. American Journal of Botany, 76(7): 1073–1080.

Baskin C.C., Baskin J.M., 2003. When breaking seed dormancy is a problem. Try a move-along experiment. Native Plants|Spring, 4: 17–21.

Baskin J.M., Baskin C.C., 2004. A classification system for seed dormancy. Seed Science

Research, 14: 1–16.

Baskin C.C., Baskin J.M., 2014. Seeds: ecology, biogeography, and evolution of dormancy and germination. San Diego: Academic Press.

Bewley J.D., Black M., 1994. Seeds: Physiology of development and germination. New York: Plenum Press.

Bewley J.D., 1997. Seed germination and dormancy. Plant Cell, 9: 1055–1066.

Billings W.D., Mooney H.A., 1968. The ecology of arctic and alpine plants. Biological Reviews, 43: 481–529.

Bonner F.T., 1984. Glossary of seed germination terms for tree seed workers. General Technical Report SO-49. New Orleans: USDA Forest Service, Southern Forest Experiment Station, 4 p.

Cañadas E.M., Fenu G., Peñas J., Lorite J., Mattana E., Bacchetta G., 2014. Hotspots within hotspots: Endemic plant richness, environmental drivers, and implications for conservation. Biological Conservation, 170: 282–291.

Carmignani L., Oggiano G., Barca S., Conti P., Salvadori I., Eltrudis A., Funedda A., Pasci S., 2001. Geologia della Sardegna; Note Illustrative della Carta Geologica della Sardegna in scala 1:200.000. Memorie Descrittive della Carta Geologica d'Italia, 60, Servizio Geologico d'Italia, Roma, 283 pp.

Cavieres L.A., Arroyo M.T.K., 2000. Seed germination response to cold stratification period and thermal regime in *Phacelia secunda* (Hydrophyllaceae) - Altitudinal variation in the mediterranean Andes of central Chile. Plant Ecology, 149: 1–8.

Cavieres L.A., Badano E.I., Sierra-Almeida A., Gómez-González S., Molina-Montenegro M.A., 2006. Positive interactions between alpine plant species and the nurse cushion plant *Laretia acaulis* do not increase with elevation in the Andes of central Chile. New Phytologist, 169: 59–69.

Daws M.I., Burslem D.F.R.P., Crabtree L.M., Kirkman P., Mullins C.E., Dalling J.W., 2002. Differences in seed germination responses may promote coexistence of four sympatric Piper species. Functional Ecology, 16: 258–267.

Daws M.I., Lydall E., Chmielarz P., Leprince O., Matthews S., Thanos C.A., Pritchard H.W., 2004. Developmental heat sum influences recalcitrant seed traits in *Aesculus hippocastanum* across Europe. New Phytologist, 162: 157–166.

Debussche M., Garnier E., Thompson J.D., 2004. Exploring the causes of variation in phenology and morphology in Mediterranean geophytes: A genus-wide study of Cyclamen. Botanical Journal of the Linnean Society, 145: 469–484.

Doussi M.A., Thanos C.A., 2002. Ecophysiology of seed germination in Mediterranean geophytes. 1. *Muscari* spp. Seed Science Research, 12: 193–201.

Ellis R.H., Covell S., Roberts E.H., Summerfield R.J., 1986. The influence of temperature on seed germination rate in grain legumes. II. Intraspecific variation in chickpea (*Cicer arietinum* L.) at constant temperatures. Journal of Experimental Botany, 37: 1503–1515.

Ellis R.H., Simon G., Covell S., 1987. The influence of temperature on seed germination rate in grain legumes. III. A comparison of five faba bean genotypes at constant temperatures using a new screening method. Journal of Experimental Botany, 38: 1033–1043.

Fenner M., Thompson K., 2005. The Ecology of Seeds. Cambridge University Press, Cambridge.

Fenu, G., Fois, M., Cañadas, E.M., Bacchetta, G., 2014. Using endemic-plant distribution, geology and geomorphology in biogeography: the case of Sardinia (Mediterranean Basin). Systematics and Biodiversity, 12(2): 181–193.

Fernández-Pascual E., Jiménez-Alfaro B., Caujapé-Castells J., Jaén-Molina R., Díaz T.E., 2013. A local dormancy cline is related to the seed maturation environment, population genetic composition and climate. Annals of Botany, 112: 937–945.

Finch-Savage W.E., Leubner-Metzge G., 2006. Seed dormancy and the control of germination. New Phytologist, 171: 501–523.

Finch-Savage W.E., Cadman C.S.C., Toorop P.E., Lynn J.R., Hilhorst H.W.M., 2007. Seed dormancy release in *Arabidopsis thaliana* by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing. The Plant Journal, 51: 60–78.

Garcia-Huidobro J., Monteith J.L., Squire G.R., 1982. Time, temperature and germination of pearl millet (*Pennisetum typhoides* S. & H.). Journal of Experimental Botany, 33: 288–296.

Giménez-Benavides L., Escudero A., Pérez-García F., 2005. Seed germination of high mountain Mediterranean species: altitudinal, interpopulation and interannual variability. Ecological Research, 20: 433–444.

Giménez-Benavides L., Escudero A., Iriondo J.M., 2007. Local adaptation enhances seedling recruitment along an altitudinal gradient in a high mountain Mediterranean plant. Annals of Botany, 99: 723–734.

Gresta F., Cristaudo A., Onofri A., Restuccia A., Avola G., 2010. Germination response of four pasture species to temperature, light, and post-harvest period. Plant Biosystems, 144(4): 849–856.

Grubb P.J., 1977. The maintenance of species-richness in plant communities: the importance of the regeneration niche. Biological Reviews, 52: 107–145.

Galmés J., Flexas J., Savé R., Medrano H., 2007. Water relations and stomatal characteristics of Mediterranean plants with different growth forms and leaf habits: responses to water stress and recovery. Plant Soil, 290: 139–155.

Joffre R., Rambal S., Damesin C., 1999. Functional attributes in Mediterranean-type ecosystems. In: Pugnaire FI, Valladares F, eds. Handbook of functional plant ecology. NewYork: Marcel Dekker, 347–380.

Kadis C., Georghiou K., 2010. Seed dispersal and germination behavior of three threatened endemic labiates of Cyprus. Plant Species Biology, 25: 77–84.

Karlsson L.M., Tamado T., Milberg P., 2008. Inter-species comparison of seed dormancy and germination of six annual Asteraceae weeds in an ecological context. Seed Science Research, 18: 35–45.

Kucera B., Cohn M.A., Leubner-Metzger L., 2005. Plant hormone interactions during seed dormancy release and germination. Seed Science Research, 15: 281–307.

Liu K., Baskin J.M., Baskin C.C., Bu H., Du G., Ma M., 2013. Effect of diurnal fluctuating versus constant temperatures on germination of 445 species from the Eastern Tibet Plateau. PLoS ONE 8(7): e69364. doi:10.1371/journal.pone.0069364.

Luna B., Pérez B., Céspedes B., Moreno J.M., 2008. Effect of cold exposure on seed germination of 58 plant species comprising several functional groups from a mid-mountain Mediterranean area. Ecoscience, 15(4): 478–484.

Mattana E., Daws M.I., Bacchetta G., 2009. Effects of temperature, light and prechilling on germination of *Rhamnus persicifolia*, an endemic tree species of Sardinia (Italy). Seed Science and Technology, 37: 758–764.

Mattana E., Daws M.I., Bacchetta G., 2010. Comparative germination ecology of the endemic *Centranthus amazonum* (Valerianaceae) and its widespread congener *Centranthus ruber*. Plant Species Biology, 25(3): 165–172.

Mattana E., Fenu G., Bacchetta G., 2012. Seed production and *in situ* germination of *Lamyropsis microcephala* (Asteraceae), a threatened mediterranean mountain species. Arctic, Antarctic, and Alpine Research, 44(3): 343–349.

McDonald J.H., 2008. Handbook of Biological Statistics Sparky House Publishing, Baltimore.

Medrano H., Flexas J., Galmés J., 2009. Variability in water use efficiency at the leaf level among Mediterranean plants with different growth forms. Plant Soil, 317: 17–2.

Michalet R., 2006. Is facilitation in arid environments the result of direct or complex interactions? New Phytologist, 169: 3–6.

Mooney H.A., Hillier R.D., Billings W.D., 1965. Transpiration rates of alpine plants in the Sierra Nevada of California. American Midland Naturalist, 74: 375–386.

Peishi Z., Plummer J., Turner D., Choengsaat D., Bell D., 1999. Low- and high-temperature storage effects on viability and germinability of seeds of three Australian Asteraceae. Australian Journal of Botany, 47: 265–275.

Porceddu M., Mattana E., Pritchard H.W., Bacchetta G., 2013. Thermal niche for in situ seed germination in Mediterranean mountain streams: model prediction and validation for *Rhamnus persicifolia* seeds. Annals of Botany, 112(9): 1887–1897.

Poschlod P., Abedi M., Bartelheimer M., Drobnik J., Rosbakh S., Saatkamp A., 2013. Seed ecology and assembly rules in plant communities. Vegetation Ecology (eds E. van der Maarel and J. Franklin), pp. 164–202. John Wiley and Sons, Chichester.

Pritchard H.W. Wood J.A., Manger K.R., 1993. Influence of temperature on seed germination and nutritional requirements for embryo growth in *Arum maculatum* L. New Phytologist, 123: 801–809.

Probert R.J., 2000. The role of temperature in the regulation of seed dormancy and germination. In: Fenner M., ed. Seeds: the ecology of regeneration in plant communities. Wallingford, UK: CAB International, pp. 261–292.

Rosbakh S., Poschlod P., 2014. Initial temperature of seed germination as related to species occurrence along a temperature gradient. Functional Ecology, doi: 10.1111/1365-2435.12304.

Royal Botanic Gardens Kew., 2014. Seed Information Database (SID). Version 7.1. Available from: http://data.kew.org/sid/.

Rundel P.W., Gibson A.C., Midgley G.S., Wand S.J.E., Palma B., Kleier C., Lambrinos J., 2003. Ecological and ecophysiological patterns in a prealtiplano shrubland of the Andean Cordillera in northern Chile. Plant Ecology, 169: 179–193.

Santo A., Mattana E., Grillo O., Bacchetta G., 2014a. Morphocolorimetric analysis and seed germination of *Brassica insularis* Moris (Brassicaceae) populations. Plant Biology, 17: 335–343.

Santo A., Mattana E., Frigau L., Bacchetta G., 2014b. Light, temperature, dry after-ripening and salt stress effects on seed germination of *Phleum sardoum* (Hackel) Hackel. Plant Species Biology, 29: 300–305.

Schütz W., Rave G., 1999. The effect of cold stratification and light on the seed germination of temperate sedges (Carex) from various habitats and implications for regenerative strategies. Plant Ecology, 144: 215–230.

Skordilis A., Thanos C.A., 1995. Seed stratification and germination strategy in the Mediterranean pines *Pinus brutia* and *P. halepensis*. Seed Science Research, 5: 151–160.

Thanos C.A., Georghiou K., Skarou F., 1989. *Glaucium flavum* seed germination: an ecophysiological approach. Annals of Applied Botany, 63(1): 121–130.

Thanos C.A., Georghiou K., Dimitra J.D., Marangaki C.J., 1991. Photoinhibition of seed germination in Mediterranean maritime plants. Annals of Botany, 68: 469–47.

Thanos C.A., Costas C.K., Skarou F., 1995. Ecophysiology of germination in the aromatic plants thyme, savory and oregano (Labiatae). Seed Science Research, 5: 161–170.

Thompson K., Grime J.P., 1983. A comparative study of germination responses to diurnally fluctuating temperatures. Journal of Applied Ecology, 20: 141–156.

Thompson P.A., 1968. Germination of Caryophyllaceae at low temperatures in relation to geographical distribution. Nature, 217: 1156–1157.

Trudgill D.L., Squire G.R., Thompson K., 2000. A thermal time basis for comparing the germination requirements of some British herbaceous plants. New Phytologist, 145: 107–114.

Valbuena L., Vera M.L., 2002. The effects of thermal scarification and seed storage on germination of four heatland species. Plant Ecology, 161: 137–144.

Valladares F., Sánchez-Gómez D., 2006. Ecophysiological traits associated with drought in Mediterranean tree seedlings: individual responses versus interspecific trends in eleven species. Plant Biology, 8: 688–697.

Vandelook F., Van Assche J.A., 2008. Temperature Requirements for Seed Germination and Seedling Development Determine Timing of Seedling Emergence of Three Monocotyledonous Temperate Forest Spring Geophytes. Annals of Botany, 102: 865–875.

Vera M.L., 1997. Effects of altitude and seed size on germination and seedling survival of heathland plants in north Spain. Plant Ecology, 133: 101–106.

Villiers T.A., Wareing P.F., 1964. Dormancy in fruits of *Fraxinus excelsior* L. Journal of Experimental Botany, 15: 359–367.

Appendixes

Appendix 1. Seed germination results (%) of all tested species at different temperatures (5, 10, 15, 20, 25 and 25/10°C) and treatments (0, control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for 3 months; W, 25°C for 3 months; DAR, 25°C for 3 months on silica gel). Data are the mean (± S.D.) of four or three replicates. Species are arranged in order of altitude (from 5 m a.s.l. to 1800 m a.s.l.).

Species	Temperature (°C)	Germination percentages (mean ± S.D.)				
		0	GA_3	C	W	DAR
Brassica tournefortii	**5**	1.7 ± 3.3	100.0 ± 0.0	1.7 ± 1.9	7.5 ± 8.3	4.2 ± 1.7
	10	4.2 ± 3.2	100.0 ± 0.0	5.0 ± 3.3	10.8 ± 12.9	61.7 ± 1.9
	15	4.2 ± 3.2	100.0 ± 0.0	5.8 ± 3.2	5.8 ± 9.6	76.7 ± 0.0
	20	2.5 ± 1.7	100.0 ± 0.0	3.3 ± 2.7	8.3 ± 7.9	57.5 ± 6.3
	25	1.7 ± 1.9	100.0 ± 0.0	2.5 ± 3.2	6.7 ± 5.4	45.8 ± 15.2
	25/10	0.8 ± 1.7	99.2 ± 1.7	6.7 ± 4.7	4.2 ± 4.2	28.3 ± 7.9
Silene succulenta ssp. *corsica*	**5**	10.0 ± 5.0	-	6.7 ± 2.9	5.0 ± 5.0	8.3 ± 2.9
	10	30.0 ± 0.0	-	81.7 ± 11.6	18.3 ± 2.9	51.7 ± 5.8
	15	61.7 ± 15.3	-	93.3 ± 2.9	20.0 ± 10.0	71.7 ± 12.6
	20	58.3 ± 10.4	-	55.0 ± 11.8	21.7 ± 16.1	46.7 ± 15.3
	25	1.7 ± 2.9	-	21.7 ± 12.6	11.7 ± 7.6	0.0 ± 0.0
	25/10	23.3 ± 7.6	-	16.7 ± 10.4	20.2 ± 13.0	46.7 ± 7.6
Scrophularia ramosissima	**5**	73.3 ± 5.8	86.7 ± 5.8	68.3 ± 16.1	1.7 ± 2.9	78.3 ± 2.9
	10	75.0 ± 10.0	83.3 ± 14.4	70.0 ± 0.0	50.0 ± 10.0	68.3 ± 14.4
	15	60.0 ±15.0	90.0 ± 5.0	58.3 ± 12.6	32.1 ± 11.2	66.7 ± 2.9
	20	63.3 ± 5.8	81.7 ± 2.9	30.0 ± 5.0	18.6 ± 5.6	51.7 ± 7.6
	25	11.7 ± 7.3	78.3 ± 7.6	15.4 ± 9.4	11.7 ± 2.9	30.0 ± 15.0
	25/10	78.3 ± 16.1	83.3 ± 12.6	25.0 ± 18.0	73.3 ± 17.6	81.7 ± 10.4
Helianthemum caput-felis	**5**	86.7 ± 7.6	-	-	-	100.0 ± 0.0
	10	85.0 ± 5.0	-	-	-	100.0 ± 0.0
	15	91.7 ± 7.6	-	-	-	100.0 ± 0.0
	20	91.7 ± 2.9	-	-	-	100.0 ± 0.0
	25	90.0 ± 13.2	-	-	-	100.0 ± 0.0
	25/10	85.0 ± 10.0	-	-	-	100.0 ± 0.0
Rhamnus lycioides ssp. *oleoides*	**5**	0.0 ± 0.0	0.00 ± 0.0	37.8 ± 11.1	0.0 ± 0.0	1.8 ± 3.0
	10	73.3 ± 2.9	78.16 ± 15.9	35.7 ± 18.9	2.6 ± 4.4	75.0 ± 13.2
	15	78.3 ± 2.9	78.33 ± 7.6	30.6 ± 4.8	0.0 ± 0.0	64.1 ± 8.6
	20	68.3 ± 2.9	89.82 ± 10.0	29.9 ± 14.2	0.0 ± 0.0	68.0 ± 12.1
	25	51.7 ± 12.6	78.33 ± 5.8	22.8 ± 20.0	0.0 ± 0.0	55.0 ± 8.7
	25/10	65.0 ± 5.0	76.67 ± 2.9	15.0 ± 7.7	0.0 ± 0.0	57.4 ± 24.0
Dianthus morisianus	**5**	41.7 ± 10.4	-	0.0 ± 0.0	55.7 ± 19.7	33.3 ± 23.6
	10	96.7 ± 5.8	-	15.6 ± 13.9	86.5 ± 14.9	94.9 ± 5.0
	15	98.3 ± 2.9	-	47.6 ± 17.2	85.5 ± 12.7	98.3 ± 2.9
	20	67.9 ± 5.0	-	2.4 ± 4.1	37.9 ± 15.9	65.3 ± 13.8
	25	11.7 ± 7.6	-	2.2 ± 3.9	19.0 ± 21.3	6.7 ± 7.6
	25/10	98.3 ± 2.9	-	55.7 ± 18.2	84.8 ± 3.9	98.3 ± 2.9
Lupinus luteus	**5**	100.0 ± 0.0	-	-	-	98.3 ± 2.9
	10	100.0 ± 0.0	-	-	-	100.0 ± 0.0
	15	100.0 ± 0.0	-	-	-	100.0 ± 0.0
	20	100.0 ± 0.0	-	-	-	100.0 ± 0.0
	25	100.0 ± 0.0	-	-	-	100.0 ± 0.0
	25/10	100.0 ± 0.0	-	-	-	100.0 ± 0.0
Ptilostemon casabonae	**5**	98.3 ± 3.3	97.5 ± 3.2	0.0 ± 0.0	65.8 ± 25.0	95.0 ± 4.3
	10	100.0 ± 0.0	100.0 ± 0.0	9.5 ± 8.6	95.8 ± 6.3	100.0 ± 0.0
	15	99.2 ± 1.7	99.2 ± 1.7	42.3 ± 20.3	97.5 ± 1.7	100.0 ± 0.0
	20	97.5 ± 5.0	100.0 ± 0.0	90.6 ± 4.9	97.5 ± 3.2	100.0 ± 0.0
	25	2.5 ± 1.7	18.3 ± 11.4	0.0 ± 0.0	15.0 ± 23.3	10.0 ± 10.5
	25/10	100.0 ± 0.0	99.2 ± 1.7	94.9 ± 4.1	98.3 ± 1.9	100.0 ± 0.0

Santolina insularis	**5**	45.8 ± 4.1	47.0 ± 9.1	24.7 ± 6.4	32.8 ± 5.8	44.4 ± 12.4
	10	49.9 ± 11.4	58.0 ± 8.6	27.5 ± 7.0	29.1 ± 11.3	45.4 ± 7.7
	15	63.2 ± 6.6	54.3 ± 3.6	14.3 ± 12.1	43.0 ± 11.4	57.3 ± 5.3
	20	45.2 ± 6.8	46.4 ± 11.4	24.9 ± 14.1	28.0 ± 2.9	49.5 ± 8.7
	25	21.4 ± 9.1	48.1 ± 20.7	8.6 ± 6.2	28.8 ± 14.6	33.1 ± 5.9
	25/10	37.5 ± 16.6	51.9 ± 8.3	27.9 ± 9.4	44.2 ± 10.1	46.7 ± 4.9
Verbascum plantagineum	**5**	1.7 ± 2.9	88.3 ± 7.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	10	46.3 ± 12.0	93.3 ± 5.8	0.0 ± 0.0	0.0 ± 0.0	3.3 ± 5.8
	15	86.7 ± 18.9	86.7 ± 10.4	1.7 ± 2.9	0.0 ± 0.0	73.3 ± 7.6
	20	73.3 ± 10.4	93.3 ± 7.6	1.7 ± 2.9	3.3 ± 5.8	83.3 ± 2.9
	25	60.0 ± 30.4	93.3 ± 2.9	31.7 ± 10.4	18.3 ± 17.6	73.0 ± 5.17
	25/10	91.7 ± 2.9	91.7 ± 2.9	50.0 ± 21.8	88.3 ± 12.6	86.4 ± 3.14
Scrophularia trifoliata	**5**	48.7 ± 12.4	92.5 ± 8.8	21.5 ± 8.2	68.8 ± 8.9	23.3 ± 7.2
	10	63.0 ± 10.7	95.8 ± 5.0	24.2 ± 5.7	68.3 ± 16.0	31.9 ± 8.3
	15	80.8 ± 8.8	94.9 ± 6.0	12.6 ± 5.7	58.3 ± 8.8	53.3 ± 7.2
	20	78.9 ± 8.1	100.0 ± 0.0	7.5 ± 7.4	66.3 ± 10.6	67.0 ± 12.8
	25	50.0 ± 12.8	100.0 ± 0.0	14.6 ± 9.8	72.3 ± 5.4	35.0 ± 4.3
	25/10	91.7 ± 11.1	97.4 ± 1.7	19.4 ± 9.4	95.8 ± 3.2	86.7 ± 12.8
Digitalis purpurea var. *gyspergerae*	**5**	36.7 ± 7.2	93.3 ± 2.7	92.4 ± 6.9	1.7 ± 3.3	0.0 ± 0.0
	10	90.0 ± 5.4	96.7 ± 3.9	86.6 ± 2.7	64.1 ± 29.0	85.0 ± 7.9
	15	95.0 ± 4.3	96.7 ± 2.7	75.6 ± 5.7	94.1 ± 4.2	91.6 ± 6.5
	20	97.5 ± 1.7	96.7 ± 3.9	40.3 ± 13.4	98.3 ± 1.9	95.0 ± 4.3
	25	93.3 ± 7.2	95.8 ± 4.2	48.3 ± 13.7	80.0 ± 6.9	88.7 ± 5.0
	25/10	93.3 ± 4.7	98.3 ± 2.0	21.2 ± 7.5	95.0 ± 1.9	95.8 ± 4.2
Rhamnus alaternus ssp.*alaternus*	**5**	0.0 ± 0.0	-	41.2 ± 12.7	-	-
	10	30.0 ± 5.0	-	40.8 ± 3.8	-	-
	15	51.9 ± 25.2	-	34.8 ± 19.2	-	-
	20	61.5 ± 24.5	-	25.7 ± 14.5	-	-
	25	0.0 ± 0.0	-	13.4 ± 7.1	-	-
	25/10	10.9 ± 10.7	-	5.6 ± 5.9	-	-
Scrophularia trifoliata	**5**	77.5 ± 12.6	89.2 ± 12.0	75.9 ± 6.9	89.2 ± 5.7	75.7 ± 5.4
	10	73.3 ± 6.7	90.8 ± 5.0	44.5 ± 7.1	97.5 ± 3.2	92.5 ± 5.0
	15	86.7 ± 4.71	93.3 ± 7.2	35.8 ± 11.0	89.9 ± 2.9	92.5 ± 5.0
	20	80.0 ± 11.2	95.8 ± 3.2	19.4 ± 9.1	83.8 ± 5.4	78.3 ± 7.9
	25	33.3 ± 8.6	82.2 ± 5.5	3.5 ± 2.7	47.5 ± 5.7	37.2 ± 7.8
	25/10	83.3 ± 11.2	98.3 ± 1.9	77.5 ± 13.8	92.5 ± 5.0	84.2 ± 5.7
Helichrysum microphyllum ssp. *tyrrhenicum*	**5**	77.5 ± 6.3	94.2 ± 5.7	50.7 ± 13.2	52.9 ± 14.6	68.3 ± 14.5
	10	85.0 ± 11.4	95.0 ± 3.3	63.4 ± 10.0	92.0 ± 5.8	86.7 ± 13.6
	15	93.3 ± 7.2	93.3 ± 4.7	74.1 ± 15.6	88.3 ± 12.1	93.3 ± 3.9
	20	95.0 ± 4.3	96.7 ± 2.7	57.4 ± 19.4	91.4 ± 7.8	92.5 ± 8.8
	25	83.0 ± 0.6	92.5 ± 5.7	82.9 ± 14.4	80.3 ± 2.0	85.2 ± 10.5
	25/10	90.0 ± 11.2	96.7 ± 4.7	73.6 ± 20.7	89.0 ± 4.3	94.2 ± 1.7
Ptilostemon casabonae	**5**	92.5 ± 3.1	96.7 ± 4.7	16.7 ± 6.7	96.7 ± 2.7	75.0 ± 5.8
	10	95.0 ± 1.9	97.5 ± 1.7	80.8 ± 7.4	97.5 ± 3.2	100.0 ± 0.0
	15	96.6 ± 2.8	96.7 ± 0.0	92.5 ± 3.2	99.2 ± 1.7	100.0 ± 0.0
	20	90.8 ± 9.2	98.3 ± 1.9	89.1 ± 3.1	96.6 ± 0.1	97.5 ± 3.2
	25	0.8 ± 1.7	38.9 ± 21.1	0.8 ± 1.7	47.5 ± 20.3	0.0 ± 0.0
	25/10	97.5 ± 3.2	100.0 ± 0.0	84.2 ± 5.0	97.5 ± 3.2	98.3 ± 1.9
Santolina insularis	**5**	57.7 ± 7.2	73.9 ± 4.5	44.7 ± 12.7	45.3 ± 5.5	54.8 ± 14.8
	10	75.9 ± 7.3	69.2 ± 3.7	54.7 ± 11.7	50.1 ± 7.2	71.6 ± 8.5
	15	76.6 ± 5.4	74.8 ± 4.9	57.9 ± 10.9	60.2 ± 4.0	74.7 ± 20.0
	20	94.2 ± 4.7	92.6 ± 3.7	66.3 ± 6.9	49.1 ± 7.1	69.8 ± 2.4
	25	78.2 ± 6.9	85.4 ± 7.2	68.5 ± 8.1	60.3 ± 5.6	64.2 ± 6.8
	25/10	96.6 ± 4.2	97.8 ± 2.5	69.8 ± 4.1	64.2 ± 7.3	77.1 ± 6.9

Clematis vitalba	**5**	0.0 ± 0.0	89.2 ± 5.0	85.8 ± 9.7	0.0 ± 0.0	0.0 ± 0.0
	10	0.0 ± 0.0	79.9 ± 5.3	91.0 ± 6.2	2.7 ± 1.8	0.0 ± 0.0
	15	0.0 ± 0.0	68.8 ± 9.1	86.8 ± 6.1	0.0 ± 0.0	0.0 ± 0.0
	20	0.0 ± 0.0	68.1 ± 6.3	94.5 ± 4.4	0.0 ± 0.0	0.0 ± 0.0
	25	0.0 ± 0.0	68.8 ± 8.0	80.9 ± 7.4	0.0 ± 0.0	0.0 ± 0.0
	25/10	86.9 ± 7.0	71.9 ± 8.4	88.2 ± 5.6	84.2 ± 2.9	71.9 ± 8.4
Digitalis purpurea var. *gyspergerae*	**5**	0.0 ± 0.0	80.8 ± 10.0	78.1 ± 11.2	0.8 ± 1.7	0.0 ± 0.0
	10	67.5 ± 6.9	87.2 ± 6.5	75.4 ± 7.4	62.3 ± 8.7	70.6 ± 8.4
	15	85.0 ± 1.9	88.8 ± 7.8	63.3 ± 2.4	66.4 ± 17.9	87.7 ± 7.1
	20	98.3 ± 1.9	94.8 ± 4.3	27.6 ± 20.2	81.2 ± 6.1	94.1 ± 7.9
	25	86.0 ± 3.4	86.6 ± 9.0	42.3 ± 6.7	83.8 ± 8.3	82.6 ± 8.7
	25/10	95.0 ± 5.8	94.8 ± 6.0	15.6 ± 7.9	72.2 ± 22.6	92.5 ± 5.7
Nepeta foliosa	**5**	0.0 ± 0.0	56.2 ± 14.6	64.2 ± 15.7	9.5 ± 11.5	0.0 ± 0.0
	10	2.6 ± 4.4	66.4 ± 7.5	43.1 ± 10.3	9.3 ± 3.6	0.0 ± 0.0
	15	20.6 ± 13.1	63.3 ± 16.1	41.3 ± 10.3	24.9 ± 7.5	0.0 ± 0.0
	20	41.9 ± 17.1	85.0 ± 13.5	80.0 ± 7.0	89.6 ± 18.0	51.4 ± 21.9
	25	65.0 ± 5.0	85.1 ± 8.3	76.4 ± 6.0	54.3 ± 12.5	59.4 ± 4.1
	25/10	46.4 ± 17.3	87.3 ± 7.2	47.2 ± 15.4	42.2 ± 11.1	37.8 ± 4.0
Scrophularia trifoliata	**5**	7.5 ± 4.2	95.0 ± 4.3	21.7 ± 11.4	36.2 ± 10.4	1.7 ± 3.3
	10	16.0 ± 6.7	95.8 ± 5.1	11.7 ± 1.9	47.7 ± 26.9	8.3 ± 5.8
	15	47.5 ± 5.0	94.9 ± 3.3	10.8 ± 5.7	39.8 ± 16.6	28.3 ± 11.7
	20	57.5 ± 4.2	96.7 ± 4.7	9.2 ± 5.0	45.4 ± 4.2	49.1 ± 13.1
	25	41.6 ± 12.3	92.5 ± 5.0	4.2 ± 5.0	41.7 ± 10.4	42.8 ± 8.2
	25/10	83.3 ± 7.2	94.9 ± 4.3	27.7 ± 7.9	89.2 ± 17.3	83.0 ± 7.2
Ptilostemon casabonae	**5**	84.2 ± 5.0	100.0 ± 0.0	32.5 ± 19.8	92.2 ± 5.9	80.8 ± 8.23
	10	96.7 ± 2.7	99.2 ± 1.7	62.0 ± 16.0	100.0 ± 0.0	98.3 ± 1.9
	15	96.7 ± 0.0	98.3 ± 1.9	89.3 ± 6.5	98.1 ± 2.2	98.3 ± 1.9
	20	96.7 ± 2.7	95.0 ± 4.3	94.7 ± 6.7	99.1 ± 1.8	93.3 ± 6.7
	25	40.8 ± 6.1	84.2 ± 7.4	0.0 ± 0.0	79.2 ± 15.1	35.8 ± 18.9
	25/10	95.0 ± 5.8	95.0 ± 3.3	94.5 ± 4.8	99.2 ± 1.7	97.5 ± 3.2
Santolina insularis	**5**	20.3 ± 11.8	75.6 ± 10.5	34.6 ± 21.4	43.4 ± 14.0	36.7 ± 9.0
	10	83.6 ± 6.9	94.1 ± 4.9	66.4 ± 8.7	84.3 ± 4.7	74.1 ± 12.4
	15	94.9 ± 4.3	92.4 ± 3.3	86.9 ± 7.8	88.2 ± 3.6	85.8 ± 5.7
	20	92.4 ± 4.2	90.8 ± 3.2	88.4 ± 8.8	93.8 ± 4.4	80.6 ± 10.1
	25	93.0 ± 5.8	89.5 ± 4.3	81.7 ± 11.1	91.6 ± 9.7	83.3 ± 11.9
	25/10	95.7 ± 3.3	90.5 ± 3.1	86.8 ± 10.1	84.2 ± 11.1	85.0 ± 12.3
Ruta lamarmorae	**5**	0.0 ± 0.0	63.4 ± 13.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	10	8.7 ± 4.9	66.5 ± 8.6	6.0 ± 5.9	1.9 ± 3.2	7.2 ± 2.5
	15	9.6 ± 8.4	46.8 ± 11.5	7.3 ± 7.2	0.0 ± 0.0	32.2 ± 8.5
	20	19.9 ± 3.2	63.3 ± 2.9	5.6 ± 4.9	0.0 ± 0.0	56.3 ± 11.2
	25	12.2 ± 7.3	48.3 ± 10.4	0.0 ± 0.0	0.0 ± 0.0	26.3 ± 7.3
	25/10	1.8 ± 3.0	66.0 ± 8.6	5.6 ± 4.9	4.2 ± 3.8	14.6 ± 8.5
Lamyropsis microcephala	**5**	6.4 ± 5.5	-	8.5 ± 7.5	-	3.0 ± 5.2
	10	12.5 ± 7.8	-	3.7 ± 6.4	-	19.5 ± 9.4
	15	18.3 ± 10.9	-	10.7 ± 11.1	-	28.4 ± 15.1
	20	26.6 ± 14.4	-	55.8 ± 9.4	-	37.5 ± 5.9
	25	69.5 ± 4.8	-	73.8 ± 8.6	-	84.1 ± 11.0
	25/10	33.3 ± 30.6	-	63.3 ± 20.8	-	32.1 ± 20.8
Digitalis purpurea var. *gyspergerae*	**5**	0.0 ± 0.0	74.3 ± 7.6	66.4 ± 2.5	3.0 ± 2.0	0.0 ± 0.0
	10	7.5 ± 1.7	61.2 ± 2.7	42.7 ± 3.2	1.8 ± 3.6	1.7 ± 2.0
	15	35.8 ± 5.0	71.9 ± 3.0	60.4 ± 2.2	15.4 ± 5.1	47.8 ± 7.0
	20	52.5 ± 16.6	75.5 ± 6.8	27.7 ± 7.4	16.6 ± 12.0	63.0 ± 21.6
	25	42.5 ± 13.2	61.7 ± 8.8	38.2 ± 6.8	21.3 ± 7.7	46.0 ± 9.5
	25/10	56.7 ± 9.8	74.1 ± 7.8	38.7 ± 8.1	8.6 ± 1.8	63.0 ± 8.6

Appendix 2. GLM results for seed germination (%) of all tested species for the effect on seed germination of the following factors: "Treatment" (0, control; C, 5°C for 3 months; W, 25°C for 3 months; GA_3, 250 mg L^{-1} in the germination substrate; DAR, 25°C for 3 months on silica gel), "Temperature" (5, 10, 15, 20, 25 and 25/10°C). Species are arranged in order of altitude (from 5 m a.s.l. to 1800 m a.s.l.).

Species	**Germination (%)**	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
Brassica tournefortii	NULL			199	10090.2		
	Treatment	4	9027.7	115	1062.5	590.063	< 0.001
	Temperature	5	483.1	110	579.4	25.2584	< 0.001
	Treatment:Temperatur	20	210.8	90	368.6	2.7556	< 0.001
Silene succulenta ssp. *corsica*	NULL			71	2714.5		
	Treatment	3	408.9	68	2305.6	26.4557	< 0.001
	Temperature	5	1530.2	63	775.5	59.4045	< 0.001
	Treatment:Temperatur	15	516.5	48	259.0	6.6834	< 0.001
Scrophularia ramosissima	NULL			89	3178.6		
	Treatment	4	1228.9	85	1949.8	50.4186	< 0.001
	Temperature	5	822.7	80	1127.1	27.0065	< 0.001
	Treatment:Temperatur	20	742.5	60	384.6	6.0934	< 0.001
Helianthemum caput-felis	NULL			35	418.8		
	Treatment	1	304.14	34	114.7	84.8292	< 0.001
	Temperature	5	14.7	29	100.0	0.8212	> 0.05
	Treatment:Temperatur	5	-	-	-	-	-
Rhamnus lycioides ssp. *oleoides*	NULL			89	5062.1		
	Treatment	4	2695.4	85	2366.7	127.779	< 0.001
	Temperature	5	1289.8	80	1076.9	48.9143	< 0.001
	Treatment:Temperatur	20	747.9	60	329.1	7.0908	< 0.001
Dianthus morisianus	NULL			71	5070.1		
	Treatment	3	1168.3	68	3901.8	40.075	< 0.001
	Temperature	5	3070.3	63	831.6	63.191	< 0.001
	Treatment:Temperatur	15	297.8	48	533.8	2.043	< 0.05
Lupinus luteus	NULL			35	36.1		
	Treatment	1	6.9	34	29.1	16.3747	< 0.001
	Temperature	5	18.0	29	11.2	8.4901	< 0.001
	Treatment:Temperatur	5	-	-	-	-	-
Ptilostemon casabonae	NULL			119	10582.3		
	Treatment	4	1728.7	115	8853.6	73.2384	< 0.001
	Temperature	5	7844.3	110	1009.3	265.870	< 0.001
	Treatment:Temperatur	20	430.7	90	578.6	3.6496	< 0.001
Santolina insularis	NULL			119	1369.9		
	Treatment	4	578.6	115	791.3	31.3837	< 0.001
	Temperature	5	172.5	110	618.9	7.4836	< 0.001
	Treatment:Temperatur	20	189.8	90	429.1	2.0588	< 0.05
Verbascum plantagineum	NULL			89	7466.1		
	Treatment	4	3276.2	85	4189.9	113.852	< 0.001
	Temperature	5	2560.8	80	1629.0	71.1945	< 0.001
	Treatment:Temperatur	20	1148.7	60	480.3	7.9838	< 0.001
Scrophularia trifoliata	NULL			119	5684.2		
	Treatment	4	4073.3	115	1610.9	210.720	< 0.001
	Temperature	5	591.1	110	1019.8	24.4625	< 0.001
	Treatment:Temperatur	20	551.0	90	468.8	5.7012	< 0.001

Digitalis purpurea var. *gyspergerae*	NULL			119	5945.1		
	Treatment	4	1123.3	115	4821.8	58.737	< 0.001
	Temperature	5	1591.6	110	3230.2	66.578	< 0.001
	Treatment:Temperatur	20	2742.0	90	488.1	28.676	< 0.001
Rhamnus alaternus ssp. *alaternus*	NULL			35	1094.2		
	Treatment	1	0.71	34	1093.5	0.0775	> 0.05
	Temperature	5	480.4	29	613.1	10.5411	< 0.001
	Treatment:Temperatur	5	372.8	24	240.3	8.1809	< 0.001
Scrophularia trifoliata	NULL			119	4177.2		
	Treatment	4	1658.5	115	2518.7	97.1362	< 0.001
	Temperature	5	1588.6	110	930.1	74.4348	< 0.001
	Treatment:Temperatur	20	520.7	90	409.4	6.0993	< 0.001
Helichrysum microphyllum ssp. *tyrrhenicum*	NULL			119	2109.4		
	Treatment	4	721.8	115	1387.6	23.9530	< 0.001
	Temperature	5	393.5	110	994.2	10.4457	< 0.001
	Treatment:Temperatur	20	245.2	90	749.0	1.6271	> 0.05
Ptilostemon casabonae	NULL			119	7616.4		
	Treatment	4	722.9	115	6893.5	51.9723	< 0.001
	Temperature	5	6059.0	110	834.5	348.471	< 0.001
	Treatment:Temperatur	20	489.9	90	344.6	7.0438	< 0.001
Santolina insularis	NULL			119	1651.0		
	Treatment	4	654.0	115	997.1	48.0456	< 0.001
	Temperature	5	389.9	110	607.2	22.9153	< 0.001
	Treatment:Temperatur	20	279.2	90	328.0	4.1025	< 0.001
Clematis vitalba	NULL			119	10788.1		
	Treatment	4	5981.1	115	4807.0	859.134	< 0.001
	Temperature	5	3101.1	110	1705.9	356.352	< 0.001
	Treatment:Temperatur	20	1519.1	90	186.8	43.641	< 0.001
Digitalis purpurea var. *gyspergerae*	NULL			119	5885.5		
	Treatment	4	972.5	115	4913.0	43.564	< 0.001
	Temperature	5	1593.5	110	3319.5	57.109	< 0.001
	Treatment:Temperatur	20	2743.6	90	575.9	24.581	< 0.001
Nepeta foliosa	NULL			119	4131.4		
	Treatment	4	1276.0	115	2855.5	45.3582	< 0.001
	Temperature	5	1637.3	110	1218.2	46.5620	< 0.001
	Treatment:Temperatur	20	761.9	90	456.3	5.4165	< 0.001
Scrophularia trifoliata	NULL			119	6585.7		
	Treatment	4	3962.4	115	2623.3	164.579	< 0.001
	Temperature	5	1366.3	110	1257.0	45.401	< 0.001
	Treatment:Temperatur	20	693.9	90	563.1	5.764	< 0.001
Ptilostemon casabonae	NULL			119	5003.6		
	Treatment	4	1225.6	115	3777.9	64.9447	< 0.001
	Temperature	5	2876.7	110	901.3	121.943	< 0.001
	Treatment:Temperatur	20	425.9	90	475.4	4.5134	< 0.001
Santolina insularis	NULL			119	2942.4		
	Treatment	4	228.2	115	2714.2	9.7783	< 0.001
	Temperature	5	1875.3	110	839.0	64.2976	< 0.001
	Treatment:Temperatur	20	274.5	90	564.4	2.353	< 0.01

Ruta lamarmorae	NULL			89	3450.6		
	Treatment	4	2539.2	85	911.4	197.415	< 0.001
	Temperature	5	194.8	80	716.7	12.113	< 0.001
	Treatment:Temperatur	20	485.6	60	231.1	7.550	< 0.001
Lamyropsis microcephala	NULL			53	2101.3		
	Treatment	2	30.8	51	2070.5	1.4871	> 0.05
	Temperature	5	1450.2	46	620.3	27.9795	< 0.001
	Treatment:Temperatur	10	189.1	36	431.2	1.8240	> 0.05
Digitalis purpurea var. *gyspergerae*	NULL			119	4210.1		
	Treatment	4	1958.1	115	2252.0	145.51	< 0.001
	Temperature	5	588.9	110	1663.1	35.01	< 0.001
	Treatment:Temperatur	20	1349.1	90	314.1	20.05	< 0.001

Appendix 3. Base temperature (T_b), and thermal constant for 50% germination (S) results for all the tested species after the different treatments (0, control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for 3 months; W, 25°C for 3 months; DAR, 25°C for 3 months on silica gel). Species are arranged in order of altitude (from 5 m a.s.l. to 1800 m a.s.l.).

Species	Treatments									
	0		GA_3		C		W		DAR	
	T_b	S	T_b	S	T_b	S	T_b	S	T_b	S
Brassica tournefortii	5*	-	4.4	17.0	5*	-	5*	-	5*	-
Silene succulenta ssp. *corsica*	5*	-			5*	-	5*	-	5*	-
Scrophularia ramosissima	3.0	178.6	2.4	125.0	3.3	97.1	5*	-	2.6	212.8
Helianthemum caput-felis	1.1	22.2							0.4	41.3
Rhamnus lycioides ssp. *oleoides*	-4.9	357.1	5.6	123.5	5*	-	10*	-	-2.4	312.5
Dianthus morisianus	1.5	58.1			10*	-	1.8	58.5	0.1	66.7
Lupinus luteus	-9.1	92.6							-4.5	81.3
Ptilostemon casabonae	0.2	104.2	0.8	107.5	10*	-	0.9	138.9	2.0	85.5
Santolina insularis	3.2	208.3	-0.8	196.1	5*	-	5*	-	5*	-
Verbascum plantagineum	4.5	98.0	2.5	85.5			5*	-	6.3	77.6
Scrophularia trifoliata	2.3	119.1	0.8	103.1	5*	-	0.9	238.1	5*	-
Digitalis purpurea var. *gyspergerae*	-4.9	243.9	0.7	123.5	0.6	204.1	7.4	55.6	-4.7	217.4
Rhamnus alaternus ssp. *alaternus*	10*	-			5*	-				
Scrophularia trifoliata	-0.6	133.3	-0.6	133.3	5*	-	0.3	147.1	0.3	158.7
Helichrysum microphyllum ssp. *tyrrhenicum*	-1.0	95.2	-2.8	85.5	3.0	55.3	1.6	126.6	1.5	78.7
Ptilostemon casabonae	-2.7	158.7	-0.6	96.2	5*	-	2.0	98.0	0.2	108.7
Santolina insularis	-1.2	169.5	-4.6	166.7	2.3	73.0	5*	-	-0.9	178.6
Clematis vitalba			-0.0	344.8	1.5	24.5	10*	-		
Digitalis purpurea var. *gyspergerae*	0.4	119.1	-4.0	158.7	3.7	69.4	9.4	69.4	7.9	67.6
Nepeta foliosa	10*	-	4.1	227.3	1.3	212.8	5*	-	20*	-
Scrophularia trifoliata	5*	-	1.0	96.2	5*	-	3.3	131.6	5*	-
Ptilostemon casabonae	2.0	71.4	0.4	78.1	3.0	99.0	1.6	90.9	-1.6	108.7
Santolina insularis	3.5	59.9	2.3	52.9	6.5	33.1	0.2	68.0	4.4	62.5
Ruta lamarmorae	10*	-	3.7	92.6	10*	-	10*	-	10*	-
Lamyropsis microcephala	5*	-			5*	-			5*	-
Digitalis purpurea var. *gyspergerae*	10*	-	2.0	81.3	0.5	126.6	5*	-	12.0	39.5

(*) Represent estimated values.

Chapter 2: Thermal time requirements for seed dormancy loss and germination of three *Rhamnus* species along an altitudinal gradient

Introduction

By definition, germination commences with the uptake of water by the dry seed and it is completed when a part of the embryo, usually the radicle, extends to penetrate the structures that surround it (Bewley, 1997). In some species germination at unfavorable times is prevented by a mechanism that is commonly described as dormancy. Dormancy is a physiological state in which a seed disposed to germinate does not, even in the presence of favorable environmental conditions (Bonner, 1984). Seed dormancy has probably evolved differently across species, through adaptation to various habitats, climatic conditions and prevailing environments, so that germination occurs when conditions for establishing a new plant generation are likely to be suitable (Fenner and Thompson, 2005; Finch-Savage *et al*., 2007; Baskin and Baskin, 2014). Baskin and Baskin (2004) have proposed a comprehensive classification system which includes five classes of seed dormancy: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) and combinational (PY + PD).

PD is the most prevalent form of seed dormancy (Baskin and Baskin, 2004, 2014) and can be triggered by divergent environmental cues. Along an altitudinal gradient, PD is expected to be more represented in species of high altitudes due to more severe autumn-winters temperature, and in many species chilling, followed by favourable temperatures, represents a natural dormancy breaking mechanism (Baker, 1989; Probert, 1992; Vera, 1997). In particular, several authors have reported that cold stratification improves germination performance in many arctic and alpine species, promoting germination in late spring, under higher temperatures, with a lower probability of damage the new seedlings because of frost (Billings and Mooney, 1968; Cavieres and Arroyo, 2000; Körner, 2003; Schwienbacher *et al*., 2011; Baskin and Baskin, 2014). However, in several Mediterranean species, cold stratification is not supposed to enhance seed germination and in some cases may have also a detrimental effect and/or induce secondary dormancy (Skordilis and Thanos, 1995; Mattana *et al.*, 2012; Giménez-Benavides and Milla, 2013).

By contrast, PY is more common in species from Mediterranean lowland habitats where high fluctuating soil temperatures (McKeon and Mott, 1982) and fire can break it (Thanos and Georghiou, 1988; Doussi and Thanos, 1994; Baskin and Baskin, 2003, 2014).

Different classes of dormancy are reported for different species belonging to the same family; in this sense, regarding members of the Rhamnaceae family, the seeds can be non-dormant (ND) or can show PD, PY or PY + PD (Nikolaeva, 1977; Baskin and Baskin, 2004, 2014). Walck *et al*. (2012) reported that PY is the most represented dormancy class in this family (present in ca. 61% of the investigated species), followed by PY + PD (22%), PD (12%) and ND (6%).

In the genus *Rhamnus*, the sclerophyllous woodland species mostly have PY while the temperate deciduous species have PD (Baskin and Baskin, 2014; Mattana *et al*., 2009; Porceddu *et al.*, 2013). In particular, PD was identified for *R. alaternus* L. (García-Fayos *et al*., 2001), *R. alnifolia* L'Hér (Hubbard, 1974), *R. caroliniana* Walter (Nokes, 1986), *R. frangula* L. (Heit, 1968), *R. lanceolata* Pursh (Sharma and Graves, 2005), *R. persicifolia* Moris (Porceddu *et al.*, 2013) and *R. purshiana* DC. (Radwan, 1976); PY for *R. californica* Eschsch. and *R. crocea* Nutt (Keeley, 1987) and ND for *R. cathartica* (Tylkowsky, 2007).

There are many studies on the change in the rate of germination with increase in temperature, which reveal that the thermal responses of species and local populations of plants vary (Thompson, 1970). When seeds are subjected to temperatures above a base temperature for germination (T_b), the germination rate increases linearly with temperature up

to optimum temperature (T_o), above which germination rate decreases (i.e. the sub-optimal temperature range). Therefore, when this relation is linear, the thermal time or accumulated temperature for germination is a constant which can be used to describe and compare germination in seed lots of different species, from varying climates and disparate locations (Garcia-Huidobro *et al*., 1982).

Treatments for dormancy release can modify the T_b for seed germination (Pritchard *et al*., 1999; Steadman and Pritchard, 2004; Orrù *et al*., 2012; Porceddu *et al*., 2013), and the widening of the range of temperatures for germination could be used as a surrogate for the efficient removal of dormancy. Using germination as a "substitute" measure of dormancy, seeds' performance is became a descriptor of change in seedlots dormancy status. With this approach the thermal niche requirements for dormancy release and germination were quantified (Cochrane *et al*., 2011; Baskin and Baskin, 2014).

In this study, the germination ecophysiology of three different *Rhamnus* species that grow in Sardinia: *R. alaternus* ssp. *alaternus*, *R. lycioides* L. ssp. *oleoide*s (L.) Jahand. & Maire and *R. persicifolia*, which differ for their distribution according to their ecology, is investigated. Recent studies on the germination ecology of *R. persicifolia* have reported that chilling released PD (Mattana *et al*., 2009) by reducing T_b values and thermal times requirements (θ_{50}) (Porceddu *et al*., 2013). However, there is no data available about thermal requirements for seed germination of the other two *Rhamnus* species.

The aims of this work were to: (1) characterize the requirements for physical and physiological dormancy loss and germination of *R. lycioides* ssp. *oleoides*, *R. alaternus* ssp. *alaternus* and *R. persicifolia* which differ on habitat and climatic conditions; and (2) evaluate the effect of cold stratification on the thermal requirements for dormancy loss and germination of these three species.

Material and methods

Study species

In this study, three dioecious species of *Rhamnus* genus were investigated; each produces fleshy fruits and seeds that are covered by an endocarp that opens when the fruit pulp is removed.

Rhamnus lycioides ssp. *oleoides* (hereafter *R. oleoides*) is a perennial shrub, naturally occurring in the Western Mediterranean Basin (de Bolòs *et al*., 2005). It can be found from sea level up to 1000 m a.s.l., usually in Mediterranean thermo-xerophilous shrublands. It flowers in spring and fruits (berries) ripen during late spring and early summer and usually bear one to three seeds (Gulías and Traveset, 2012).

Rhamnus alaternus ssp. *alaternus* (hereafter *R. alaternus*) is a perennial shrub distributed along the Mediterranean Basin. It is common in shrublands and oak woods of the Mediterranean region (Bas *et al*., 2005), and it has also the ability to survive in xeric environments. The flowering occurs during late winter and early spring, with a peak in mid-February. It produces fleshy fruits that ripen in late spring and early summer (Gulías *et al*., 2004). Fruits usually bear two or three seeds (Bas *et al*., 2002).

Rhamnus persicifolia is a small tree or shrub. It is endemic to Central-Eastern Sardinia, occurring at 500-1500 m a.s.l. on both limestone and siliceous substrata. Mainly it grows in riparian woods or hygrophilous scrubs along mountainous waterways and deep gorges (Arrigoni, 1977; Mattana *et al*., 2009). The flowering occurs in late spring and fruiting only in late summer and early autumn. *R. persicifolia* is included in the Italian Red Book as vulnerable (Conti *et al*., 1992, 1997), mainly for its estimated narrow distribution and population decline induced by human activities, which generated a continued degradation of the riparian forest of Sardinia (Arrigoni, 1977), and by climate change (Porceddu *et al.*, 2013).

Seed lot details

Fruits of *R. lycioides* and *R. alaternus* were collected directly from the mother plants in their natural populations at the time of fruit ripening (Table 1). In *R. lycioides* the fruits usually bear one to three seeds (Gulías and Traveset, 2012) while in *R. alaternus* every fruit has 2–5 seeds (Bas *et al*., 2002). In both species, seeds are covered by an endocarp that opens when the fruit pulp is removed, therefore consist in stones / pyrenes (Bas *et al*., 2002, 2005; Gulías and Traveset, 2012; Aou-ouad *et al*., 2014). In *R. persicifolia* drupes contain 3(4) pyrenes (hereafter seeds) each, with the fourth generally being aborted / empty (Mattana *et al*., 2009). After collection, seeds were immediately separated from the fleshy fruits by rubbing through sieves under running water. The cleaned seeds were then spread out and left to dry at ca. 20°C and 40% RH, until the experiments started, as specified below.

Data on seed germination of *R. persicifolia* were taken from Porceddu *et al*. (2013). Seeds of *R. persicifolia*, collected in 2011 and stored at the Sardinian Germplasm Bank (BG-SAR), were removed from the active collection (+5ºC) and used for the analysis of the imbibition rate; fruit collecting and seed processing methods used for this species were the same applied for the other two species object of study.

Table 1 – Seed lots details and ecological information on the three investigated *Rhamnus* species.

Species	Locality	Coordinates (WGS84)	Altitude (m a.s.l.)	Substrate	Bioclimate	Habitat	Seed collecting
Rhamnus oleoides	Perdu Collu (Pula, Cagliari), SW Sardinia	N 39°00’ E 8°56’	60	Alluvials	Thermomediterranean / Dry	Mediterranean shrublands	10/08/2012
Rhamnus alaternus	Monte Santa Barbara (Capoterra, Cagliari), SW Sardinia	N 39°08’ E 8°56’	505	Granites	Mesomediterranean / Subhumid	Mediterranean woodlands	12/07/2013
Rhamnus persicifolia	Rio Correboi (Villagrande Strisaili), CE Sardinia	N 40°04’ E 9°20’	1120	Metamorphic rocks	Supramediterranean / Humid	Mediterranean riparian woods	30/09/2011

Imbibition curve

Imbibition tests were performed for seeds of the three *taxa* in order to study the rate of seed imbibition in a fixed period. Three replicates of 20 seeds each were used to evaluate the rate of water uptake in scarified (by chipping the seeds with a scalpel) and non-scarified seeds. Seeds were placed in small glass bottles with distilled water and incubated in the light (12 h light / 12 h dark) at 20°C. Initial seed mass (t_0) was determined and then changes in mass were monitored: seeds were weighted at 1-h intervals for the first 12 h, and then every 24 h for the next 96 h (four days). Percentage water uptake was calculated following Hidayati *et al*. (2001) in relation to seed mass at t_0:

$$\%W_s = [W_i - W_d)/W_d] \times 100 \quad (1)$$

where W_s = increase in mass of seeds, W_i = mass of seeds after a given interval of imbibition, and W_d = seed mass at t_0.

Germination tests

Three replicates of 20 seeds of *R. oleoides* and *R. alaternus* were sown on the surface of 1% agar water in 90-mm diameter plastic Petri dishes. Seeds were incubated in the light (12 h light / 12 h dark) for a maximum of four months at a range of constant (10, 15, 20 and 25°C) and an alternating germination temperatures (25/10°C). Further replicates were given for pre-chilling treatment (C) where seeds were incubated for three months at 5°C in 1% agar water in 90 mm diameter plastic Petri dishes. At the end of this pre-treatment, seeds were incubated at the germination temperatures above mentioned.

Germination, defined as visible radicle emergence (> 1 mm), was recorded three times a week. At the end of the germination tests, when no additional germination had occurred for two weeks, a cut-test was carried out to determine the viability of the remaining seeds and the number of empty seeds.

In this work, germination data achieved from *R. oleoides* and *R. alaternus* were compared with results reported in literature for *R. persicifolia* by Porceddu *et al.* (2013).

Data analysis

Final germination percentages were calculated on the basis of the total number of filled seeds as the mean of the three replicates (± S.D.). During C pre-treatment, seed germination was recorded and when seeds germinated before moving to the incubation temperatures, these where not considered in the final germination percentages.

A thermal time approach was used to describe the temperature dependence of germination for seeds germinating at constant temperatures for untreated (0, Control) and chilled (C) seeds.

Estimates of time (t_g, d) taken for cumulative germination to reach different percentiles (g) for successive increments of 10% germination were then interpolated from the germination progress curves (Covell *et al*., 1986).

The germination rate ($1/t_g$) was regressed, using linear models to estimate the base temperature (T_b) [see eqn (1)] at which the germination rate is equal to zero, by averaging the x-intercept for the suboptimal temperature ranges (Ellis *et al*., 1986). When the rate is linear, extrapolation defines a base temperature (T_b) and the thermal response of germination can be expressed as the following equation (Garcia-Huidobro *et al*., 1982):

$$1/t_g(d^{-1}) = (T_g - T_b)/\theta \quad (2)$$

Linear regression equations were then recalculated for each percentile, but constrained to pass through T_b (Hardegree, 2006).

A comparison of regressions was then made between this model and one in which the T_b were allowed to vary for all the percentiles, and the best estimate was considered to be that which resulted in the smallest residual variance (Covell *et al*., 1986). Thermal time (θ, °Cd) estimates for 0 and C were then calculated separately as the inverse of the suboptimal regression equations (Covell *et al*., 1986).

Germination percentages were transformed to probits using tabular data from Finney (1971). Linear regression was used to express probit (g) as a function of thermal time (θ_g) and the form of cumulative germination response of seeds described by the equation (Covell *et al*., 1986):

$$\text{probit}(g) = K + \theta_g / \sigma \quad (3)$$

where K is an intercept constant when θ_g is zero, and σ is the standard deviation of the response to θ_g (i.e. the reciprocal of the slope), and represents the sensitivity of the population to θ_g (Covell *et al*., 1986). θ_g may be normal or log-normal distributed (and the best model was evaluated on the basis of the r^2 values; Hardegree, 2006).

The thermal constant (S) expressed in ºCd, given by the reciprocal of the slope of the linear regression (Garcia-Huidobro *et al*., 1982; Trudgill *et al*., 2000), was also calculated for each seed lot, both for 0 and C treated seeds.

Regression analyses were carried out using SigmaPlot Version 11.0 (Systat Software, Inc., San Jose California USA).

Estimated T_b and θ_{50} obtained for *R. oleoides* and *R. alaternus* were then compared with the values of *R. persicifolia* (Porceddu *et al.*, 2013). T_b values estimated for each species were fitting using a linear regression in order to evaluate the relationship between T_b and stratification time.

Statistical analysis

Generalized Linear Models (GLMs) were used to compare final germination percentages and base temperature (T_b) for germination. GLM with a logit link function and quasibinomial error structure was used for analysing germination percentages, while a GLM with a log link function and quasipoisson error structure was used for analysing T_b values. Quasipoisson and quasibinomial error structures and F tests with an empirical scale parameter instead of chi-squared on the subsequent analysis of variance (ANOVA) were used in order to overcome residual overdispersion (Crawley, 2007). Significant differences were analysed by a *post-hoc* pairwise comparisons *t*-test (with Bonferroni adjustment). All statistical analyses were carried out with R v. 3.0.3 (R Development Core Team, 2014).

Results

Imbibition tests

During the first 10 hours of the imbibition test, scarified seeds of each species imbibed faster in than the non-scarified ones. However, in the remaining hours, the rate of increasing of mass between them was similar and tended to reach a same limit point regardless of the scarification. Specifically, during the first 10 hours of imbibition test, non-scarified seeds of *R. oleoiodes* had imbibed water equal to 46% of the initial weight while scarified seeds increased their mass up to 57% (Fig. 1). A lower increase in mass was reached by *R. alaternus*, where non-scarified seeds increased in mass up to ca. 38% whereas scarified seeds reached approximately 42%. Finally, non-scarified seeds of *R. persicifolia* had imbibed water up to 50%, while scarified seeds increased their mass up to 60% (Fig. 1).

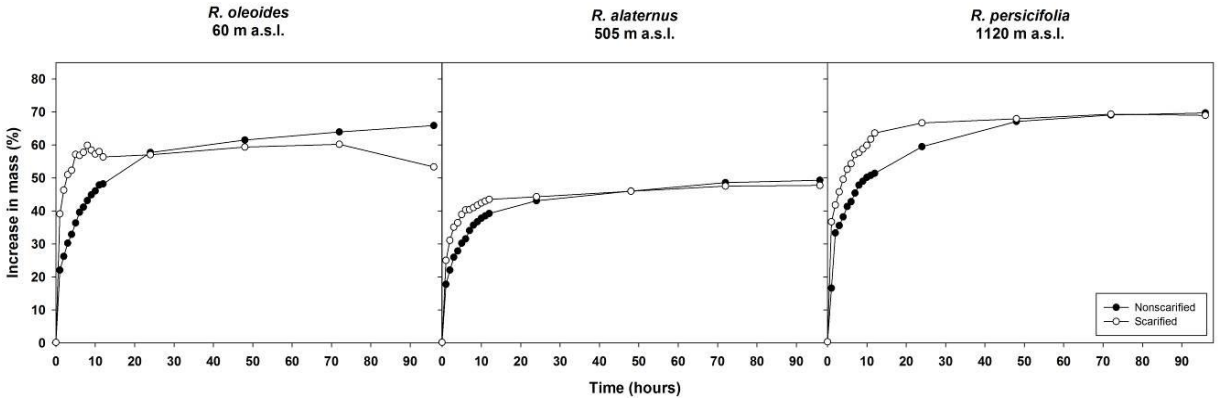

Figure 1 - Water uptake in non-scarified and scarified seeds of *R. oleoides*, *R. alaternus* and *R. persicifolia*, collected at 60, 505 and 1120 m a.s.l., respectively. Seeds incubated at room temperature (ca. 20°C) on deionized water for a total of 96 hours.

Seed germination

GLMs highlighted a statistically significant effect of the treatment, temperature and species and on their interactions ($P < 0.001$; Table 2).

Table 2 - GLMs results for the final seed germination (%) for the following factors: "Treatment" (0, Control; C, 5°C for three months), "Temperature" (10, 15, 20, 25 and 25⁄10°C), "Species" (*R. oleoides*, *R. alaternus*, *R. persicifolia*) and their interactions.

Germination (%)	Df	Deviance	Resid. Df	Resid. Dev	F	P (>F)
NULL			89	3444.9		
Treatment (Treat)	1	63.73	88	3381.2	9.2792	0.003441 **
Temperature (Temp)	4	301.40	84	3079.8	10.9718	9.629e-07 ***
Species	2	1080.29	82	1999.5	78.6505	< 2.2e-16 ***
Treat:Temp	4	261.03	78	1738.5	9.5021	5.107e-06 ***
Treat:Species	2	665.84	76	1072.6	48.4763	2.961e-13 ***
Temp:Species	8	357.64	68	715.0	6.5095	4.431e-06 ***
Treat:Temp:Species	8	273.46	60	441.5	4.9773	9.380e-05 ***

R. oleoides seeds germinated between ca. 52% (25°C) and 78% (15°C), while *R. alaternus* seeds reached values from ca. 10 to 62%, for 25/10°C and 20°C respectively, with no germination at 25°C (Fig. 2).

Cold stratification (C) negatively affected germination of *R. oleoides* and *R. alaternus* seeds, indeed in both cases was < 50%. On the contrary, a positive effect of C was reported for seeds of *R. persicifolia* (Fig. 2). In addition, the effect of C for each *Rhamnus* species was more evident at low temperatures, showing the highest germination percentage at 10°C (36 ± 19%, 41 ± 4%, and 92 ± 8%, for *R. oleoides*, *R. alaternus* and *R. persicifolia*, respectively; Fig. 2). The lowest germination percentages (< 20%) were recorded by *R. oleoides* and *R. alaternus* at 25/10°C, whereas at the same alternate temperature *R. persicifolia* germinated with 68% (Fig. 2).

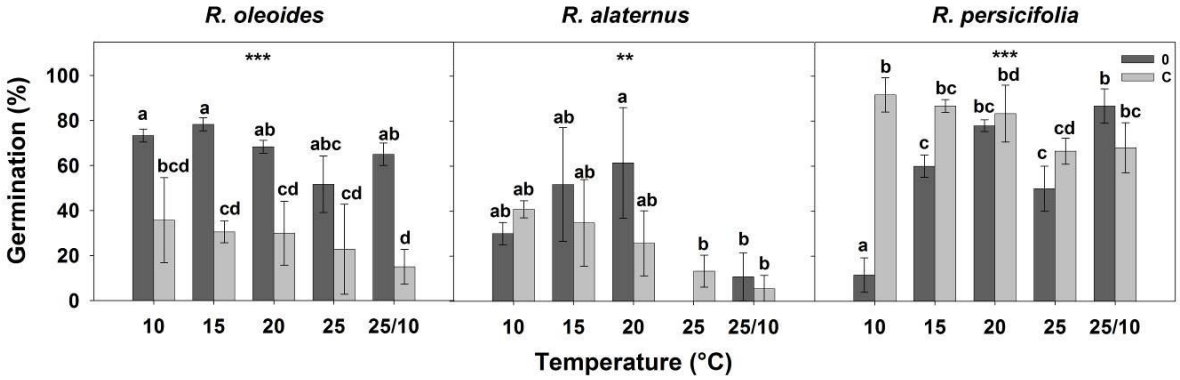

Figure 2 - Final germination percentages of *R. oleoides*, *R. alaternus* and *R. persicifolia* seeds. Data were achieved at the end of germination tests after 0 (Control) and C (5°C for three months) and are the mean of the three replicates (1 ± S.D.). GLM was carried out to test differences in values. *Post hoc* pairwise *t*-test comparisons (with Bonferroni adjustment) were carried out, and bars with different letters indicate significant ($P < 0.05$) differences.

Thermal thresholds for seed germination

GLMs highlighted a statistically significant effect of the species and treatment factors ($P < 0.001$) on T_b of the study species. Also their interactions were statistically significant ($P < 0.001$; Table 3).

Table 3 - GLMs results for base temperature of germination (T_b) of the following factors: "Species" (*R. oleoides*, *R. alaternus*, *R. persicifolia*), "Treatment" (0, Control; C, 5°C for three months) and their interactions.

Base temperature (T_b)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
Null			32	69.509		
Species	2	16.130	30	53.379	42.357	4.72e-09 ***
Treatment (Treat)	1	42.569	29	10.809	223.568	1.39e-14 ***
Species:Treat	2	4.815	27	5.995	12.643	0.0001334 ***

For untreated seeds (Control, 0), average T_b values were 4.67 ± 0.89°C, 3.25 ± 0.17°C and 10.45 ± 0.62°C for *R. oleoides*, *R. alaternus* and *R. persicifolia*, respectively (Fig. 3). The T_b values were statistically different ($P < 0.001$) by GLM, and the *post hoc* pairwise *t*-test comparison highlighted significant differences among all species (Fig. 3). Fig. 3 reported also the T_b values of C, where it is observed that for each species, chilling decreased T_b values respect to the Control (0). In particular, seeds of *R. oleoides* decreased from ca. 5 to ca. 0°C, while in *R. alaternus* T_b values were similar in both treatments with values near to 3°C (Fig. 3). T_b in seeds of *R. persicifolia* varied from approx. 10°C for non-treated seeds to approx. 3°C for cold-stratified ones. Furthermore, GLM for C treated seeds detected that T_b values were statistically different ($P < 0.05$) between the species, and the *post hoc* pairwise *t*-test comparison highlighted statistical differences among *R. oleoides* and *R. persicifolia* (Fig. 3).

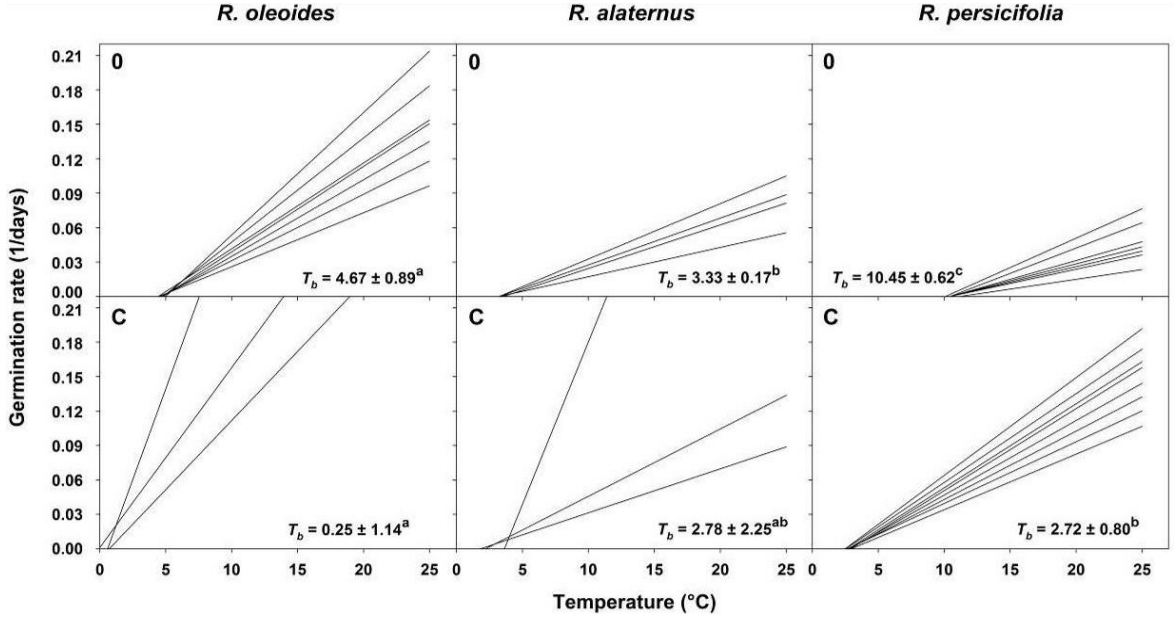

Figure 3 - Base temperatures for germination (T_b) calculated for different germination percentiles after Control test (0) and pre-chilling (C; three months at 5°C) of *R. oleoides*, *R. alaternus* and *R. persicifolia* seeds, collected at 60, 505 and 1120 m a.s.l., respectively. The linear regressions were constrained to the common value of T_b. Statistical differences among species under 0 and C treatment were analysed by GLM followed by *post hoc* pairwise *t*-test comparisons (with Bonferroni adjustment). Mean T_b values with different letters are significantly different at $P < 0.05$.

A negative correlation between T_b and the stratification period at 5°C was highlighted for *R. oleoides* and *R. persicifolia*, where distinctly T_b decreased with increasing of the stratification time in days. While, for *R. alaternus* T_b does not seem to have a decrease with the increasing of stratification period (Fig. 4).

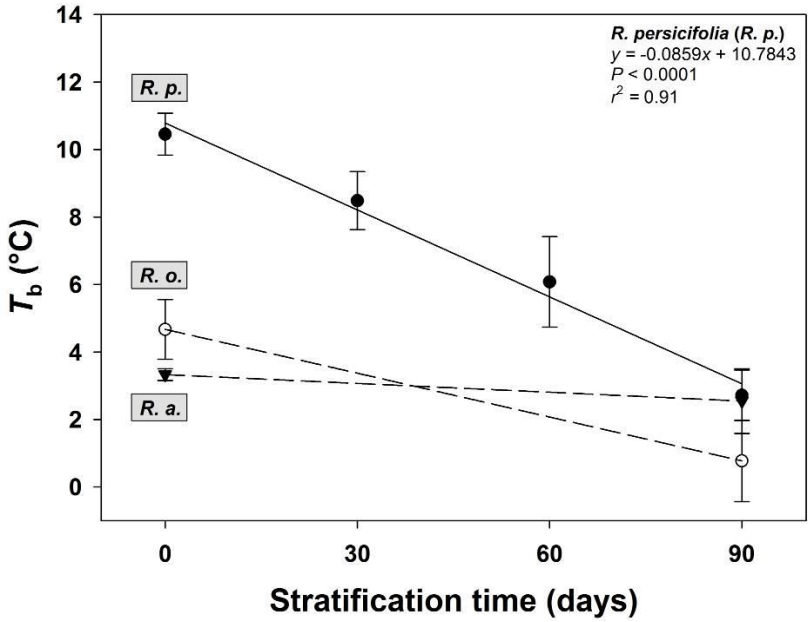

Figure 4 - Relationship between T_b and stratification time in days of *R. oleoides* (*R. o.*), *R. alaternus* (*R. a.*) and *R. persicifolia* (*R. p.*). Data are the mean ± S.D. of T_b of each percentile. Dashed lines represent the theoretical relationship between T_b and stratification time calculated at 0 and 90 days of cold stratification for R. o and R. a.

Fig. 5a shows the relationship between log thermal time (θ) and germination expressed in probits for 0, calculated according to eqn (3). The relationship between log θ and probit germination had a better fit (r^2 0.95 and 0.97, for *R. persicifolia* and *R. oleoides*, respectively) than when expressed on a linear scale (data not shown). *R. persicifolia* highlighted a greater θ_{50} (2.59 log°Cd) compared to *R. oleoides* (2.20 log°Cd), while *R. alaternus* did not reach the 50% of germination, but can be estimated to be ca. 2.63 log°Cd (Fig. 5a).

The relationship between θ and germination for C was evaluated only for *R. persicifolia*, showing a θ_{50} value of 2.19 log °Cd, unlike *R. oleoides* and *R. alaternus* which did not reach the 50% of germination, but can be estimated to be ca. 2.43 log °Cd and ca. 2.76 log °Cd; respectively (Fig. 5b).

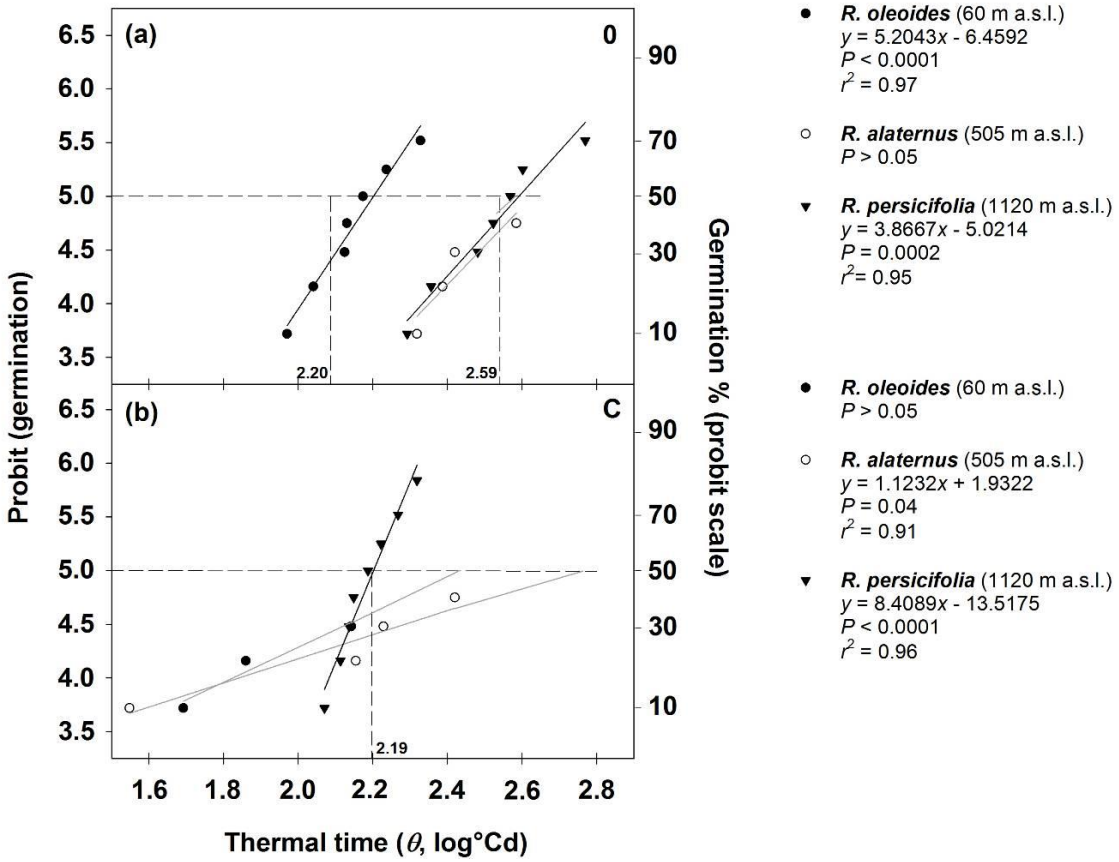

Figure 5 - Probit germination for *R. oleoides*, *R. alaternus* and *R. persicifolia* seeds, as a function of log-thermal time requirement after 0 "Control" (a), and pre-chilling "C; three months at 5°C" (b) . Thermal times to reach θ_{50} are also shown (dashed lines). Grey line corresponds to θ estimated for *R. alaternus* in (a) and for *R. oleoides* and *R. alaternus* in (b).

Discussion

Scarification was reported to release dormancy efficiently in species which present PY (Baskin and Baskin, 2004, 2014). The imbibition test carried out in the study species showed similar rate of increasing seed mass between scarified and non-scarified seeds. Thus, we detected that seeds of the study species have no PY. It is reported that many matorral shrubs have seeds (or fruits) with physical dormancy (Baskin and Baskin, 2014). In agreement, Keeley (1987) found PY for two *Rhamnus* species of sclerophyllus woodlands, *R. californica* and *R. crocea*. Since *R. oleoides and R. alaternus* are common species of Mediterranean shrublands, which sprout vigorously after fire, even if their seeds can not withstand to high fire temperatures (García-Fayos *et al*., 2001), we expected that both species were more inclined to show PY. However, this was not detected for *R. oleoides* and *R. alaternus*.

For many plant species, cold stratification is known to increase germination (Bewley and Black, 1982), while, others are not affected by cold stratification, and in the cases where they are, germination is generally lower after stratification, not higher (Luna *et al*., 2008). Germination tests performed for the three species highlighted different responses to stratification and temperature. In particular, germination of *R. oleoides* appears negatively influenced by cold stratification. This behaviour may be interpreted as a mechanism to stop germination after winter, preventing plants from late germination in spring, which would leave them facing summer drought without a deeply developed root system (Luna *et al*., 2008). By contrast, results obtained from *R. persicifolia* showed that seed germination was positively influenced by chilling (Porceddu *et al*., 2013). This is in agreement with previous studies which reported that seeds from populations encountering long periods with snow cover and adverse winter conditions would require longer periods of cold stratification for germination than those from populations exposed to milder winters (Billings and Mooney, 1968; Meyer *et al.* 1995; Pendleton and Meyer 2004; Wang *et al.*, 2010). Our results illustrate a significant variation in germination characteristics over a spatially short altitudinal gradient. Therefore, seed germination responses to stratification and temperature reflect a climate-adapted strategy on the timing of seedling emergence (Skordilis and Thanos, 1995).

Different values of T_b were detected for untreated seeds of the three *Rhamnus* species. Chilling clearly reduced T_b values in *R. oleoides* and *R. persicifolia* seeds, although the effect of pre-treatment was more evident for the latter species. This suggests that seeds from higher elevations may have a higher degree of dormancy than those from lower altitudes (Vera, 1997; Cavieres and Arroyo, 2000; Fernández-Pascual *et al*., 2013; Baskin and Baskin, 2014; Zhou and Bao, 2014).

Seeds of all the three *Rhamnus* species varied in their thermal time estimates (θ_{50}). Specifically, the estimated θ_{50} of untreated seeds of *R. oleoides* and *R. alaternus* increased with cold stratification. By contrast, Porceddu *et al*. (2013) reported an opposite trend for *R. persicifolia* where chilling reduced θ_{50} value of untreated seeds. These results highlighted that seed provenance may influence the sensitivity of the seed germination response to thermal time (Daws *et al*., 2004; Orrù *et al.*, 2012; Porceddu *et al*., 2013). Finally, these investigations contribute to understand how germination timing can be affected by environmental change.

Conclusion

The results of this study highlighted that *R. oleoides*, *R. alaternus* and *R. persicifolia* have not PY.

In addition, it is showed that seed germination responses are consistent with environmental cues which seeds are exposed. Cold stratification negatively affected germination of *R. oleoides*, and to a less extent, also of *R. alaternus*, suggesting that germination for these two species is limited to winter, during the rainy season. The analysis of the thermal time confirmed that species have different sensitivities in the accumulation of thermal units (°Cd) according their provenance. Therefore, the thermal time requirements may represent a useful instrument for evaluating plant ecological responses to climate change.

References

Anderberg A.L., 1994. Atlas of seeds and small fruits of Northwest-European plant species with morphological descriptions. Part 4 Resedaceae-Umbelliferae. Swedish Museum of Natural History, Stockholm, Sweden.

Aou-ouad H.E., Medrano H., Lamarti A., Gulías J., 2014. Seed germination at different temperatures and seedling emergence at different depths of *Rhamnus* spp. Central European Journal of Biology, 9(5): 569–578.

Arrigoni P.V., 1977. Le piante endemiche della Sardegna: 2–4. Bollettino della Società Sarda di Scienze Naturali, 16: 269–280.

Association of Officinal Seed Analysts, 1981. Rules for testing seeds. Journal of Seed Technology, 6: 1–126.

Baker G.H., 1989. Some aspects of the natural history of seed banks. In: Leck, M.A., Parker, V.T. & Simpson, R.L. (eds), Ecology of soil seed banks, Academic Press, San Diego.

Bas J.M., Gómez C., Pons P., 2002. Caracterización morfológica y estructural de los frutos del aladierno (*Rhamnus alaternus* L.) en el Noreste de la Península Ibérica. Studia Botanica, 21: 89–103.

Bas J.M., Gómez C., Pons P., 2005. Fruit production and predispersal seed fall and predation in *Rhamnus alaternus* (Rhamnaceae). Acta Oecologica, 27: 115–123.

Baskin J.M., Baskin C.C., 2004. A classification system for seed dormancy. Seed Science Research, 14: 1–16.

Baskin C.C., Baskin J.M., 2014. Seeds: ecology, biogeography, and evolution of dormancy and germination. San Diego: Academic Press.

Bewley J.D., Black M., 1982. Physiology and Biochemistry of Seeds. 2. Viability, Dormancy, and Environmental Control. Springer-Verlag, Berlin.

Bewley J.D., 1997. Seed germination and dormancy. The Plant Cell, 9: 1055–1066.

Billings W.D., Mooney H.A., 1968. The ecology of arctic and alpine plants. Biological Reviews, 43: 481–529.

Bonner F.T., 1984. Glossary of seed germination terms for tree seed workers. General Technical Report SO-49. New Orleans: USDA Forest Service, Southern Forest Experiment Station, 4 p.

Cavieres L.A., Arroyo M.T.K., 2000. Seed germination response to cold stratification period and thermal regime in *Phacelia secunda* (Hydrophyllaceae) - Altitudinal variation in the mediterranean Andes of central Chile. Plant Ecology, 149: 1–8.

Cochrane A., Daws M.I., Hay F.R., 2011. Seed-based approach for identifying flora at risk from climate warming. Austral Ecology, 36: 923–935.

Conti F., Manzi A., Pedrotti F., 1992 - Libro rosso delle piante d'Italia. WWF, Roma.

Conti F., Manzi A., Pedrotti F., 1997 - Liste rosse regionali delle piante d'Italia. Dipartimento di Botanica ed Ecologia, Università degli Studi di Camerino, Camerino.

Covell S., Ellis R.H., Roberts E.H., Summerfield R.J., 1986. The influence of temperature on seed germination rate in grain legumes. I. A comparison of chickpea, lentil, soyabean and cowpea at constant temperatures. Journal of Experimental Botany, 37: 705–715.

Crawley M.J., 2007. The R book. Chichester, UK: JohnWiley & Sons Ltd.

Daws M.I., Lydall E., Chmielarz P., Leprince O., Matthews S., Thanos C.A., Pritchard H.W., 2004. Developmental heat sum influences recalcitrant seed traits in *Aesculus hippocastanum* across Europe. New Phytologist, 162: 157–166.

Doussi M.A., Thanos C.A., 1994. Post-fire regeneration of hardseeded plants: ecophysiology of seed germination. Proc. 2nd Int. Conf. Forest Fire Research, Vol. II, D. 25, pp. 1035–1044.

De Bolós O., Vigo J., Masalles R.M., Ninot J.M., 2005. Flora Manual Dels Països Catalans, 3rd edn. Barcelona, Spain: Editorial Pórtic.

Ellis R.H., Covell S., Roberts E.H., Summerfield R.J., 1986. The influence of temperature on seed germination rate in grain legumes. II. Intraspecific variation in chickpea (*Cicer arietinum* L.) at constant temperatures. Journal of Experimental Botany, 37: 1503–1515.

Fenner M.,Thompson K., 2005. The ecology of seeds. Cambridge, UK: Cambridge University Press.

Fernández-Pascual E., Jiménez-Alfaro B., Caujapé-Castells J., Jaén-Molina R., Díaz T.E., 2013. A local dormancy cline is related to the seed maturation environment, population genetic composition and climate. Annals of Botany, 112: 937–945.

Finch-Savage W.E., Cadman C.S.C., Toorop P.E., Lynn J.R., Hilhorst H.W.M., 2007. Seed dormancy release in *Arabidopsis* Cvi by dry afterripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing. The Plant Journal, 51: 60–78.

Finney D.J., 1971. Probit analysis, 3rd edn. Cambridge: Cambridge University Press.

García-Fayos P., Gulias J., Martínez J, Marzo A., Melero J. P., Traveset A., Veintimilla P., Verdú M., Cerdán V., Gasque M., Medrano H., 2001. Bases ecológicas para la recolección, almacenamiento y germinación de semillas de especies de uso forestal de la Comunidad Valenciana. Back de Llavors Forestals, Conselleria de Medi Ambient, Generalitat Valenciana, Valencia, España.

Garcia-Huidobro J., Monteith J.L., Squire G.R., 1982. Time, temperature and germination of pearl millet (*Pennisetum typhoides* S. & H.). Journal of Experimental Botany, 33: 288–296.

Godwin H., 1943. Rhamnaceae. Journal of Ecology, 31: 66–68.

Gulías J., Traveset A., Riera N., Mus M., 2004. Critical Stages in the Recruitment Process of *Rhamnus alaternus* L. Annals of Botany, 93: 723–731.

Gulías J., Traveset A., 2012. Altitudinal variation in the reproductive performance of the Mediterranean shrub *Rhamnus lycioides* L. Journal of Plant Ecology, 5(3): 330–336.

Hardegree SP., 2006. Predicting germination response to temperature. I. Cardinal temperature models and subpopulation-specific regression. Annals of Botany, 97: 1115–1125.

Heit C.E., 1968. Propagation from seed. 15. Fall planting of shrub seeds for successful seedling production. American Nurseryman, 128(4): 8–10, 70–80.

Hidayati, S.N., Baskin J.M. And Baskin C.C., 2001. Dormancy-breaking and germination requirements of *Symphoricarpos orbiculatus* (Caprifoliaceae). American Journal of Botany, 88: 1444–1451.

Hubbard R.L., 1974. *Rhamnus* L. Buckthorn. In "Seeds of Woody Plants in the United States" (C. S. Schopmeyer, Tech. Coord.), pp. 704–708. USDA Forest Service Agriculture Handbook No. 450.

International Seed Testing Association (ISTA), 1985. International rules for seed testing. Edition 1985. Seed Science and Technology, 13: 299–513.

Keeley J.E., 1987. Role of fire in seed germination of woody *taxa* in California chaparral. Ecology, 68: 434–443.

Körner C., 2003. Alpin Plant Life: Functional Plant Ecology of High Mountain Ecosystems, 2nd ed. Springer, Berlin-Heidelberg.

Luna B., Pérez B., Céspedes B., Moreno J.M., 2008. Effect of cold exposure on seed germination of 58 plant species comprising several functional groups from a mid-mountain Mediterranean area. Ecoscience, 15(4): 478–484.

Martin A.C., 1946. The comparative internal morphology of seeds. American Midland Naturalist, 36: 513–660.

Mattana E., Daws M.I., Bacchetta G., 2009. Effects of temperature, light and prechilling on germination of *Rhamnus persicifolia*, an endemic tree species of Sardinia (Italy). Seed Science and Technology, 37: 758–764.

Mattana E., Pritchard H.W., Porceddu M., Stuppy W.H., Bacchetta G., 2012. Interchangeable effects of gibberellic acid and temperature on embryo growth, seed germination and epicotyl emergence in *Ribes multiflorum* ssp. *sandalioticum* (Grossulariaceae). Plant Biology, 14: 77–87.

McKeon G.M., Mott J.J., 1982. The effect of temperature on the field softening of hard seed of *Stylosanthes humilis* and *S. hamata* in a dry monsoonal climate. Australian Journal of Agricultural Research, 33: 75–85.

Meyer S., Kitchen S., Beckstead J., 1995. Seed germination timing patterns in intermountain *Penstemon* (Scrophulariaceae). American Journal of Botany, 82: 377–389.

Nikolaeva M.G., 1977. Factors controlling the seed dormancy pattern. In The physiology and biochemistry of seed dormancy and germination, (ed. A.A. Khan), 51–74, North-Holland, Amsterdam.

Nokes J., 1986. "How to Grow Native Plants of Texas and the Southwest." Texas Monthly Press, Austin.

Orrù M., Mattana E., Pritchard H.W., Bacchetta G., 2012. Thermal thresholds as predictors of seed dormancy release and germination timing: altitude-related risks from climate warming for the wild grapevine *Vitis vinifera* subsp. *sylvestris*. Annals of Botany, 110: 1651–1660.

Pendleton B.K., Meyer S.E., 2004. Habitat-correlated variation in blackbrush (*Coleogyne ramosissima*: Rosaceae) seed germination response. Journal of Arid Environments, 59: 229–243.

Porceddu M., Mattana E., Pritchard H.W., Bacchetta G., 2013. Thermal niche for in situ seed germination in Mediterranean mountain streams: model prediction and validation for *Rhamnus persicifolia* seeds. Annals of Botany, 112(9): 1887–1897.

Pritchard H.W., Steadman K.J., Nash J.V., Jones C., 1999. Kinetics of dormancy release and the high temperature germination response in Aesculus hippocastanum seeds. Journal of Experimental Botany, 50: 1507–1514.

Probert R.J., 2000. The role of temperature in the regulation of seed dormancy and germination. In: Fenner M., ed. Seeds: the ecology of regeneration in plant communities. Wallingford, UK: CAB International, pp. 261–292.

R Development Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Radwan M.A., 1976. Germination of cascara seed. Tree Plant, Notes, 27(2): 20–23.

Royal Botanic Gardens Kew., 2014. Seed Information Database (SID). Version 7.1. Available from: http://data.kew.org/sid/.

Schwienbacher E., Navarro-Cano J.A., Neuner G., Erschbamer B., 2011. Seed dormancy in alpine species. Flora, 206: 845–856.

Sharma J., Graves W.R., 2005. Propagation of *Rhamnus alnifolia* and *Rhamnus lanceolata* by seeds and cuttings. Journal of Environmental Horticulture, 23: 86–90.

Skordilis A., Thanos C.A., 1995. Seed stratification and germination strategy in the Mediterranean pines *Pinus brutia* and *P. halepensis*. Seed Science Research, 5: 151–160.

Steadman K.J., Pritchard H.W., 2004. Germination of *Aesculus hippocastanum* seeds following cold-induced dormancy loss can be described in relation to a temperature-dependent reduction in base temperature (T_b) and thermal time. New Phytologist, 161: 415–425.

Thanos C.A., Georghiou K., 1988. Ecophysiology of fire-stimulated seed germination in *Cistus incanus* ssp. *creticus* (L.) Heywood and *C. salvifolius* L. Plant, Cell and Environment, 11: 841–849.

Thompson P.A., 1970. A comparison of the germination character of species of Caryophyllaceae collected in Central Germany. Journal of Ecology, 58: 699–711.

Vera M.L., 1997. Effects of altitude and seed size on germination and seedling survival of heathland plants in north Spain. Plant Ecology, 133: 101–106.

Walck J.L., Cofer M.S., Jayasuriya K.M.G.G., Fernando M.T.R., Hidayati S.N., 2012. A temperate rhamnaceous species with a non-enclosing stone and without physical dormancy. Seed Science Research, 22: 269–278.

Wang J.H., Baskin C.C., Du Chen W.G.Z., 2010. Variation in seed germination between populations of five sub-alpine woody species from eastern Qinghai-Tibet Plateau following dry storage at low temperatures. Ecological Research, 25: 195–203.

Zhou Z., Bao W., 2014. Changes in seed dormancy of *Rosa multibracteata* Hemsl. & E. H. Wilson with increasing elevation in an arid valley in the eastern Tibetan Plateau. Ecological Research, 29: 693–700.

Chapter 3: Intra-specific differences on thermal thresholds for seed germination along an altitudinal gradient

Introduction

Once seeds have been matured and dispersed, survival of the species requires that they germinate at a time and place favourable for growth and survival of the seedlings. Germination commences with the uptake of water by the dry seed (imbibition), followed by embryo expansion and the elongation of the embryonic axis, and is completed when a part of the embryo, usually the radicle, extends to penetrate the structures that surround it (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006). Environmental conditions during seed maturation influence germination with temperature being the major environmental factor responsible for changes in dormancy states of seeds (Baskin and Baskin, 2014). At maturity, seeds may be non-dormant (they germinate over the broadest set of conditions) or dormant (they fail to germinate at all, or do so under a narrow set of environmental conditions; Walk *et al*., 2011).

Seed dormancy has probably evolved differently across species, through adaptation to various habitats and prevailing environments, so that the completion of germination occurs when conditions for establishing a new plant generation are likely to be suitable (Fenner and Thompson, 2005; Finch-Savage and Leubner-Metzger, 2006; Baskin and Baskin, 2014). Dormancy breaking and germination requirements are specific for each species and depend on phylogeny, distribution and habitat (Finch-Savage and Leubner Metzger, 2006; Baskin and Baskin, 2004, 2014). Even closely related species, either growing in a variety of habitats (e.g. Vandelook *et al.*, 2008) or co-occurring in a given habitat, may differ in germination response to environmental signals (e.g. Daws *et al*., 2002; Karlsson *et al*., 2008).

Baskin and Baskin (2004) have proposed a comprehensive classification system which includes five classes of seed dormancy: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) and combinational (PY + PD). PD is the most prevalent form of seed dormancy and can be triggered by divergent environmental cues (Finch-Savage *et al.*, 2007; Baskin and Baskin 2014). Seed germination responses following the application of warm and/or cold stratification as well as the plant hormones (in particular gibberellins) help determine the class of dormancy, and frequently these treatments are used to release PD (Finch-Savage and Leubner-Metzger, 2006; Baskin and Baskin, 2004, 2014). Also dry after-ripening treatment is a common method used to relieve this type of dormancy (Bewley, 1997; Finch-Savage *et al.*, 2007), and may represent a natural mechanism that can control dormancy in dry climates (Probert, 2000).

In non-dormant seeds, the germination response to accumulated temperature has been modelled using thermal time (θ) approach (e.g. García-Huidobro *et al*., 1982; Covell *et al*., 1986; Ellis *et al*., 1986, 1987; Pritchard *et al*., 1996; Hardegree, 2006). In this model, seeds accumulate units of thermal time (°Cd) to germinate to a percentile (g) of the population. When seeds are subjected to temperatures (T) above a base temperature for germination (T_b), at which the germination rate is zero, and below an optimum temperature (T_o), above which germination rate does not increase anymore (sub-optimal temperature range), germination rate increases linearly with temperature (García-Huidobro *et al*., 1982).

Germination behaviour may vary greatly within the same species from one population to another and from year to year (Urbanska and Schütz, 1986), as reported for several *taxa* (e.g. Andersson and Milberg, 1998; Holm, 1994; Pérez-García *et al*., 2003, 2006; Giménez-Benavides *et al*., 2005; Baskin and Baskin, 2014)., as well as variation in seed dormancy among individuals and populations of the same species from different sites (e.g. Schütz and Rave, 2003; Fernández-Pascual *et al*., 2013; Vera 1997). Intra-specific variation among seed

populations may depend on genetic differences, local weather during growth of mother plant, soil quality or other naturally occurring factors (Karlsson and Milberg, 2007).

In this study, seed germination characteristics of three different populations of four species that grow in Sardinia at different altitudes (from ca. 135 to 1810 m a.s.l.) under a Mediterranean climate: *Digitalis purpurea* L. var. *gyspergerae* (Rouy) Fiori, *Scrophularia trifoliata* L., *Santolina insularis* (Gennari *ex* Fiori) Arrigoni and *Ptilostemon casabonae* (L.) Greuter were investigated. These species are all endemics and their populations are located in a wide range of altitudes, showing indifference to the edaphic factors. We expected to find a pattern showing that the level of seed dormancy increases with increasing altitudes, due to higher levels of T_b. Therefore, the main aim of this study was to evaluate the effect of altitude and different treatments, on the thermal requirements for dormancy loss, mostly the physiological type, and germination of populations of the same species along an altitudinal gradient.

Materials and methods

Study species

Digitalis pupurea var. *gyspergerae* (hereafter *D. purpurea*; Scrophulariaceae) is a biennial or perennial herb, endemic to Sardinia and Corsica. In Sardinia it occurs both on limestone and siliceous substrata at an altitude ranging from ca. 200 to 1800 m a.s.l. The flowering occurs from March to June while fruiting from June to August (Bacchetta *et al*., 2008).

Scrophularia trifoliata (Scrophulariaceae) is a perennial herb, endemic to Corsica, Sardinia and the Island of Gorgona. It is a hemicryptophyte that inhabits sheltered, moist places in montane and submontane areas (Valsecchi, 1979) but also lives in sunny environments without a direct input of water, from the sea level up to 1500 m a.s.l. The flowering occurs from April to June and fruiting in May-June (Arrigoni, 1978a).

Santolina insularis (Asteraceae) is an endemic bush distributed mainly in the south and central-eastern parts of Sardinia (Italy). It is a chamaephyte or nanophanerophyte found from sea level to the highest peaks of the Sardinian massifs. Populations of this species are located in different ecological contexts and show an indifference to edaphic factors (Angiolini and Bacchetta, 2003). The flowering occurs from May to July and fruiting from July to September, depending on altitude (Arrigoni, 1978b).

Ptilostemon casabonae (Asteraceae) is a perennial herb, endemic to Sardinia, Corsica and Hyères Islands. It is a rhizomatose geophyte species. In Sardinia it is found both on limestone and siliceous substrata, showing different edaphic tolerance (Marengo *et al*., 2015). The species is located in a wide altitudinal range, from ca. 10 to ca. 1700 m a.s.l. The flowering occurs from June to August and fruiting from July to September (Marengo *et al*., 2015).

Seed lot details

Fully ripened seeds of three different populations of each investigated species were collected in the field during the summers 2012 (for *D. purpurea*) and 2013 (for the others species; Table 1). Seeds were collected directly from mother plants and randomly chosen from approximately 50 individuals per population during the time of the natural dispersal, in an area of ca. 150-200 m².

Table 1 - Collection site details of the three populations investigated of *Digitalis purpurea*, *Scrophularia trifoliata*, *Santolina insularis* and *Ptilostemon casabonae*. Abbreviations on the province: CA = Cagliari; NU = Nuoro; CI = Carbonia-Iglesias; OR = Oristano.

Taxon	**Locality**	**Mean Coordinates (Datum WGS84)**	**Mean Altitude (m a.s.l.)**	**Collection date**
Digitalis purpurea	Is Cioffus - Capoterra (CA)	39°06'N 08°57'E	360	26/06/2012
	Monte Lattias - Uta (CA)	39°08'N 08°50'E	904	23/07/2012
	Bruncu Spina - Desulo (NU)	40°00'N 09°18'E	1810	30/08/2012
Scrophularia trifoliata	Miniera Luigi - Buggerru (CI)	39°22'N 08°25'E	217	11/07/2013
	Laconi - Laconi (OR)	39°51'N 09°03'E	510	21/06/2013
	Su Thuttureli - Oliena (NU)	40°14'N 09°25'E	1230	17/07/2013
Santolina insularis	Miniera Luigi - Buggerru (CI)	39°22'N 08°25'E	147	11/07/2013
	Sugalaffricu - Laconi (OR)	39°52'N 09°00'E	500	22/07/2013
	Separadorgiu - Fonni (NU)	40°02'N 09°17'E	1531	12/09/2013
Ptilostemon casabonae	Miniera Luigi - Buggerru (CI)	39°22'N 08°25'E	135	11/07/2013
	Mitzaorxia - Laconi (OR)	39°52'N 09°04'E	686	22/07/2013
	Is Terr'e Molentes - Fonni (NU)	40°03'N 09°19'E	1300	23/07/2013

Germination tests

For each *taxon*, four replicates of 30 seeds each were sown on the surface of 1% agar water, in 60-mm diameter plastic Petri dishes (except *P. casabonae* seeds that were sown in 90-mm diameter Petri dishes) and incubated in the light (12 h light ⁄ 12 h dark) at a range of constant germination temperatures (5, 10, 15, 20 and 25°C) and under an alternating temperature regime (25⁄10°C) for a maximum of four months. In addition, different pre-treatments were also applied, i.e., pre-chilling (C, three months at 5°C), pre-warming (W, three months at 25°C) and dry after ripening (DAR, seeds were stored for three months at 25°C inside a sealed glass container with silica gel in a ratio seed / silica gel 1:1), and at the end of each pre-treatment, seeds were incubated at the above listed temperatures. During C and W stratifications, the seeds were incubated in continuous dark, by wrapping dishes in aluminium foil and then incubated to the light condition at the germination temperatures specified above (Table 2). Extra four replicates of 30 seeds each were sown on the surface of 1% agar water with 250 mg l^{-1} of GA_3 and incubated in the light (12 h light ⁄ 12 h dark), at the previously cited temperatures.

Germination, defined as visible radicle emergence (> 1 mm), was recorded three times a week, except for dark-incubated seeds that were scored at the end of the stratifications, to avoid any exposure to light. At the end of the germination tests, when no additional germination had occurred for two weeks, after a minimum of one month from sowing, a cut-test was carried out to determine the viability of the remaining seeds and the number of empty seeds (Mattana *et al.*, 2012), in particular, firm seeds were considered to be viable.

Data analysis

Final germination percentages were calculated on the basis of the total number of filled seeds as the mean of the four replicates (± S.D.) for each tested condition. During stratifications (i.e., C and W), when seeds germinated before moving to the incubation temperatures, these where not considered in the final germination percentages which were calculated on the basis of the remaining filled seeds.

A thermal time approach was carried out for seeds germinating at constant temperatures (5–25°C) after each pre-treatment. Estimates of time (t_g, d) taken for cumulative germination to reach different percentiles (g) for successive increments of 10% germination were then interpolated from the germination progress curves (Covell *et al*., 1986).

The germination rate ($1/t_g$) was regressed using linear models to estimate the base temperature (T_b) [see eqn (1)] and the ceiling temperature (T_c) [see eqn (2)] at which the germination rate is equal to zero, by averaging the x-intercept for both suboptimal and supra-optimal temperature ranges (Ellis *et al*., 1986). When the rate is linear, extrapolation in the suboptimal range defines a T_b, the thermal response of germination can be expressed as the following equation (Garcia-Huidobro *et al*., 1982):

$$1/t_g(d^{-1}) = (T_g - T_b)/\theta \qquad (1)$$

Linear regression equations were then recalculated for each percentile, but constrained to pass through T_b (Hardegree, 2006).

A comparison of regressions was then made between this model and one in which the T_b were allowed to vary for all the percentiles, and the best estimate was considered to be that which resulted in the smallest residual variance (Covell *et al*., 1986). Thermal time (θ, °Cd) estimates for each treatment were then calculated separately as the inverse of the suboptimal regression equations (Covell *et al*., 1986). The use of thermal time to describe the temperature dependence of germination can be extended to supra-optimal temperatures in the case where $1/t$ decreases linearly with an increase in temperature above the optimum, (T_o), reaching zero

at a maximum temperature, (T_c). Garcia-Huidobro *et al*. (1982) described the relationship when $T_o < T < T_c$ as a second value of thermal time θ_2:

$$1/t_g(d^{-1}) = (T_c - T_g)/\theta_2 \qquad (2)$$

Germination percentages were transformed to probits using tabular data from Finney (1971). Linear regression was used to express probit (g) as a function of thermal time (θ_g) and the form of cumulative germination response of seeds described by the equation (Covell *et al*., 1986):

$$\text{probit}(g) = K + \theta_g/\sigma \qquad (3)$$

where K is an intercept constant when θ_g is zero and σ is the standard deviation of the response to θ_g (i.e., the reciprocal of the slope), and represents the sensitivity of the population to θ_g (Covell *et al*., 1986). θ_g may be normal or log-normal distributed (and the best model evaluated on the basis of the r^2 values; Hardegree, 2006),

Regression analyses were carried out using SigmaPlot Version 11.0 (Systat Software, Inc., San Jose California USA).

Statistical analysis

Generalized Linear Models (GLMs) were used to compare final germination percentages of each investigated species. GLM_S with quasibinomial error structures and F tests with an empirical scale parameter instead of chi-squared on the subsequent analysis of variance (ANOVA) were used in order to overcome residual overdispersion (Crawley, 2007). Significant differences were analysed by a *post-hoc* pairwise comparisons *t*-test (with Bonferroni adjustment). All statistical analyses were carried out with R v. 3.0.3 (R Development Core Team, 2014).

Results

Digitalis purpurea

GLMs highlighted a statistically significant effect of the treatment, population and temperature factors and their interactions ($P < 0.001$) on seed germination of *D. purpurea* (Table 2).

Table 2 - GLMs results for seed germination (%) of the following factors: "Treatment" (0, Control; C, 5°C for three months; W, 25°C for three months; GA_3, 250 mg L^{-1} in the germination substrate; DAR, 25°C for three months on silica gel), "Temperature" (5, 10, 15, 20, 25 and 25/10°C),"Population" (360, 904 and 1810 m a.s.l.).

Germination (%)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
NULL			359	20221.1		
Treatment	4	2694.5	355	17526.6	147.0272	< 2.2e-16 ***
Population	2	4530.6	353	12996.0	494.4419	< 2.2e-16 ***
Temperature	5	2956.5	348	10039.5	129.0591	< 2.2e-16 ***
Treatment:Population	8	1066.4	340	8973.1	29.0956	< 2.2e-16 ***
Treatment:Temperature	20	6692.5	320	2280.6	73.0371	< 2.2e-16 ***
Population:Temperature	10	324.1	310	1956.5	7.0738	7.067e-10 ***
Treatm:Popul.:Temp.	40	577.6	270	1379.0	3.1516	1.638e-08 ***

During C and W stratifications in dark conditions, all three populations of *D. purpurea* achieved very low germination percentages, on the average < 2%.

Germination results for each population highlighted a decreasing overall germination with increasing altitudes for untreated seeds. Indeed, whereas untreated seeds of the lowest population germinated at all temperature regimes, the population at the middle and high altitude failed to germinate at 5°C. The highest altitude population showed the lowest germination percentage, on average < 60%, compared to the middle and the low population that showed approximately 72% and 84%, respectively (Fig. 1). GA_3 treated seeds for the three populations germinated well at all the tested temperatures with percentages on the average never less than 70% (Fig. 1). The effect of C was more evident at relatively low incubation temperatures (5 - 15°C) for all three populations, where seeds reached on the average of ca. 85, 72 and 56% (populations at low, middle and high elevation, respectively; Fig. 1). W treatment led to low germination percentages (< 6%) at 5°C for all populations. Seeds germinated mainly from seed lots from low and middle altitudes than the population of high elevation; the latter on the average reached only 11 ± 8% while the mid and the lowest population reached 61 ± 31% and 72 ± 37%, respectively (Fig. 1). For all populations, seeds treated with DAR failed to germinate at 5°C. Furthermore, DAR treated seeds highlighted a decreasing overall germination with increasing altitudes. Seeds collected from the lowest altitude population reached on average ca. 76% whereas those collected at high elevation achieved approximately only 37% (Fig. 1).

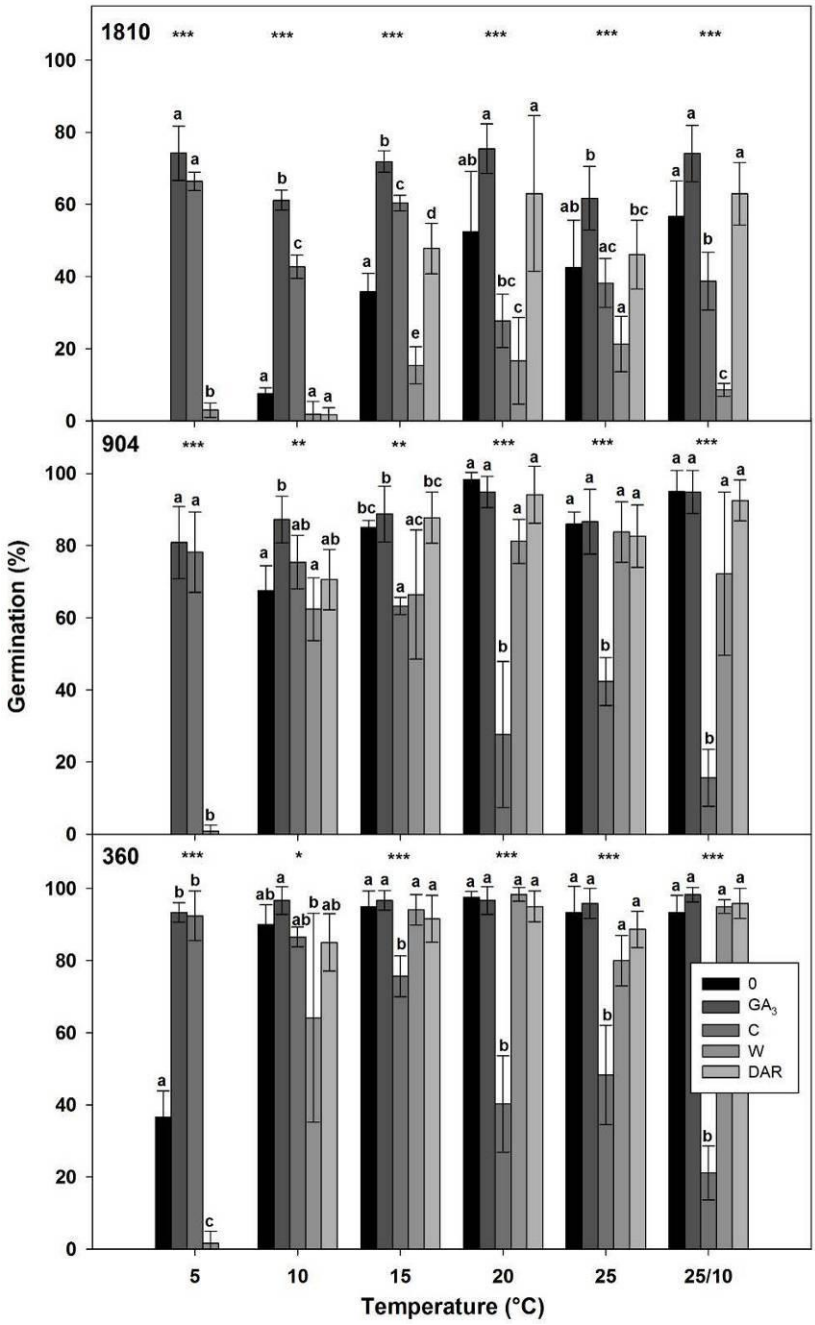

Figure 1 - Final germination percentages of *Digitalis purpurea* seeds collected at 360, 904 and 1810 m a.s.l. achieved at the end of germination tests after each pre-treatment (0, Control; C, 5°C for three months; W, 25°C for three months; GA_3, 250 mg L^{-1} in the germination substrate; DAR, 25°C for three months on silica gel). Data are the mean of four replicates (1 ± S.D.). Temperatures, treatments and their interaction are statistically significant ($P < 0.001$) by GLM. *Post hoc* pairwise *t*-test comparisons (with Bonferroni adjustment) were carried out for each germination temperature, and bars with different letters indicate significant differences ($P < 0.05$).

T_b increased with rising altitudes for untreated seeds, i.e. -0.94, 0.82 and 8.57°C for populations at low, middle and high elevation, respectively (Fig. 2), whereas GA_3 reduced these values both for the middle and high altitude population. In particular, T_b of GA_3 treated seeds of the highest population decreased of approximately 6°C, compared to the value detected for the Control (0). C positively affected the germination rate especially for the low and middle altitude population, for which was possible to calculate the optimal temperatures (T_o) and ceiling temperatures (T_c). In particular for seeds collected at low altitude T_b was -0.04°C, T_o 14.21°C and T_c 27.92°C while those collected at middle elevation showed T_b of 3.97°C, T_o of 15.37°C and T_c of 26.37°C. While, T_b of C treated seeds of the highest population decreased of approximately 8°C, compared to the value detected for the Control (0) (Fig. 2). For W treated seeds, T_b could be calculated only for the two lower altitude populations, and in both cases T_b increased of approximately 8°C compared to the values recorded for the Control. In DAR treatment T_b increased with increasing altitudes, i.e., from -6 to 9°C (population at low and high altitude, respectively). In addition, T_b for the lowest altitude population decreased of approximately 5°C compared to the values detected for the Control unlike the middle altitude population, in which DAR treatment increased T_b of ca. 7°C; while, significant differences were not noted for the highest population (T_b is approximately 9°C both for Control and DAR, Fig. 2).

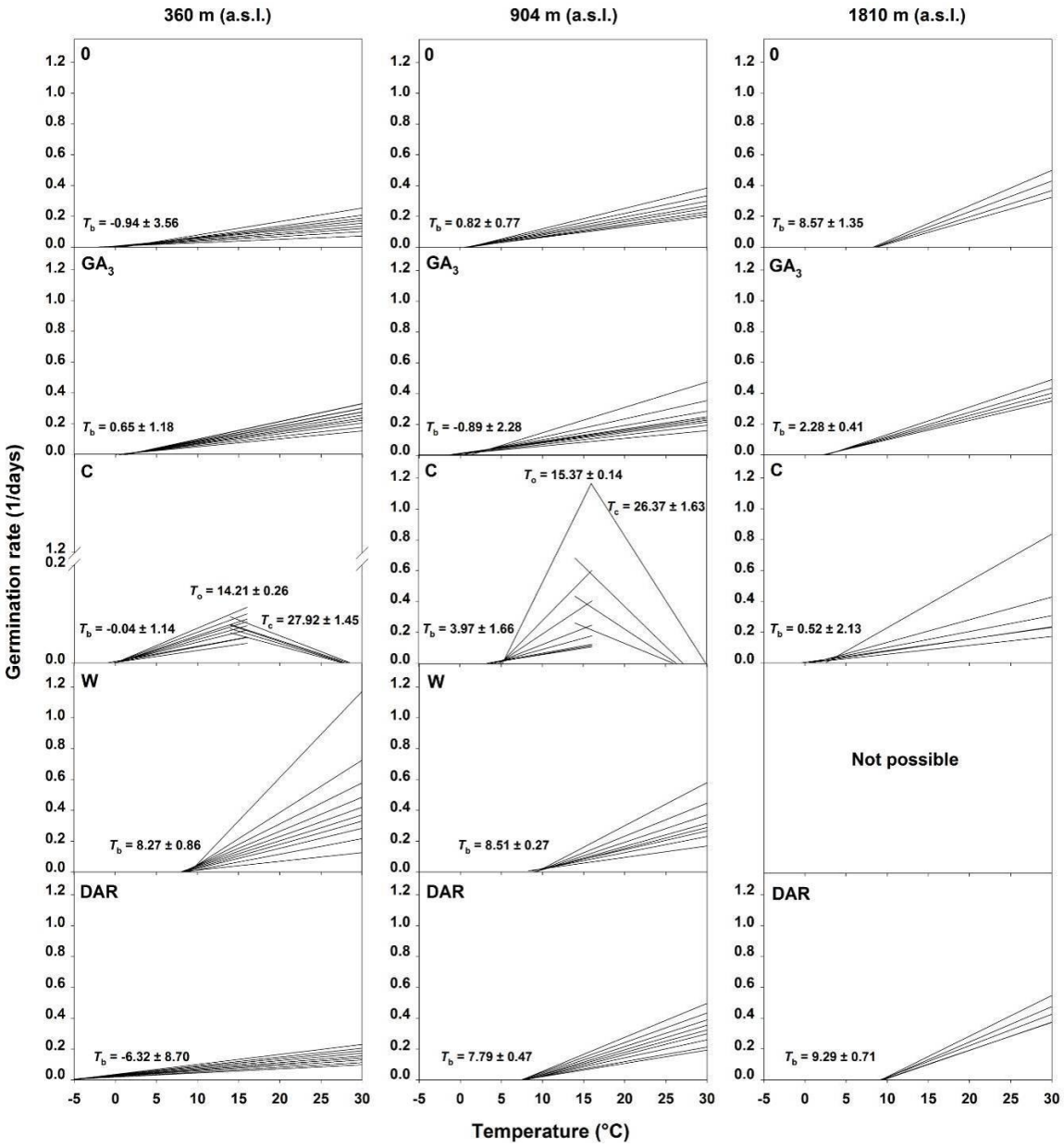

Figure 2 - Intra-specific differences on seed germination rates of *Digitalis purpurea*. Cardinal temperatures for germination (base T_b, optimum T_o and ceiling T_c temperatures) after each pre-treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel). For all treatments, the linear regressions for the different percentiles were constrained to the common value of T_b and T_c. Linear regressions of percentiles with $P > 0.05$ were not included.

Fig. 3 shows the relationship between log thermal time (θ) and germination expressed in probits for all treatments, calculated according to eqn (3). The relationship between log θ and probit germination had better fit ($r^2 > 0.94$ for each populations) than when expressed on a linear scale (data not shown). For 0, θ_{50} was greater for seeds collected at the lowest altitude population (2.32 log °Cd) compared to those of middle altitude (2.07 log °Cd), while seeds collected at high altitude did not reach the 50% of germination (Fig. 3). The populations at low and middle altitudes showed similar thermal time requirements for GA_3 treated seeds (2.08 and 2.09 log °Cd, respectively) compared with the highest population (1.90 log °Cd). The θ_{50} for the C tests was greater for the seeds of the lowest population (2.36 log °Cd) compared to those of the high and middle population (2.18 and 1.76 log °Cd, respectively; Fig. 3). For the W treatment, it was possible to calculate the θ_{50} only for seeds collected at low and middle altitude that reached 1.68 and 1.87 log °Cd, respectively. The population at low altitude showed a greater θ_{50} for DAR treated seeds (2.37 log °Cd) compared with the population at middle and high altitude (Fig. 3).

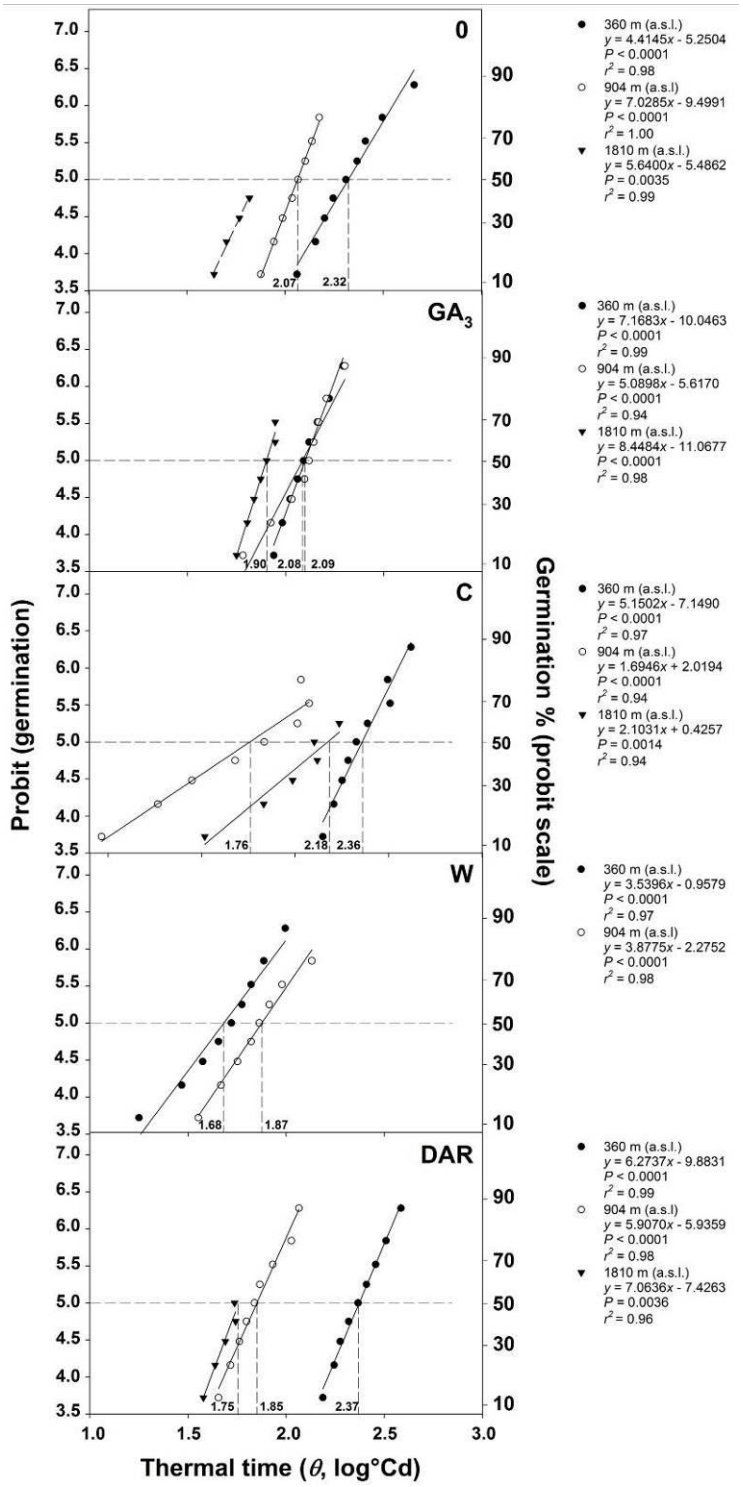

Figure 3 - Probit germination after each treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel) as a function of log-thermal time in the three populations of *Digitalis purpurea* located at different altitudes (360, 904 and 1810 m a.s.l.). Thermal times to reach θ_{50} are also shown (dashed lines).

Scrophularia trifoliata

GLMs highlighted a statistically significant effect of the treatment, population and temperature factors and their interactions ($P < 0.001$) on seed germination of *S. trifoliata* (Table 3).

Table 3 - GLMs results for seed germination (%) of the following factors: "Treatment" (0, Control; C, 5°C for three months; W, 25°C for three months; GA_3, 250 mg L^{-1} in the germination substrate; DAR, 25°C for three months on silica gel), "Temperature" (5, 10, 15, 20, 25 and 25/10°C),"Population" (217, 510 and 1230 m a.s.l.).

Germination (%)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
NULL			359	18214.4		
Treatment	4	8674.8	355	9539.6	425.8602	< 2.2e-16 ***
Population	2	2214.1	353	7325.5	217.3896	< 2.2e-16 ***
Temperature	5	2302.5	348	5022.9	90.4291	< 2.2e-16 ***
Treatment:Population	8	610.7	340	4412.2	14.9912	< 2.2e-16 ***
Treatment:Temperature	20	1086.1	320	3326.1	10.6638	< 2.2e-16 ***
Population:Temperature	10	1128.1	310	2198.0	22.1521	< 2.2e-16 ***
Treatm.:Popul.:Temp.	40	743.5	270	1454.5	3.6499	1.314e-10 ***

In dark conditions, during C pre-treatment, seeds of each population of *S. trifoliata* reached 4 ± 5% of germination, while seeds treated with W, during pre-treatment, never reached values > 1% of germination.

Germination tests for all three populations highlighted a decreasing overall germination with increasing altitudes for untreated seeds, i.e., on the average from 69 ± 18% to 42 ± 28% (population at low and high altitude, respectively; Fig. 4). GA_3 treated seeds of all populations germinated well at all the tested temperatures, with germination percentages never less than 80%. The effect of C was more evident at the middle population, especially at 5 and 25/10°C, where seeds reached and exceeded 50% of germination while other populations reached 17 ± 6 and 14 ± 9% of germination for seeds collected at low and high altitude, respectively. W treatment showed a high germination percentage at the alternating temperatures for all populations (≥ 90%), but highlighted significant differences for the other temperatures at the different altitudes. Overall, the highest germination percentages were reached from the middle altitude population (on the average ca. 83%). Also for seeds treated with DAR high germination percentages were reached at 25/10°C (> 82%). The middle altitude population showed the highest value of germination (77 ± 21%) compared to the other populations; in these latter cases, seeds highlighted a increasing overall germination with increasing temperatures (Fig. 4).

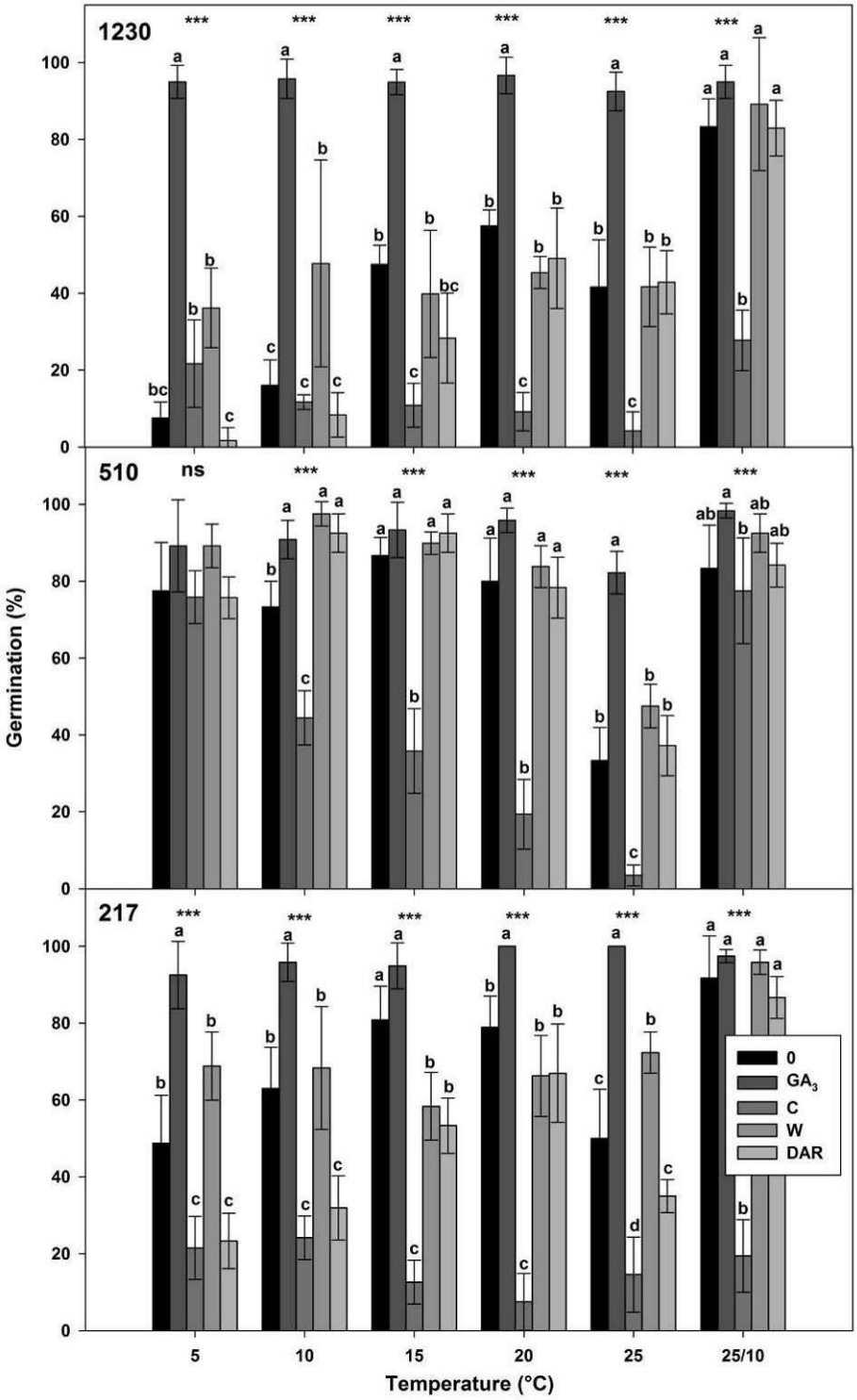

Figure 4 - Final germination percentages of *Scrophularia trifoliata* seeds collected at 217, 510 and 1230 m a.s.l. achieved at the end of germination tests after each pre-treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel). Data are the mean of four replicates (1 ± S.D.).

It was possible to calculate T_b, T_o and T_c for two populations of untreated seeds (Fig. 5). In particular, seeds collected at low altitude showed T_b = 1.80, T_o = 15.64 and T_c = 28.81°C while those collected at middle elevation showed T_b = -2.36, T_o = 15.09 and T_c = 27.20°C. While, T_b of untreated seeds of the highest population was -1.61 (Fig. 5). GA_3 treated seeds highlighted similar values of T_b independently of the altitude. Also in this case was possible calculate T_o and T_c for the middle altitude population, in particular T_b was -1.12°C, T_o was 15.58°C and T_c was 29.93°C. T_b increased with increasing altitudes for seeds treated with C, indeed its values varied from -2.31°C to 0.86°C for seeds collected at low and high elevation, respectively. Also with W treatment, T_b increased with increasing altitude, i.e., from 1.79°C at the lowest altitude to 3.09°C the highest altitude. Furthermore, for the mid-altitude population was possible to calculate not only the T_b but also T_o and T_c, in particular T_b = -0.83, T_o = 15.32 and T_c = 26.63°C. The opposite happened for seeds treated with DAR where T_b varied from 3.62 to 1.83°C (for seeds collected at low and high elevation, respectively), thus decreasing values with altitude (Fig. 5).

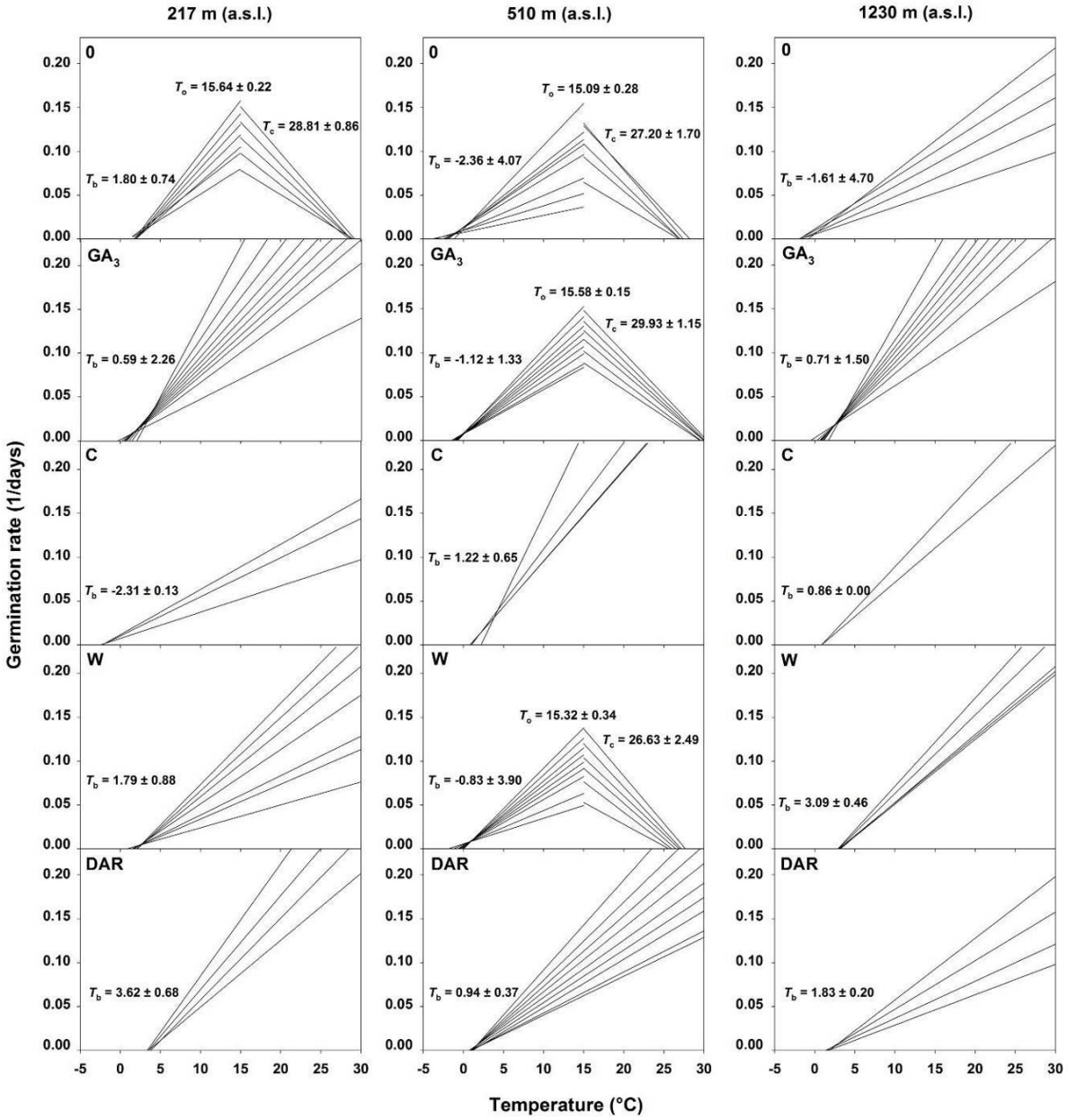

Figure 5 - Intra-specific differences on seed germination rates of *Scrophularia trifoliata*. Cardinal temperatures for germination (base T_b, optimum T_o and ceiling T_c temperatures) after each pre-treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel). For each pre-treatment, the linear regressions for the different percentiles were constrained to the common value of T_b and T_c. Linear regressions of percentiles with $P > 0.05$ were not included.

Fig. 6 shows the relationship between log thermal time (θ) and germination expressed in probits for 0, GA_3, C, W and DAR calculated according to eqn (3). The relationship between log θ and probit germination had a better fit ($r^2 > 0.85$ for all populations) than when expressed on a linear scale (data not shown). Untreated seeds gave longer thermal times for 50% of germination for the highest altitude population (2.48 log °Cd) compared to the middle and low altitude population (2.36 and 2.11 log °Cd, respectively). Similar thermal time requirements for GA_3 treatment were reached by each population (1.99 and 2.14 log °Cd), while seeds of all populations treated with C never reached 50% of germination and, therefore, the thermal time for this percentile could not be estimated. With W treatment θ_{50} was greater for the seeds of the lowest population (2.35 log °Cd) compared to those of the middle and high elevation (2.19 and 2.14 log °Cd, respectively). For DAR treated seeds, it was possible to calculate θ_{50} only for seeds collected at middle altitude (2.18 log °Cd; Fig. 6).

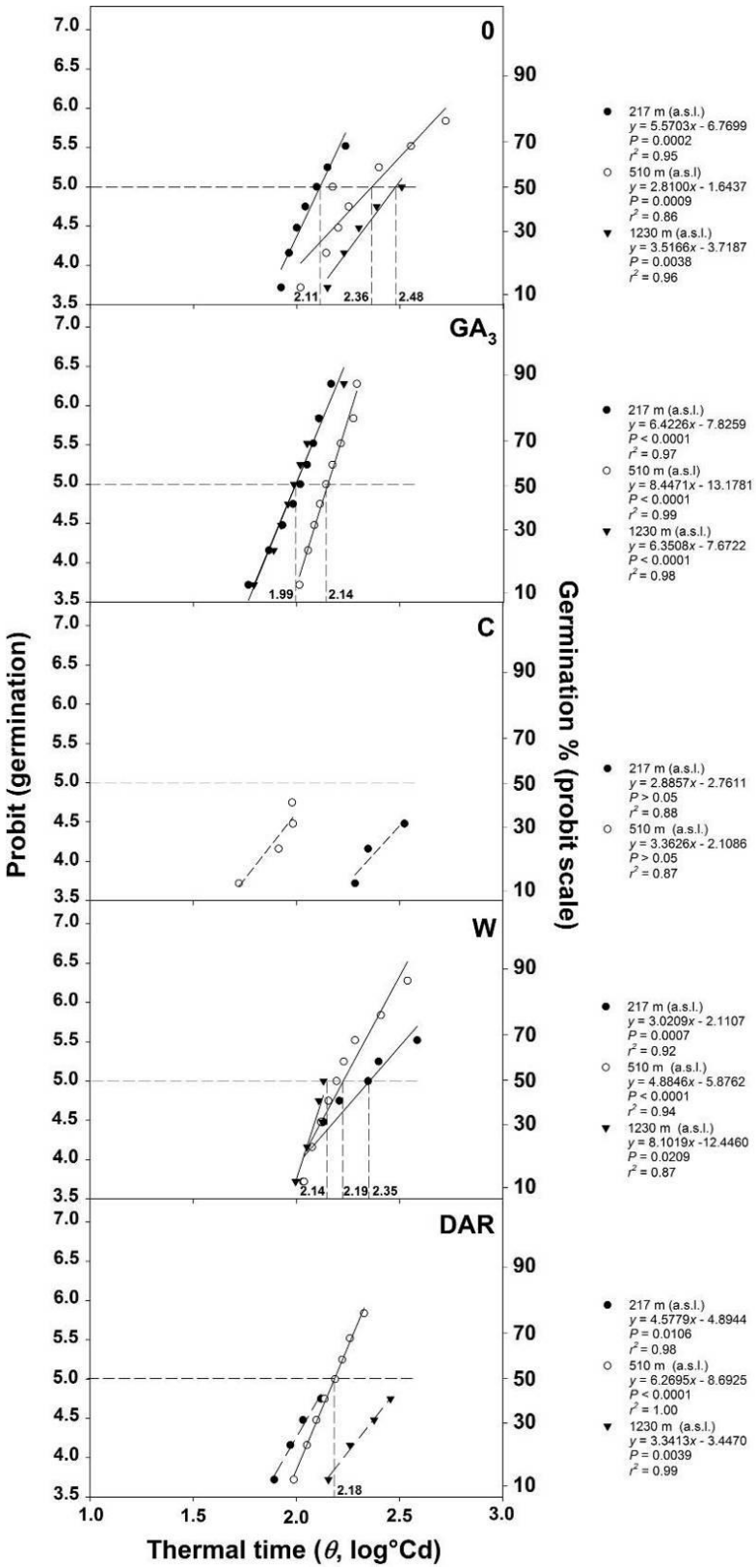

Figure 6 - Probit germination after each treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel) as a function of log-thermal time in the three populations of *Scrophularia trifoliata* located at different altitudes (217, 510 and 1230 m a.s.l.). Thermal times to reach θ_{50} are also shown (dashed lines).

Santolina insularis

GLMs highlighted a statistically significant effect of the treatment, population and temperature factors ($P < 0.001$) and their interactions ($P < 0.001$ and $P < 0.05$ on seed germination of *Santolina insularis* (Table 4).

Table 4 - GLMs results for seed germination (%) of the following factors: "Treatment" (0, Control; C, 5°C for three months; W, 25°C for three months; GA_3, 250 mg L^{-1} in the germination substrate; DAR, 25°C for three months on silica gel), "Temperature" (5, 10, 15, 20, 25 and 25/10°C),"Population" (147, 510 and 1531 m a.s.l.).

Germination (%)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
NULL			359	10486.4		
Treatment	4	954.5	355	9531.9	51.6747	< 2.2e-16 ***
Population	2	4662.5	353	4869.4	504.8637	< 2.2e-16 ***
Temperature	5	1229.9	348	3639.5	53.2717	< 2.2e-16 ***
Treatment:Population	8	375.3	340	3264.1	10.1608	2.124e-12 ***
Treatment:Temperature	20	216.9	320	3047.3	2.3482	0.001207 **
Population:Temperature	10	1176.8	310	1870.4	25.4858	< 2.2e-16 ***
Treatm.:Popul.:Temp.	40	545.9	270	1324.6	2.9553	1.083e-07 ***

During C and W stratifications, in dark conditions, all populations of *S. insularis* reached an average of 11 ± 3% of seed germination.

Germination tests for each population highlighted an increasing overall germination with increasing altitudes for untreated seeds (except at 5°C where seeds germinated with lower values), in particular those collected at high altitude showed on the average a higher germination percentage (approximately 80%), compared to the low altitude population (ca. 44%; Fig. 7). The effect of GA_3 was more evident at the middle and high populations (82 ± 11% and 89 ± 7%, respectively) while seeds collected at low elevation germinated with lower percentages (51 ± 5%). Also with C treatment, seeds collected at middle and high altitude reached higher germination percentages (60 ± 10% and 74 ± 21%, respectively) than the lowest altitude population (21 ± 8%; Fig. 7). With W, seeds of middle and high altitudes germinated better than the population at low elevation. Indeed, the latter reached on the average only 34 ± 7% while the population at middle and high altitude reached 55 ± 8% and 81 ± 19%, respectively (Fig. 7). Also with DAR, it was confirmed that *S. insularis* overall increased its germination with increasing altitudes, i.e., seeds collected at the highest elevation reached 74 ± 18% while those collected at the lowest altitude population reached only 46 ± 8% (Fig. 7).

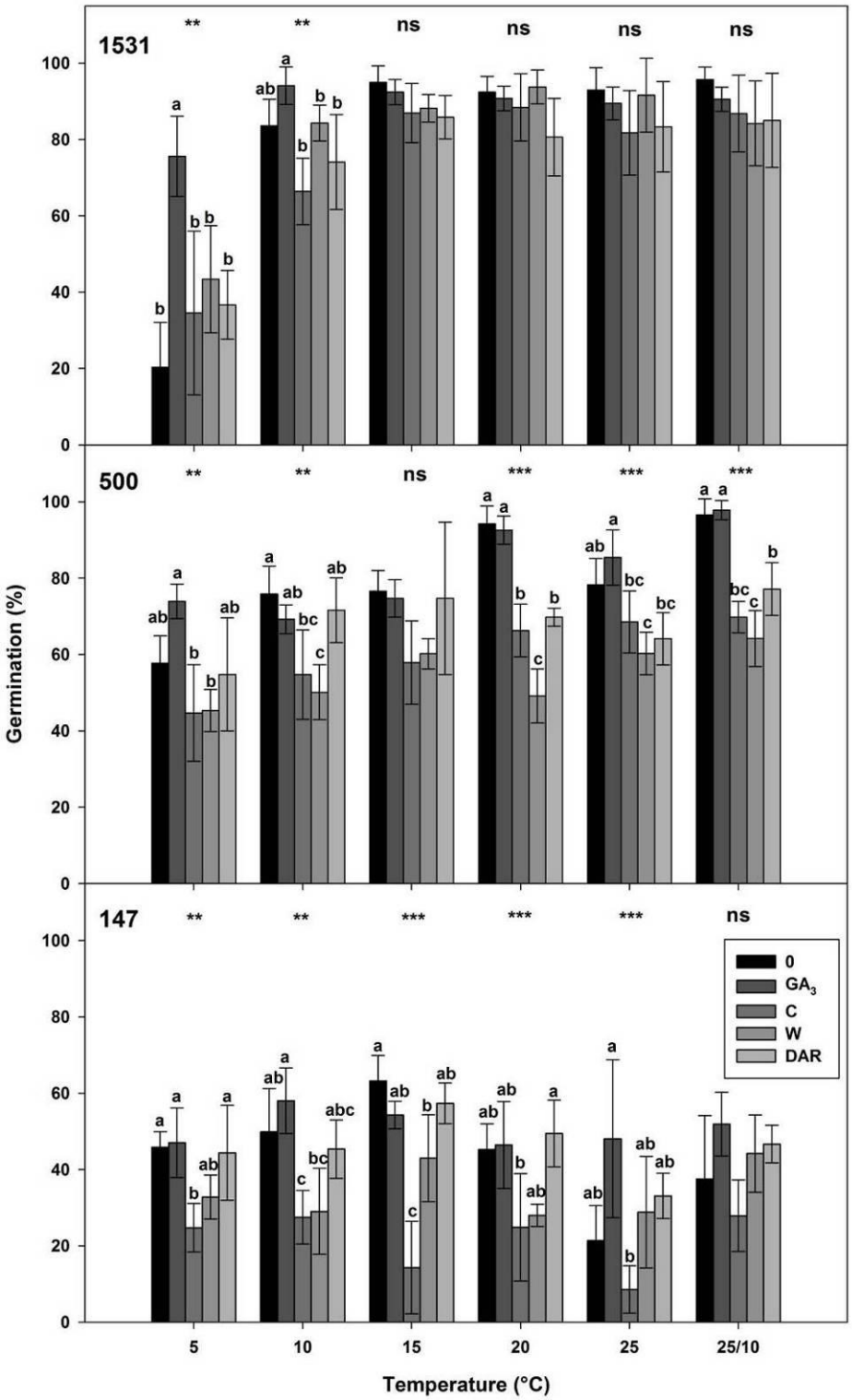

Figure 7 - Final germination percentages of *Santolina insularis* seeds collected at 147, 500 and 1531 m a.s.l. achieved at the end of germination tests after each pre-treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel). Data are the mean of four replicates (1 ± S.D.).

T_b increased with increasing altitudes for untreated (Control, 0) seeds, in particular from 1.82 to 4.84°C, for seeds collected at low and high elevation, respectively. For the lowest altitude population, it was possible to calculate T_o (15.78°C) and T_c (35.59°C; Fig. 8). With GA_3 treatment, T_b values were reduced independently of the altitude (i.e., -0.53, -3.80 and 1.80°C populations at low, middle and high elevation, respectively; Fig. 8). As well as for the Control test, T_b values increased with rising altitudes for seeds treated with C, indeed T_b varied from 1.40 to 4.76°C (for seeds collected at low and high elevation, respectively). To the contrary, seeds treated with W highlighted similar T_b independently of altitude with values included between 2.14 to 1.19°C (population at low and high altitude, respectively; Fig. 8). DAR treatment positively affected the germination rate especially for the high altitude population, allowing to calculate also T_o (15.21°C) and T_c (42.31°C). With DAR treatment, T_b values increased with greater altitude; i.e., 1.40, -2.21 and 4.43°C (populations at low, middle and high elevation, respectively; Fig. 8).

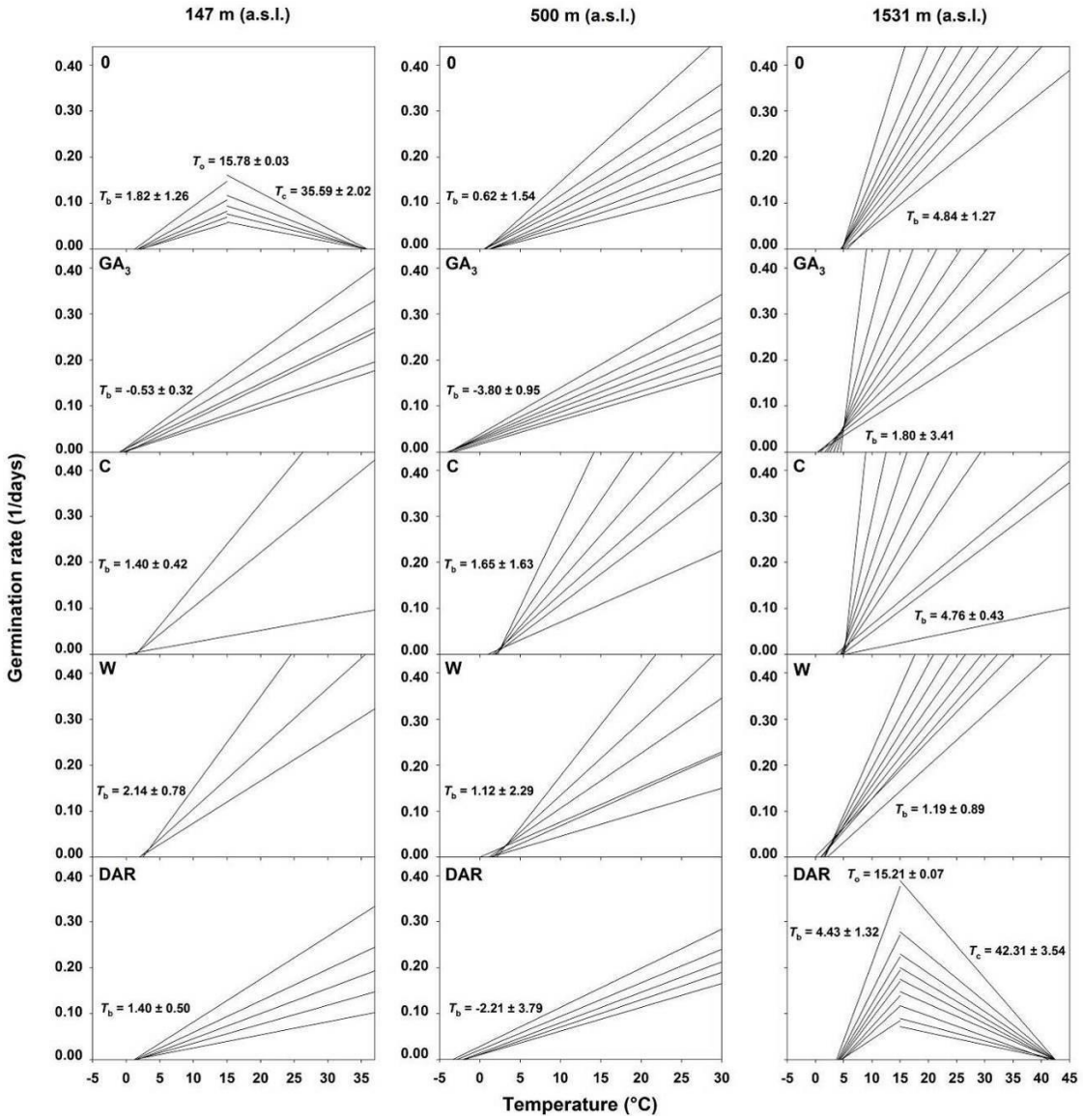

Figure 8 - Intra-specific differences on seed germination rates of *Santolina insularis*. Cardinal temperatures for germination (base T_b, optimum T_o and ceiling T_c temperatures) after each pre-treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel). For each pre-treatment, the linear regressions for the different percentiles were constrained to the common value of T_b and T_c. Linear regressions of percentiles with $P > 0.05$ were not included.

The relationship between log thermal time (θ) and germination expressed in probits was calculated for 0, GA_3, C, W and DAR according to eqn (3) (Fig. 9). A probit scale was used for the thermal time analysis, because the relationship between log θ and probit germination had a better fit ($r^2 > 0.96$ for all populations) than when expressed on a linear scale (data not shown). Thermal time for 50% of germination for untreated seeds was greater for the population at low elevation (2.35 log °Cd) than the mid and high altitude population (2.12 and 1.73 log °Cd, respectively; Fig. 9). Similar thermal time requirements for GA_3 treatment were observed for seeds collected at the lowest and the middle altitude population (2.27 and 2.21 log °C, respectively) than the population at high elevation (1.67 log °C; Fig. 9). With C and W treatments, the population at middle altitude reached a greater θ_{50} than the highest population (1.94 for C and 2.17 log °C for W; Fig. 9). While, seeds collected at low elevation never reached the θ_{50} (Fig. 9). It was possible to calculate θ_{50} for all populations treated with DAR. In particular the population at low altitude showed a greater θ_{50} (2.52 log °Cd) than the population at middle and high altitude (2.28 and 1.82 log °Cd , respectively; Fig. 9).

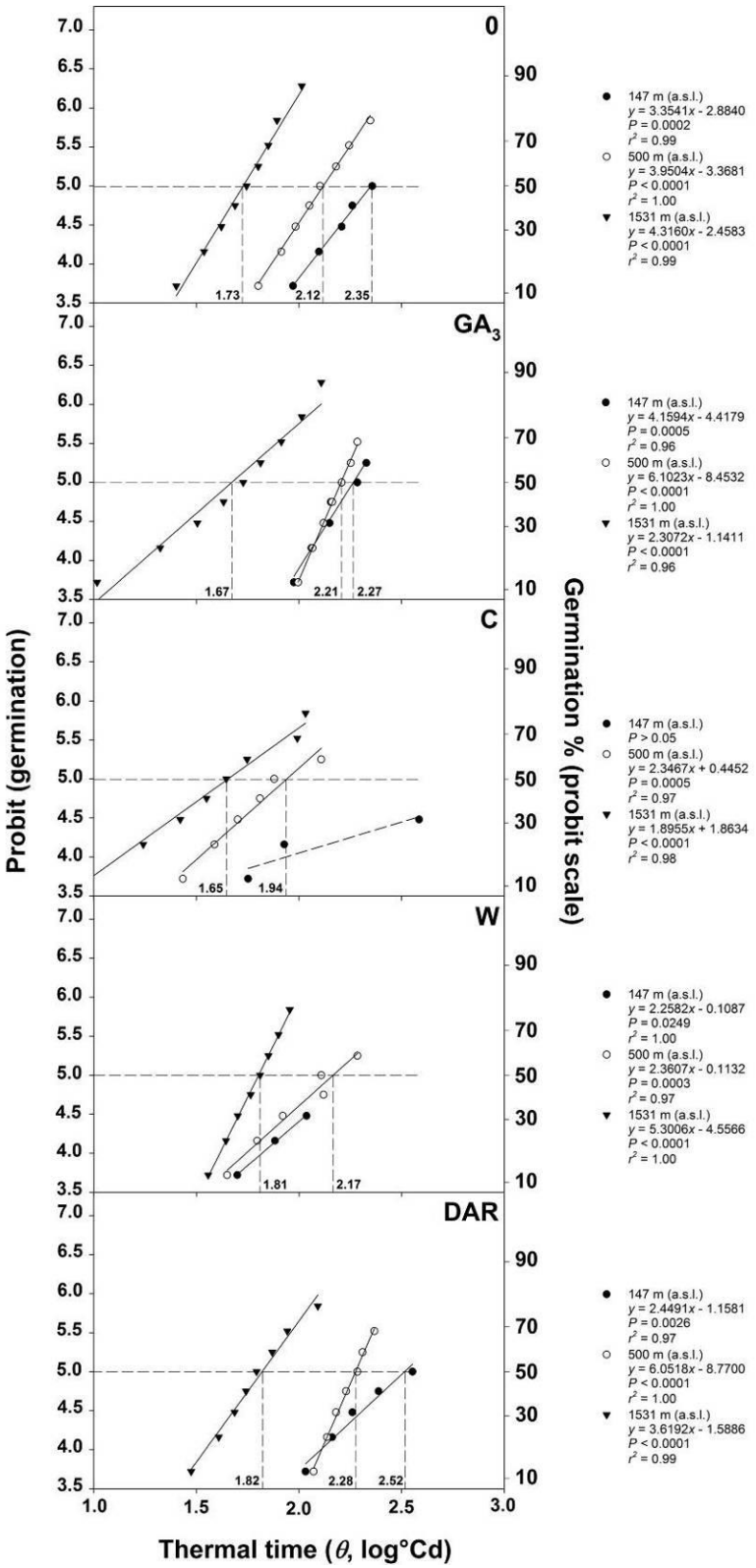

Figure 9 - Probit germination after each treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel) as a function of log-thermal time in the three populations of *Santolina insularis* located at different altitudes (147, 500 and 1531 m a.s.l.). Thermal times to reach θ_{50} are also shown (dashed lines).

Ptilostemon casabonae

GLMs highlighted a statistically significant effect of the treatment, population and temperature factors and of their interactions ($P < 0.001$) on seed germination of *Ptilostemon casabonae* (Table 5).

Table 5 - GLMs results for seed germination (%) of the following factors: "Treatment" (0, Control; C, 5°C for three months; W, 25°C for three months; GA_3, 250 mg L^{-1} in the germination substrate; DAR, 25°C for three months on silica gel), "Temperature" (5, 10, 15, 20, 25 and 25/10°C),"Population" (135, 686 and 1300 m a.s.l.).

Germination (%)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
NULL			359	23698.1		
Treatment	4	3239.2	355	20458.9	170.1889	< 2.2e-16 ***
Population	2	418.4	353	20040.5	43.9677	< 2.2e-16 ***
Temperature	5	15590.6	348	4449.8	655.3166	< 2.2e-16 ***
Treatment:Population	8	691.2	340	3758.7	18.1576	< 2.2e-16 ***
Treatment:Temperature	20	870.0	320	2888.6	9.1425	< 2.2e-16 ***
Population:Temperature	10	1033.8	310	1854.8	21.7270	< 2.2e-16 ***
Treatm.:Popul.:Temp.	40	450.2	270	1404.6	2.3656	2.754e-05 ***

In dark conditions, during C pre-treatment, seeds of each population of *P. casabonae* reached 10 ± 8% of germination as an average, while seeds treated with W, during pre-treatment, never reached values > 2%.

Untreated seeds of all three populations germinated with percentages never less than 84% at all tested temperatures except at 25°C, in this case, seeds reached 3, 1 and 41% of germination for populations at low, middle and high elevation, respectively. As well as for the Control test, GA_3 treated seeds of all populations had high germination percentages (on the average always > 85%) at all tested temperatures and also in this case, the minimum values were reached at 25°C (Fig. 10). With C treatment, seeds of all populations germinated with higher values at 15, 20 and 25/10°C. Seeds stored at 5, 10 and 15°C had increased germination with higher altitudes; specifically, from 0 to 32% at 5°C, from 7 to 60% at 10°C and from 40 to 90% at 15°C, for seeds collected at low and high elevation, respectively (Fig. 10). A similar trend of germination for seeds treated with W was shown for all tested populations. In particular, high germination was reached at all tested temperatures (never less than 78% as an average) with the lowest values at 25°C. The same was evident after DAR, where seeds of all populations reached very low percentages at 25°C, i.e., 10, 0 and 36%, for populations at low, middle and high elevation, respectively, while at the other temperatures germination was ≥ 75% (Fig. 10).

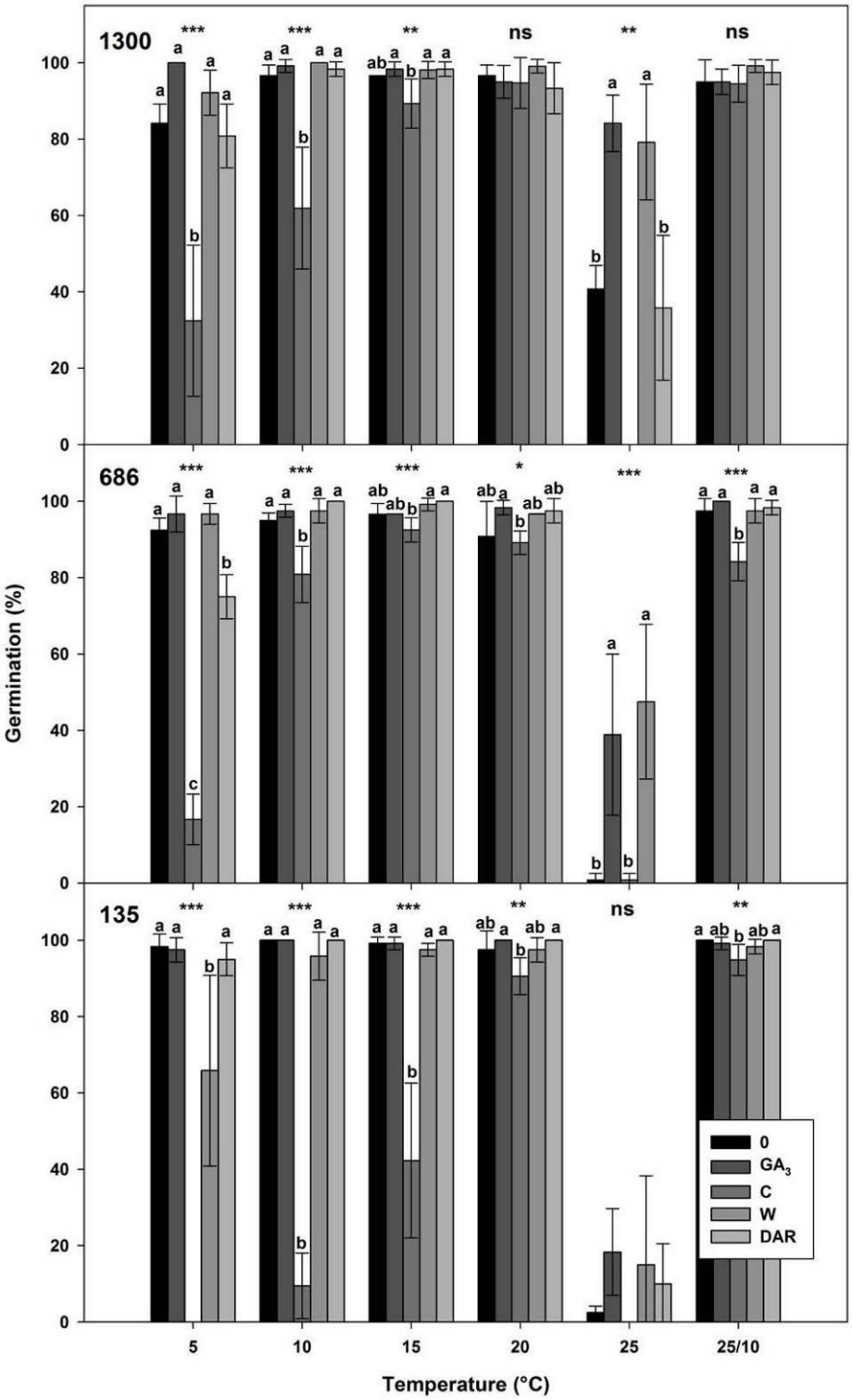

Figure 10 - Final germination percentages of *Ptilostemon casabonae* seeds collected at 135, 686 and 1300 m a.s.l. achieved at the end of germination tests after each pre-treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel). Data are the mean of four replicates (1 ± S.D.).

T_b values increased with increasing altitudes for untreated seeds (Control, 0), varying from 0.47 to 1.80°C for seeds collected at low and high elevation, respectively (Fig. 11). For GA_3 treated seeds collected at the lowest altitude population, it was possible to calculate T_b (0.66°C), T_o (15.12°C) and T_c (26.84°C) as well as for seeds collected at middle elevation which had T_b = -0.67°C, T_o = 15.08°C and T_c = 26.29°C. While, T_b of GA_3 treated seeds of the highest population was 0.59 (Fig. 11). Seeds treated with C had an increasing T_b with higher altitudes, rising from -2.73 to 3.18°C, for seeds collected at middle and high elevation, respectively). T_b was not reported for the lowest altitude population, because of the low germination at temperatures ≤ 15°C; nevertheless it was possible to calculate the ceiling temperature (T_c = 27.43°C; Fig. 11). With W treatment T_b, T_o and T_c were estimated for all populations. In particular, T_b values became higher with increasing altitude, varying from 0.39 to 1.75°C for seeds collected at low and high elevation, respectively. The population at low altitude had a T_o = 14.94°C and T_c = 29.45°C; the middle population T_o = 15.48°C and T_c = 28.40°C, while the highest altitude population had T_o = 15.02°C and T_c = 30.70°C (Fig. 11). DAR treated seeds showed T_b values between ca. -0.1 (populations at middle and high elevation) and ca. 2°C (the lowest altitude population; Fig. 11).

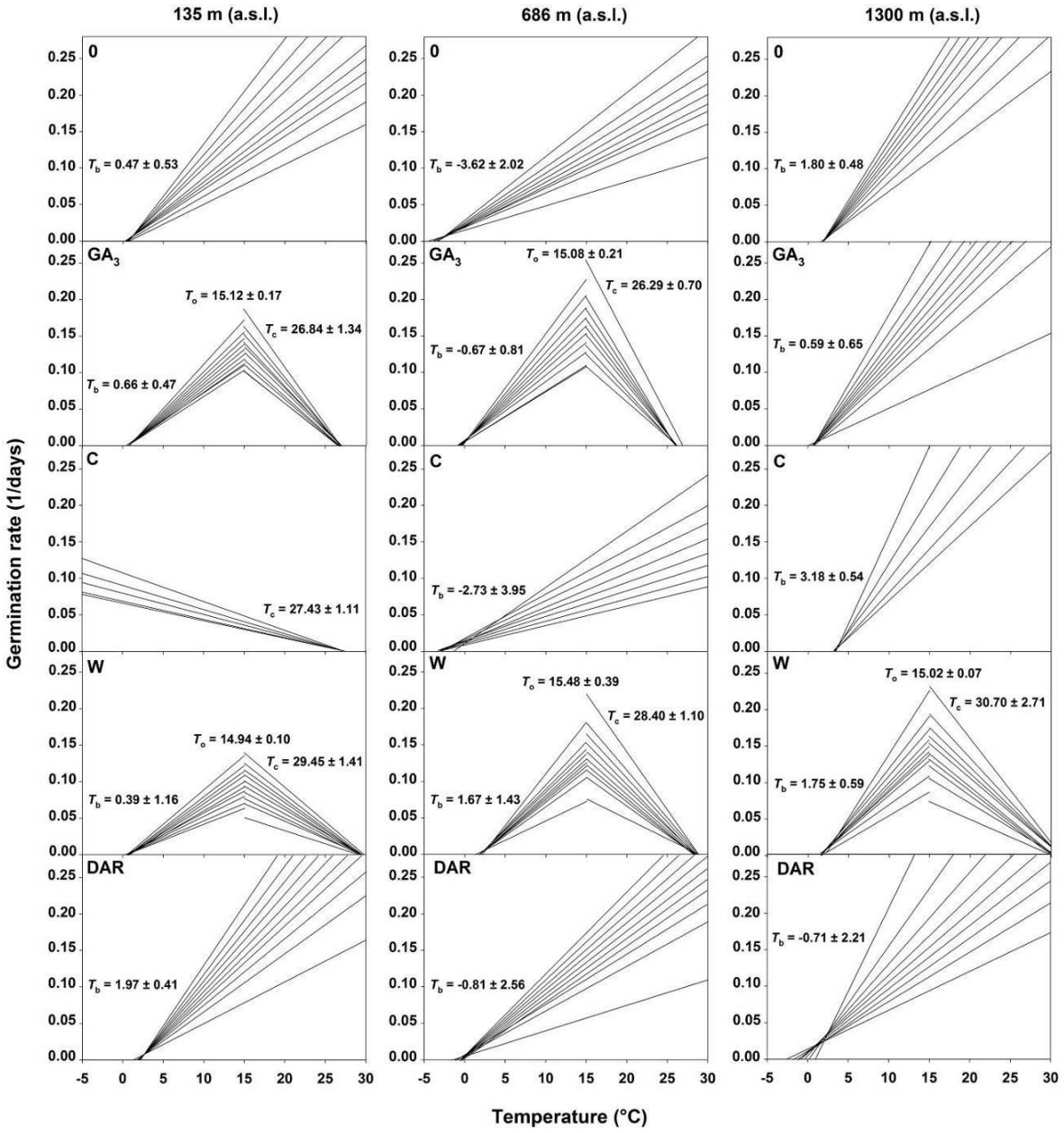

Figure 11 - Intra-specific differences on seed germination rates of *Ptilostemon casabonae*. Cardinal temperatures for germination (base T_b, optimum T_o and ceiling T_c temperatures) after each pre-treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel). For each pre-treatment, the linear regressions for the different percentiles were constrained to the common value of T_b and T_c. Linear regressions of percentiles with $P > 0.05$ were not included.

Fig. 12 shows the relationship between log thermal time (θ) and germination expressed in probits, calculated for 0, GA_3, C, W and DAR according to eqn (3). A probit scale was used for the thermal time analysis, because the relationship between log θ and probit germination had a better fit ($r^2 > 0.94$ for all populations) than when expressed on a linear scale (data not shown). For 0 seeds, the θ_{50} was greater for the population at middle altitude (2.22 log °Cd) compared to the low and high population (2.01 and 1.89 log °Cd, respectively; Fig. 12). Similar thermal time requirements for GA_3 treatment were observed for the lowest and middle altitude populations (2.04 and 1.98 log °C, respectively) compared to the population at high elevation (1.89 log °C; Fig. 12). For the C test, it was possible to calculate the θ_{50} only for the populations at middle and high altitude, which reported 2.40 and 1.99 log °Cd, respectively (Fig. 12). With W treatment, θ_{50} was greater for the population at low altitude (2.16 log °Cd) compared to the middle and high population (1.99 and 1.94 log °Cd, respectively). Finally, with DAR treatment, the population at middle altitude showed a greater θ_{50} (2.07 log °Cd) than the low and high altitude population (1.93 and 1.99 log °Cd, respectively; Fig. 12).

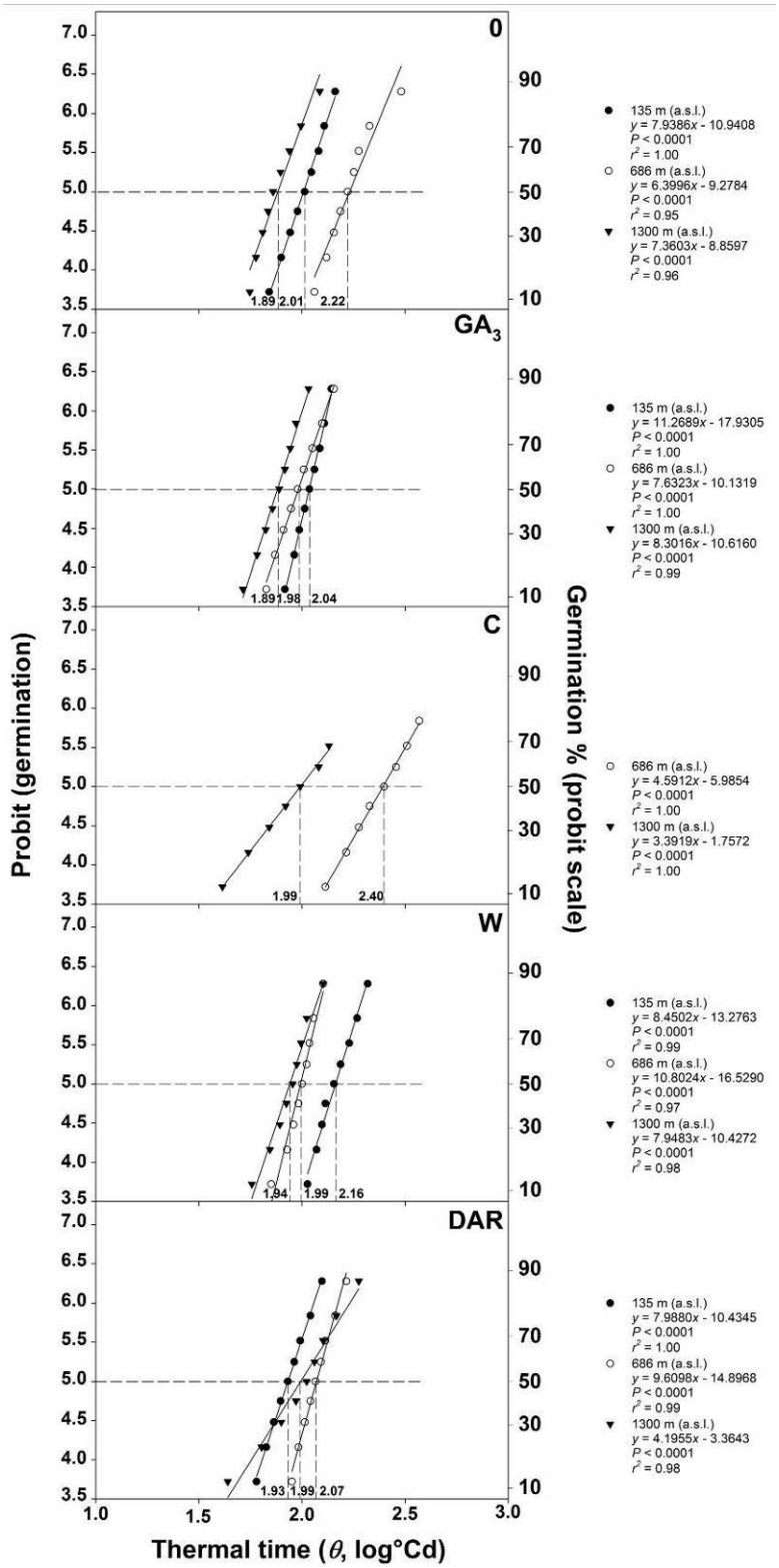

Figure 12 - Probit germination after each treatment (0, Control; GA_3, 250 mg L^{-1} in the germination substrate; C, 5°C for three months; W, 25°C for three months; DAR, 25°C for three months on silica gel) as a function of log-thermal time in the three populations of *Ptilostemon casabonae* located at different altitudes (135, 686 and 1300 m a.s.l.). Thermal times to reach θ_{50} are also shown (dashed lines).

Comparative analysis between T_b and altitude

A positive correlation between T_b and altitude for 0 was highlighted for *D. purpurea*, *S. insularis* and *P. casabonae*. However, although the linear regressions do not highlighted a statistical trend ($P > 0.05$), *S. trifoliata* seems to have a decreasing of T_b with increasing altitude (Fig. 13).

T_b in GA_3 treated seeds of *D. purpurea* and *S. insularis* increased with increasing altitude, while in seeds of *P. casabonae* and *S. trifoliata*, T_b showed no correlation with altitude ($P > 0.05$; Fig. 13). In C treated seeds of *P. casabonae* and *S. insularis*, T_b increased with the elevation. However, this pattern was not detected for *D. purpurea* and *S. trifoliata* ($P > 0.05$; Fig. 13).

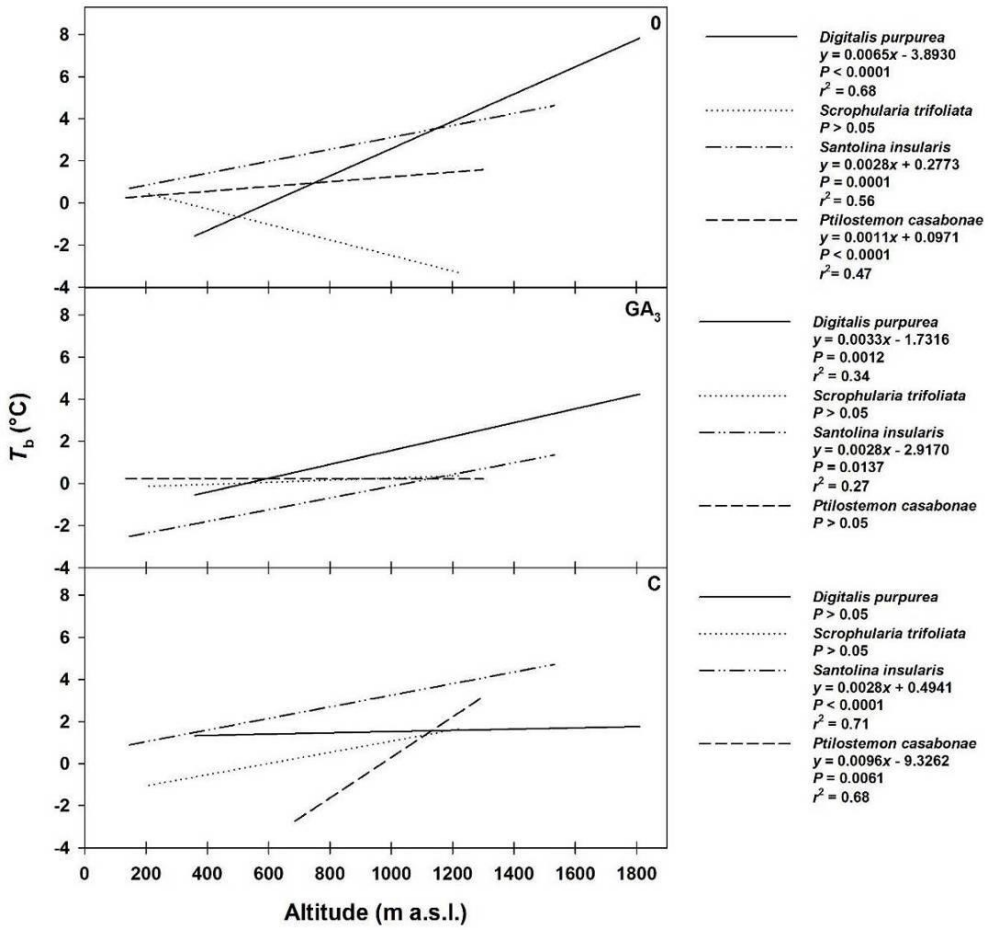

Figure 13 - Relationship between T_b and altitude among seeds of *Digitalis purpurea*, *Scrophularia trifoliata*, *Santolina insularis* and *Ptilostemon casabonae* without any pre-treatment (0; Control), for gibberellic acid (GA_3; 250 mg L^{-1} in the germination substrate) treated seeds and after pre-chilling (C; 5°C for three months). For all species were considered average values of θ_{50} of the three different populations investigated.

Comparative analysis between θ and altitude

A negative correlation between log thermal time (θ) and altitude for 0 was reported for *D. purpurea* and *S. insularis*. On the contrary, a positive correlation was highlighted for *S. trifoliata* which seeds increased the θ_{50} with increasing altitude. However, no correlation was highlighted for seeds of *P. casabonae* ($P > 0.05$; Fig. 14). The relationship between θ_{50} and altitude for seeds treated with GA_3 and altitude highlighted a negative correlation for *D. purpurea*, *P. casabonae* and *S. insularis.* In particular, θ_{50} values of the following species decreased with increasing elevation. However, no correlation was highlighted for seeds of *S. trifoliata* ($P > 0.05$; Fig. 14). Seeds treated with C of *P. casabonae* and *S. trifoliata* showed a negative correlation between θ_{50} and altitude. Although the linear regressions do not highlighted a statistical trend ($P > 0.05$), *D. purpurea* and *S. insularis* seem to have the same pattern (Fig. 14).

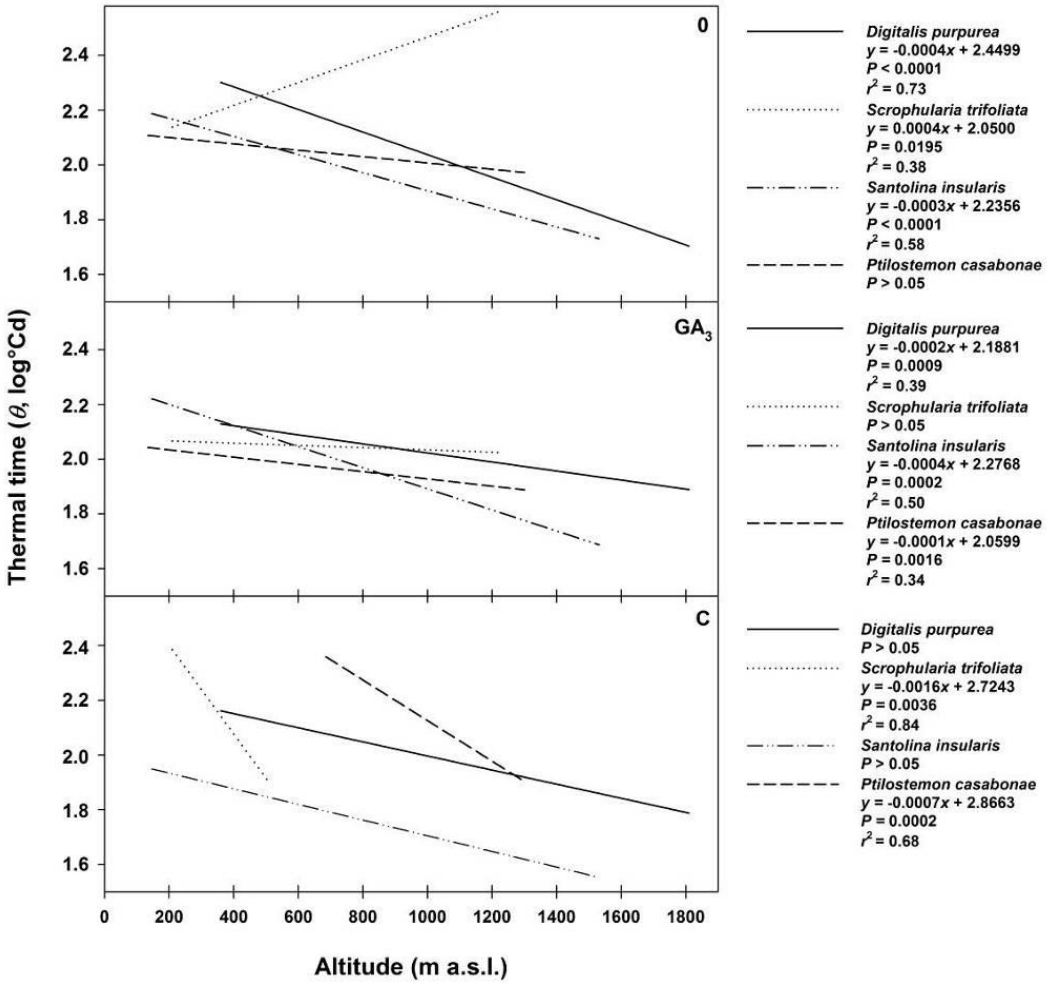

Figure 14 - Relationship between log thermal time (θ) and altitude among seeds of *Digitalis purpurea*, *Scrophularia trifoliata*, *Santolina insularis* and *Ptilostemon casabonae* without any pre-treatment (0; Control), for gibberellic acid (GA_3; 250 mg L^{-1} in the germination substrate) treated seeds and after pre-chilling (C; 5°C for three months). For all species were considered average values of θ_{50} of the three different populations investigated.

Discussion

Intra-specific differences in final germination percentages were highly significant in the four study species both for Control and for treated seeds (GA_3, C, W and DAR). In most of the four species, seeds germinated without treatments, however different germination responses were achieved among populations. GA_3 significantly enhanced seed germination in some accessions compared to the Control and this indicates that different degree of physiological dormancy (PD) could be found in the investigated populations. Also the effect of the cold and warm wet stratifications was different among populations. In some cases, for example seeds never germinated after the warm treatment; in others, seed germination increased with increasing elevation, or sometimes, seeds reached very high germination percentages regardless altitude. Finally, also the experiments with dry after-ripening, showed inter- and intra-specific variations in seed germination of the four studied species. The variability of germination characteristics could be interpreted as one of the most important survival strategies for species growing under unpredictable environmental conditions (Gutterman, 1994; Kigel, 1995). Several environmental factors (e.g. light, moisture, temperature, as well as altitude) can cause variability in germination behaviour among populations of one species (Fenner, 1991; Gutterman, 1992; Holm, 1994; Vera, 1997). In this study, the inter-population differences in germination responses could reflect local adaptation to particular environmental conditions. Correlations between higher altitude sites and higher dormancy levels have been reported by several authors (Holm, 1994; Vera *et al*., 1997; Cavieres and Arroyo, 2000). Fernández-Pascual *et al.* (2013) investigated the local patterns of seed dormancy in *Centaurium somedanum* species. In their study reported that *C. somedanum* plant, growing at lower altitudes, under a generally milder climate, produce seeds that will germinate earlier, benefiting from a longer growing season. While, plants from higher altitudes, where winters are harsher, produce seeds which will not germinate until the unfavourable season is over.

The analysis carried out in this study highlighted intra-specific differences on base temperatures (T_b) for germination of the four species. In some cases, it was possible to calculate also the optimal and the ceiling temperatures for germination. The significantly change of T_b among populations may reflect a local adaptation to environmental conditions along the altitudinal gradient (Pérez-García *et al*., 2003). For example, untreated seeds of *D. purpurea* increased T_b with increasing altitude, and chilling reduced these values, showing that seeds from higher elevations may have a higher degree of PD (with > T_b values) than those from lower altitudes. This suggests that germination for seeds collected at the high altitude, occurs preferentially after seeds have experienced low winter temperatures (Mondoni *et al*., 2012). This device prevents young seedlings from being damaged by freezing temperatures (Billings *et* Mooney 1968). Several authors (Covell *et al*., 1986; Ellis *et al*., 1987; Pritchard and Manger, 1990) reported that T_b is a constant within a population, while this value may slightly or significantly change among populations of the same species (i.e., Ellis *et al*., 1987; Daws *et al*., 2004). Our results showed that thermal thresholds for seeds germination are related clearly to the elevation of each population and treatments can modify the T_b in each population. We agree with previous studies (Pérez-García *et al*., 2003; Pérez-García *et al*., 2006) according to which extrapolation of seed germination requirements from a single population must be interpreted with caution due to the frequent variability on seed germination responses within one species.

Seeds of all the populations of the study species varied in their thermal time estimates (θ_{50}), thus, these results highlighted that seed lot provenance may influences the sensitivity of the seed germination response to thermal time (Daws *et al*., 2004; Orrù *et al.*, 2012; Porceddu *et al*., 2013). In particular, in most of the four species, the lowest θ_{50} was achieved for seeds collected at the highest altitude population. These findings are in agreement with Orrù *et al*. (2012) which reported that sensitivity to the accumulated thermal units (°Cd) of four

populations of *Vitis sylvestris*, located at different elevations, increased with altitude, leading to reduced θ_{50} values in the highest populations. This strategy represent an ecological advantage for species of high altitude that start to germinate immediately after snowmelt in spring, and need a faster germination due to the shorter growing season (Baskin and Baskin, 2003; Fenner and Thompson, 2005; Orrù *et al.*; 2012; Porceddu *et al.*, 2013). In contrast, seeds from the lowest populations due to their high θ_{50} can start to germinate in winter, during the rainy season, so that the developing seedlings exploit most of the mild winter and following spring before the onset of summer drought (Thanos *et al*., 1991; Doussi and Thanos, 2002).

The comparative analysis between T_b and altitude among seeds of the studied species highlighted inter-specific differences both in relation to altitude and treatments. The positive correlation between T_b and altitude detected for untreated seeds of *D. purpurea*, *S. insularis* and *P. casabonae* can be explained as an adaptation strategy against the long period of snow cover for high-elevation populations (Meyer *et al.*, 1995; Pendleton and Meyer, 2004). While, GA_3 seems to be more effective at lowering T_b for seeds at high altitudes, allowing a widening of the temperature range over which germination can occur (Vera, 1997; Cavieres and Arroyo, 2000; Baskin and Baskin, 2014). However, the opposite trend detected for Control and GA_3 treated seeds of *S. trifoliata*, suggests a different ecological adaptation for this species to the local environmental conditions. Inter-specific differences were also detected with C treatment. In *D. purpurea* chilling lowered T_b values of seed of high altitude of compared to the Control; however seems to have an opposite trend in *S. trifoliata* and *P. casabonae*; while, T_b of *S. insularis* was similar to the Control. Therefore, a variation in T_b values among species is apparent; this is not surprising since thermal thresholds for seed germination may be significantly influenced by the local climatic conditions they experience (Rosbakh and Poschlod, 2014; Dürr *et al*., 2015).

Also the analysis carried out to compare log thermal time (θ_{50}) and altitude showed inter-specific differences in relation to the treatments. In particular, chilled treated seeds of all species showed a negative correlation between θ_{50} and altitude. These results confirmed what is reported by different authors (Orrù *et al*., 2012; Porceddu *et al*., 2013) where chilling led to a reduction in θ_{50} in different Mediterranean species, especially from high mountain. Thus, high mountain Mediterranean plants do not seem to differ in germination characteristics from other alpine plants where spring germination prevails mainly due to a requirement of cold stratification over winter (Giménez-Benavides *et al*., 2005; Mondoni *et al.*, 2012; Baskin and Baskin, 2014).

Conclusion

Our results demonstrate high intra-specific variation for the germination pattern of seeds from different populations of *D. purpurea*, *S. trifoliata*, *S. insularis* and *P. casabonae* and provide further information about seed germination of four Mediterranean species along an altitudinal gradient. These results highlight that the origin of seed samples should always be taken into account when defining models of germination behaviour, especially in wild species with high degree of physiological variability.

References

Andersson L., Milberg P., 1998. Variation in seed dormancy among mother plants, populations and year of seed collection. Seed Science Research, 8: 29–38.

Angiolini C., Bacchetta G., 2003. Analisi distributiva e studio fitosociologico delle comunità a *Santolina insularis* (Gennari *ex* Fiori) Arrigoni della Sardegna meridionale (Italia). Fitosociologia, 40 (1): 109–127.

Arrigoni P.V., 1978a. Le piante endemiche della Sardegna: 117. Bollettino della Società Sarda di Scienze Naturali, 21*:* 438–441.

Arrigoni P.V., 1978b. Le piante endemiche della Sardegna: 99. Bollettino della Società Sarda di Scienze Naturali, 21*:* 338–343.

Bacchetta G., Fenu G., Mattana E., 2008. Studi di biologia della conservazione di specie vegetali endemiche della Sardegna nell'ambito del progetto "GENMEDOC". Webbia: Journal of Plant Taxonomy and Geography, 63(2): 293–307.

Baskin C.C., Baskin J.M., 2003. When breaking seed dormancy is a problem. Try a Move-along Experiment. Native Plants|Spring, 4: 17–21.

Baskin J.M., Baskin C.C., 2004. A classification system for seed dormancy. Seed Science Research, 14: 1–16.

Baskin C.C., Baskin J.M., 2014. Seeds: ecology, biogeography, and evolution of dormancy and germination. San Diego: Academic Press.

Bewley J.D., 1997. Seed germination and dormancy. Plant Cell, 9: 1055–1066.

Billings W.D., Mooney H.A., 1968. The ecology of arctic and alpine plants. Biological Review, 43: 481–529.

Cavieres L.A., Arroyo M.T.K., 2000. Seed germination response to cold stratification period and thermal regime in *Phacelia secunda* (Hydrophyllaceae) - Altitudinal variation in the mediterranean Andes of central Chile. Plant Ecology, 149: 1–8.

Covell S., Ellis R.H., Roberts E.H., Summerfield R.J., 1986. The influence of temperature on seed germination rate in grain legumes. I. A comparison of chickpea, lentil, soyabean and cowpea at constant temperatures. Journal of Experimental Botany, 37: 705–715.

Crawley M.J., 2007. The R book. Chichester, UK: JohnWiley & Sons Ltd.

Daws M.I., Burslem D.F.R.P., Crabtree L.M., Kirkman P., Mullins C.E., Dalling J.W., 2002. Differences in seed germination responses may promote coexistence of four sympatric *Piper* species. Functional Ecology, 16: 258–267.

Daws M.I., Lydall E., Chmielarz P., Leprince O., Matthews S., Thanos C.A., Pritchard H.W., 2004. Developmental heat sum influences recalcitrant seed traits in *Aesculus hippocastanum* across Europe. New Phytologist, 162: 157–166.

Doussi M.A., Thanos C.A., 2002. Ecophysiology of seed germination in Mediterranean geophytes. 1. *Muscar*i spp. Seed Science Research, 12: 193–201.

Dürr C., Dickie J.B., Yang X.-Y., Pritchard H.W., 2015. Ranges of critical temperature and water potential values for the germination of species worldwide: Contribution to a seed trait database. Agricultural and Forest Meteorology, 200: 222–232.

Ellis R.H., Covell S., Roberts E.H., Summerfield R.J., 1986. The influence of temperature on seed germination rate in grain legumes. II. Intraspecific variation in chickpea (*Cicer arietinum* L.) at constant temperatures. Journal of Experimental Botany, 37: 1503–1515.

Ellis R.H., Simon G., Covell S., 1987. The influence of temperature on seed germination rate in grain legumes. III. A comparison of five faba bean genotypes at constant temperatures using a new screening method. Journal of Experimental Botany, 38: 1033–1043.

Fenner M., 1991. The effects of the parent environment on seed germinability. Seed Science Research, 1: 75–84.

Fenner M., Thompson K., 2005. The ecology of seeds. Cambridge, UK: Cambridge University Press.

Fernández-Pascual E., Jiménez-Alfaro B., Caujapé-Castells J., Jaén-Molina R., Díaz T.E., 2013. A local dormancy cline is related to the seed maturation environment, population genetic composition and climate. Annals of Botany, 112: 937–945.

Finch-Savage W.E., Leubner-Metzger G., 2006. Seed dormancy and the control of germination. New Phytologist, 171: 501–523.

Finch-Savage W.E., Cadman C.S.C., Toorop P.E., Lynn J.R. Hilhorst H.W.M., 2007. Seed dormancy release in *Arabidopsis* Cvi by dry after ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing. The Plant Journal, 51: 60–78.

Garcia-Huidobro J., Monteith J.L., Squire G.R., 1982. Time, temperature and germination of pearl millet (Pennisetum typhoides S. & H.). Journal of Experimental Botany, 33: 288–296.

Giménez-Benavides L., Escudero A., Pérez-García F., 2005. Seed germination of high mountain Mediterranean species: altitudinal, interpopulation and interannual variability. Ecological Research, 20: 433–444.

Gutterman Y., 1992. Maternal effects on seeds during development. In: Fenner, M., ed. Seeds: the ecology of regeneration in plant communities. C.A.B. International, Wallingford, UK, pp. 27–59.

Gutterman Y., 1994. Strategies of seed dispersal and germination in plants inhabiting deserts. Botanical Review, 60: 373–425.

Hardegree SP., 2006. Predicting germination response to temperature. I. Cardinal temperature models and subpopulation-specific regression. Annals of Botany, 97: 1115–1125.

Hernández E.I., Vilagrosa A., Pausas J.G., Bellot J., 2010. Morphological traits and water use strategies in seedlings of Mediterranean coexisting species. Plant ecology, 207: 233–244.

Holm S.O., 1994. Reproductive patterns of *Betula pendula* and *B. pubescens* coll. along a regional altitudinal gradient in northern Sweden. Ecography, 17: 60–72.

Joffre R., Rambal S., Damesin C., 1999. Functional attributes in Mediterranean-type ecosystems. In: Pugnaire FI, Valladares F, eds. Handbook of functional plant ecology. NewYork: Marcel Dekker, 347–380.

Karlsson L.M., Milberg P., 2007. A comparative study of germination ecology of four *Papaver* taxa. Annals of Botany, 99: 935–946.

Karlsson L.M., Tamado T., Milberg P., 2008. Inter-species comparison of seed dormancy and germination of six annual Asteraceae weeds in an ecological context. Seed Science Research, 18: 35–45.

Kigel J., 1995. Seed germination in arid and semiarid regions. In: Kigel J, Galili G (eds) Seed development and germination. Marcel Dekker, New York, 645–699.

Linington S., Bean E.W., Tyler B.F., 1979. The effects of temperature upon seed germination in *Festuca pratensis* var. *apennina*. Journal of Applied Ecology, 16: 933–938.

Mattana E., Pritchard H.W., Porceddu M., Stuppy W.H., Bacchetta G., 2012. Interchangeable effects of gibberellic acid and temperature on embryo growth, seed germination and epicotyl emergence in *Ribes multiflorum* ssp. *sandalioticum* (Grossulariaceae). Plant Biology, 14: 77–87.

Marengo A., Fenu G., Gennai M., Cogoni D., Fois M., Bacchetta G., 2015. *Ptilostemon casabonae* (L.) Greuter. Informatore Botanico Italiano, in press.

Meyer S., Kitchen S., Beckstead J., 1995. Seed germination timing patterns in intermountain *Penstemon* (Scrophulariaceae). American Journal of Botany, 82:377–389.

Mondoni A., Rossi G., Orsenigo S., Probert R.J., 2012. Climate warming could shift the timing of seed germination in alpine plants. Annals of Botany, 110: 155–164.

Orrù M., Mattana E., Pritchard H.W., Bacchetta G., 2012. Thermal thresholds as predictors of seed dormancy release and germination timing: altitude-related risks from climate warming for the wild grapevine *Vitis vinifera* subsp. *sylvestris*. Annals of Botany, 110: 1651–1660.

Pendleton B.K., Meyer S.E., 2004. Habitat-correlated variation in blackbrush (*Coleogyne ramosissima*: Rosaceae) seed germination response. Journal of Arid Environments, 59:229–243.

Pérez-García F., Hornero J., González-Benito M.E., 2003. Interpopulation variation in seed germination of five Mediterranean Labiatae shrubby species. Israel Journal of Plant Sciences, 51: 117–124.

Pérez-García F., Huertas M., Mora E., Peña B., Varela F., González-Benito M.E., 2006. *Hypericum perforatum* L. seed germination: interpopulation variation and effect of light, temperature, presowing treatments and seed desiccation. Genetic Resources and Crop Evolution, 53: 1187–1198.

Porceddu M., Mattana E., Pritchard H.W., Bacchetta G., 2013. Thermal niche for in situ seed germination in Mediterranean mountain streams: model prediction and validation for *Rhamnus persicifolia* seeds. Annals of Botany, 112(9): 1887–1897.

Pritchard H.W., Manger K.R., 1990. Quantal response of fruit and seed germination rate in *Quercus robur* L. and *Castanea sativa* Mill, to constant temperatures and photon dose. Journal of Experimental Botany, 41 (233): 1549–1557,

Pritchard H.W., Tompsett P.B., Manger K.R., 1996. Development of a thermal time model for the quantification of dormancy loss in *Aesculus hippocastanum* seeds. Seed Science Research, 6: 127–135.

Probert R.J., 2000. The role of temperature in the regulation of seed dormancy and germination. In: Fenner M., ed. Seeds: the ecology of regeneration in plant communities. Wallingford, UK: CAB International, 261–292.

R Development Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Rosbakh S., Poschlod P., 2014. Initial temperature of seed germination as related to species occurrence along a temperature gradient. Functional Ecology, doi: 10.1111/1365-2435.12304.

Schütz W., Rave G., 2003. Variation in seed dormancy of the wetland sedge, *Carex elongata*, between populations and individuals in two consecutive years. Seed Science Research, 13: 315–322.

Thanos C.A., Georghiou K., Dimitra J.D., Marangaki C.J., 1991. Photoinhibition of seed germination in Mediterranean maritime plants. Annals of Botany, 68: 469–47.

Urbanska K.M., Schütz M., 1986. Reproduction by seed in alpine plants and re-vegetation research above timberline. Botanica Helvetica, 96: 43–60.

Valsecchi F., 1979. Observations sur quelques espèces du genre "*Scrophularia*" L. en Sardaigne. Webbia, 34: 265–288.

Vandelook F., Van Assche J.A., 2008. Temperature Requirements for Seed Germination and Seedling Development Determine Timing of Seedling Emergence of Three Monocotyledonous Temperate Forest Spring Geophytes. Annals of Botany, 102: 865–875.

Vera M.L., 1997. Effects of altitude and seed size on germination and seedling survival of heathland plants in north Spain. Plant Ecology, 133: 101–106.

Walck J.L., Hidayati S.N., Dixon K.W., Thompson K., Poschlod P., 2011. Climate change and plant regeneration from seed. Global Change Biology, 17: 2145–2161.

Chapter 4: Biophysical and physiological characteristics of seeds of Mediterranean species along an altitudinal gradient

Introduction

When a seed arrives at the soil surface, it may germinate immediately, or alternatively, persist in the soil or on the soil surface for a shorter or longer period with the primary purpose to synchronize germination with a season suitable for the establishment and growth (Thompson, 2000). The ability to form a persistent or semi-persistent soil seed bank can be a crucial survival mechanism of many rare or declining species (Keddy and Reznicek, 1982; Rowell *et al.*, 1982; Quilichini and Debussche, 2000; Eckstein *et al.*, 2006), conferring a degree of resilience to the intensive land use (Thompson *et al.*, 1993) and protecting populations from local extinction when vegetation is removed (Arroyo *et al.*, 2006). Yu *et al.* (2007) suggested that differences in climate may determine diverse seed persistence patterns and the local climatic conditions and may significantly influence the sensitivity of the thermal thresholds for seed germination, i.e. the base temperature of germination (T_b) (Rosbakh and Poschlod, 2014). Indeed, germination characteristics of seeds sampled from different geographical environments can vary in many ways, in particular, depending on the species, germination responses may vary with latitude, elevation, soil moisture, light and temperature (Holm, 1994; Vera, 1997; Baskin and Baskin, 2014).

Under a Mediterranean climate, characterised by a highly seasonal alternation of favourable and unfavourable conditions, plant growth and reproduction must occur in the window of favourable conditions that may vary in length and in which environmental cues and constraints play a central role (Thanos *et al.*, 1995; Doussi and Thanos, 2002; Gresta *et al.*, 2010). In this environment there are long periods of drought during the summer along with high temperatures, which combined impose severe abiotic stresses that limit plant growth and subsequently compromise survival (Medrano *et al*., 2009). Thus, the arid summer in Mediterranean environments represents the most dangerous season for seedling germination and growth (Fenner and Thompson, 2005). On the contrary, in temperate, boreal and arctic climates, seed germination occurs immediately after snowmelt in spring, in order to avoid the low, freezing, temperatures during seedling establishment in the autumn and early winter period (Thompson, 2000; Baskin and Baskin, 2003; Fenner and Thompson, 2005; Cochrane *et al*., 2011; Rosbakh and Poschlod, 2014). Sardinia represents an interesting study area in the Mediterranean, due to its geographical isolation, variable climate and geological diversity that have created a wide range of habitats and high levels of endemism (Bacchetta *et al*., 2012; Médail and Quézel, 1997).

Annual average temperatures vary with altitude and length of the winter period, and temperature during the period of seed development can affect oil composition. Whilst the fatty acid composition of the oil from safflower and castor bean was not affected by the maternal temperature between 10 and 26°C, in sunflower, flax and rape seed the amount of the more highly unsaturated fatty acids decreased as the temperature was increased, accompanied by an increase in oleic acid (Canvin, 1965). Similarly, high temperature during the development of sunflower seed caused a marked reduction in the percentage of linoleic acid (Harris *et al*., 1978). As alpine species tend to produce small seeds and small seeds efficiently store energy in the form of lipid, it is possible that an altitudinal gradient in the maternal environment could impact on fatty acid composition. Such a change might be visualised via seed thermally-dependent transformations which are known to vary in oil rich seeds with oil composition (Nadarajan and Pritchard, 2014). Consequently, seeds all thirteen species investigated here were subjected to biophysical analysis using a differential scanning calorimetry (DSC) to determine their lipid thermal fingerprints, including first (crystallisation,

melting) and second order transformations (glass transitions) during heating / cooling programmes.

As reported by Benson *et al*. (1996) transitions between liquid, amorphous glassy and ice states can be detected by heat flow data manifested as an exothermic peak during cooling and endothermic peak during re-warming. DSC is also used to aid the development of cryopreservation methods in relation to safe moisture levels for seed freezing (Dumet *et al*., 2000) and could provide insight to seed responses in the soil subjected to cold winter temperatures. Consequently, specific attention was given to the water thermal finger prints and water activity of *Digitalis purpurea* L. var. *gyspergerae* (Rouy) Fiori and *Ptilostemon casabonae* (L.) Greuter sampled from two different populations. These species were chosen because of their endemism and wide range population distribution at different altitudes and varying in their edaphic factors.

The aims of this work were to; (1) evaluate lipid thermal profile of the dry seeds and find out if they are correlated with altitudinal gradients; (2) correlate the lipid thermal fingerprints of the seeds with the actual climate data of soil temperature along the altitudinal gradient; (3) correlate the lipid thermal fingerprints with the base temperature (T_b) for 50% of germination (see Chapter 1) along the altitudinal gradient; (4) to study the biophysical adaptation of the two selected species at two different extreme altitudes through their freezing behaviour and water activity.

Material and methods

Seed collecting and soil temperature recording

Seeds of the selected species were collected from close to the sea level up to the highest mountain regions of the Island, ranging from ca. 25 to ca. 1800 m a.s.l. Specifically, 18 seed lots of 13 species were collected directly from the mother plants at the time of natural dispersal (Table 1). Several collecting trips were carried out each year from late spring (May) to the autumn (October) in 2012-2013 (Table 1). For the species listed in the annexes of the Habitat Directive, as required by the European and national laws (articles 9 and 10 of DPR 357/97 modified by DPR 120/03), seeds were collected after obtaining permits from the "Ministero dell'Ambiente e della Tutela del Territorio e del Mare".

To study and monitor the annual trend of soil temperature, 24 data-loggers (TidbiT® v2 Temp logger, Onset Computer Corporation, Cape Cod, MA, U.S.A.) were buried at a depth of 2–3 cm, at different altitudes (see Table 2), at times over two years. The loggers recorded the soil temperature at 90-min intervals and most of them covered at least two winter and summer seasons.

Thermal analysis

Lipid thermal profiles of the dry seeds were assessed using a differential scanning calorimetry DSC 1 (Mettler-Toledo, Switzerland), with a TC125-MT Intracooler (Huber, Germany), controlled by STAR^e software. For each species, samples of three replicates of seeds previously equilibrated to 15% RH weighing between 15-25 mg, were placed in pre-weighed aluminium pans, non-hermetically sealed using a Mettler-Toledo crimper and the fresh weights were recorded. Samples were then cooled from 25 to -80°C and then rewarmed to 25°C at a cooling/warming rate of ± 10°C min^{-1} and held for 2 min at isothermal temperatures. The onset, peak and end temperature and enthalpy values for lipid crystallization during cooling and lipid melting during warming were calculated using the STAR^e software (Table 3).

The lipid thermal fingerprints of the seeds were correlated with the actual climatic data obtained by recording of soil temperatures along the altitudinal gradient. For a congruent correlation, only 13 seed lots which are collected close to the data loggers were considered (i.e. *Clematis vitalba*, *Digitalis purpurea* var. *gyspergerae* (hereafter *Digitalis purpurea*; all the three populations), *Helianthemum caput-felis*, *Nepeta foliosa*, *Ptilostemon casabonae* (both the lowest and the highest altitude population), *Rhamnus alaternus*, *Rhamnus lycioides* ssp. *oleoides* (hereafter *Rhamnus oleoides*), *Santolina insularis* (the lowest altitude population), *Scrophularia trifoliata* (both the lowest and the highest altitude population).

As reported in Chapter 1, soil temperatures were analysed for winter and summer seasons. Winter period was considered from the 21st of December to the 20th of March while summer ran from the 21st of June to the 21st of September. The minimum daily temperature, the mean daily temperature and the duration of the winter period (i.e., number of days with mean daily temperatures ≤ 5°C) were calculated for the winter season. The maximum daily temperature, the mean daily temperature and the duration of the summer period (i.e. days with mean daily temperatures ≥ 25°C) were calculated for the summer season.

DSC lipid thermal profiles of dry seeds were also correlated with theoretical base temperature per germination (T_b) at which the germination rate is equal to zero (Ellis *et al*., 1986). As reported in Chapter 1, T_b was calculated by determining the seed germination rate, defined as the reciprocal of time to reach 50% of actual germination for the tests carried out at constant temperatures (5–25°C). Data was analysed using a liner regression model, by averaging the *x*-intercept for the suboptimal temperature range. When 50% of final germination was not reached, the T_b value was estimated *sensu* Trudgill *et al*. (2000),

reporting the lowest incubation temperature at which seed germination was recorded (see details in Chapter 1).

Thermal analysis of water phase behaviour was carried out for the wet seeds of two different populations of two species that are sampled from the lowest and the highest altitudinal ranges (i.e. from ca. 300 to 1800 m a.s.l for *Digitalis purpurea*; and from ca. 100-1300 m a.s.l. for *Ptilostemon casabonae*). The seeds were equilibrated to 80% relative humidity (RH) in a humidity control environment before the analysis. Samples were cooled from 20 to -40°C and then rewarmed to 20°C at a cooling/warming rate of ± 0.05°C min^{-1} and held for 2 min at isothermal temperatures, mimicking the actual environmental conditions. The onset, peak and end temperature and enthalpy values for ice crystallization during the cooling were calculated using the STAR^e software. After the thermal analysis, sample pan lids were pierced and the pans together with the samples were dried in an oven at 103°C for 17 h to determine sample dry weight. This allowed the calculation of the total water content of the samples and subsequently the proportion of frozen and unfrozen water. According to Block (2003) thermodynamically 1 g of water releases 334.5 joules of heat energy when converted into ice and *vice versa*; thus, following Nadarajan *et al.* (2008) the osmotically active water content of the sample (g water osmotically active water per g sample dry weight) was calculated from the exothermic-heat changes derived from the crystallization endotherm during the cooling cycle along with the total water content of the sample. The quantity of osmotically inactive water was calculated as the difference between total water and osmotically active water contents.

Table 1 – Seeds lots details and information on the species collected. *Sources: Bacchetta, 2006; Bacchetta *et al*., 2012; Fenu *et al*., 2014. Abbreviations on the endemic species distribution: SA = Sardinia; CO = Corsica; BL = Balearic Islands; GA = France; H = Hyères Islands; AT = Tuscan Archipelago. Abbreviations on the province: CA = Cagliari; CI = Carbonia-Iglesias; NU = Nuoro; OR = Oristano. Species were collected close to the data loggers (see Table 2).

N°	***Taxon***	**Family**	**Distribution***	**Locality**	**Altitude (m a.s.l.)**	**Collection date**
1	*Brassica tournefortii* Gouan	Brassicaceae	Medit –Saharo-Sind.	Poetto - Cagliari (CA)	0	07/05/2012
2	*Clematis vitalba* L.	Ranunculaceae	Europ-Caucas.	Monte Padenteddu - Pula (CA)	760	12/10/2012
3	*Dianthus morisianus* Vals.	Caryophyllaceae	Endem. SA	Portixeddu - Buggerru (CI)	65	24/07/2012
4	*Digitalis purpurea* L. var. *gyspergerae* (Rouy) Fiori	Scrophulariaceae	Endem. SA-CO	Is Cioffus - Capoterra (CA)	360	26/06/2012
5	*Digitalis purpurea* L. var. *gyspergerae* (Rouy) Fiori	Scrophulariaceae	Endem. SA-CO	Monte Lattias - Uta (CA)	904	23/07/2012
6	*Digitalis purpurea* L. var. *gyspergerae* (Rouy) Fiori	Scrophulariaceae	Endem. SA-CO	Bruncu Spina - Desulo (NU)	1810	30/08/2012
7	*Helianthemum caput-felis* Boiss.	Cistaceae	SW-Medit.	Sa Mesa Longa - S. V. Milis (OR)	38	29/07/2012
8	*Lupinus luteus* L.	Fabaceae	W-Medit.	Buggerru (CI)	103	29/05/2012
9	*Nepeta foliosa* Moris	Lamiaceae	Endem. SA	Prados - Oliena (NU)	1146	28/08/2012
10	*Ptilostemon casabonae* (L.) Greuter (2)	Asteraceae	Endem. SA-CO-H-AT	Miniera Luigi - Buggerru (CI)	135	22/07/2013
11	*Ptilostemon casabonae* (L.) Greuter	Asteraceae	Endem. SA-CO-H-AT	Laconi (OR)	686	11/07/2013
12	*Ptilostemon casabonae* (L.) Greuter	Asteraceae	Endem. SA-CO-H-AT	Is Terre ‘e Molentes - Fonni (NU)	1300	23/08/2013
13	*Rhamnus alaternus* L.	Rhamnaceae	Euri-Medit.	S. Barbara - Capoterra (CA)	505	12/07/2013
14	*Rhamnus lycioides* L. ssp. *oleoides* (L.) Jahand. & Maire	Rhamnaceae	S-Medit.	Perdu Collu - Pula (CA)	60	10/08/2012
15	*Santolina insularis* (Gennari *ex* Fiori) Arrigoni	Asteraceae	Endem. SA	Separadorgiu - Fonni (NU)	1531	12/09/2013
16	*Scrophularia trifoliata* L.	Scrophulariaceae	Endem. SA-CO-AT	Miniera Luigi - Buggerru (CI)	217	11/07/2013
17	*Scrophularia trifoliata* L.	Scrophulariaceae	Endem. SA-CO-AT	Su Thuttureli - Oliena (NU)	1238	17/07/2013
18	*Verbascum plantagineum* Moris	Scrophulariaceae	Endem. SA	Monte Nieddu - Pula (CA)	200	20/06/2012

Table 2 - Site information and data-loggers for soil temperature measurement details. Abbreviations on the province: VS = Medio Campidano; OR = Oristano; CI = Carbonia-Iglesias; NU = Nuoro; OG = Ogliastra.

Locality	Coordinates (WGS84)	Altitude (m a.s.l.)	Start date	End date	Measurements duration (days)
Is Arenas - Arbus (VS)	N 39°31' E 8°25'	25	14/02/2011	09/06/2013	846
Sa Mesa Longa - San Vero Milis (OR)	N 40°02' E 8°23'	38	18/06/2012	27/12/2012	192
Buggerru - Portixeddu (CI)	N 39°26' E 8°26'	63	23/01/2011	24/07/2012	548
Rio Siddo - Ghilarza (OR)	N 40°08' E 8°50'	128	17/06/2012	24/09/2014	828
Domusnovas Canales - Norbello (OR)	N 40°08' E 8°52'	227	17/06/2012	22/03/2013	278
Su Costarbu - Abbasanta (OR)	N 40°09' E 8°46'	357	17/06/2012	23/09/2014	828
Su Monte 'e su Cavalleri - Abbasanta (OR)	N 40°08' E 8°43'	430	17/06/2012	23/09/2014	828
Genna Ferracesus - Gonnosfanadiga (VS)	N 39°27' E 8°39'	569	17/05/2012	29/04/2013	346
Canale Perda Pibera - Gonnosfanadiga (VS)	N 39°26' E 8°39'	700	20/08/2012	30/09/2014	771
Iscala 'e Prados - Oliena (NU)	N 40°15' E 9°24'	700	08/08/2012	11/10/2014	794
Canale Perda Pibera - Gonnosfanadiga (VS)	N 39°26' E 8°39'	818	17/05/2012	30/09/2014	866
Rio Olai - Orgosolo (NU)	N 40°08' E 9°21'	945	22/04/2011	12/09/2013	874
Iscala 'e Prados - Oliena (NU)	N 40°15' E 9°25'	1040	08/08/2012	11/10/2014	794
Prados - Oliena (NU)	N 40°15' E 9°25'	1146	09/04/2009	11/10/2014	2011*
Rio Correboi - Villagrande (OG)	N 40°04' E 9°20'	1200	22/04/2011	04/07/2013	804
Monte Novo S. Giovanni - Orgosolo (NU)	N 40°07' E 9°24'	1255	15/05/2011	30/08/2012	473
Rio Correboi - Villagrande (OG)	N 40°03' E 9°20'	1267	22/04/2011	04/07/2013	804
Monte Spada - Fonni (NU)	N 40°04' E 9°16'	1340	22/04/2011	30/08/2012	496
Rio Correboi - Villagrande (OG)	N 40°03' E 9°20'	1344	22/04/2011	04/07/2013	804
Palumbrosa - Oliena (NU)	N 40°14' E 9°25'	1361	04/10/2010	17/07/2013	1017
Punta Corrasi - Oliena (NU)	N 40°14' E 9°25'	1412	05/08/2011	11/10/2014	1163
Bae e Laccos - Fonni (NU)	N 40°00' E 9°19'	1520	26/08/2010	25/08/2011	364
Rio Aratu - Desulo (NU)	N 40°01' E 9°17'	1665	26/08/2010	20/08/2013	1090
Punta Bruncu Spina - Desulo (NU)	N 40°00' E 9°18'	1810	02/09/2012	11/10/2014	769

*Data deficient from 19/08/2011 to 08/08/2012

Results

Correlation between altitude and lipid thermal profile during cooling and warming of the dry seeds

The highest onset temperature of lipid crystallization (-17°C) was observed in *Helianthemum caput-felis* seeds which were collected at 38 m a.s.l., while the lowest onset temperature of lipid crystallization (ca. -33°C) was recorded by seeds of *Nepeta foliosa* sampled at 1146 m a.s.l. (Fig. 1A) Similarly, the highest end temperature of lipid crystallization (ca. -27°C) was recorded in seeds of *Helianthemum caput-felis* and *Rhamnus alaternus*, collected at 38 m a.s.l. and 505 m a.s.l.; respectively. Whereas, the lowest end temperature of lipid crystallization (ca. -46°C) was recorded by *Nepeta foliosa* (Fig. 1B). Though the temperatures of lipid crystallization (both for onset and end temperatures) seem to be higher at lower elevation, no statistically significant correlation was detected between the lipid crystallization onset and end temperatures and altitude of the seeds studied ($P > 0.05$; Fig. 1A, 1B).

The highest onset temperature of lipid melt (-1°C) was reached by *Brassica tournefortii* seeds which were collected at 0 m a.s.l. While, the lowest onset temperature (ca. -42) of lipid melt was seen in seeds of *Verbascum plantagineum* (sampled at 200 m a.s.l.) and *Nepeta foliosa*. Similarly, the highest end temperatures of lipid melt (ca. -2°C) were reached by seeds of *Helianthemum caput-felis* and *Ptilostemon casabonae* (sampled at 686 m a.s.l.), (Fig. 2A). While the lowest end temperature of lipid melt (ca. -24°C) was observed in seeds of *Scrophularia trifoliata* (both populations at 200 and 1230 m a.s.l.), (Fig. 2B). The results highlighted that there was no signifcant correlation between the lipid melt events (both for onset and end temperatures) and altitude of the species studied ($P > 0.05$; Fig. 2).

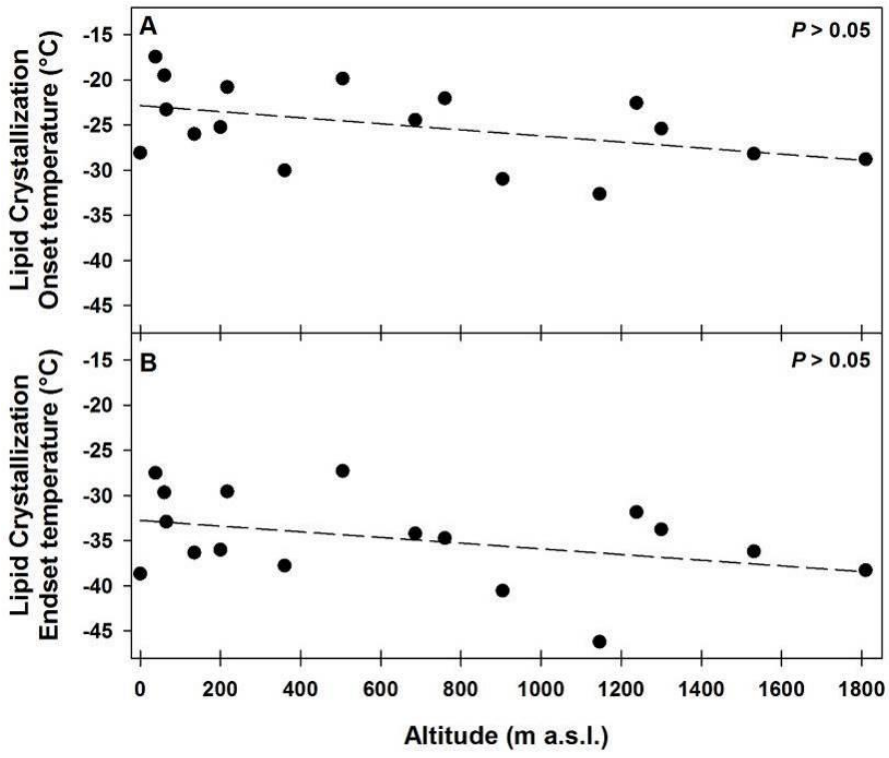

Figure 1 - Relationship between lipid crystallization onset temperatures and altitude (A), and between lipid crystallization end temperatures and altitude of the investigated species (B).

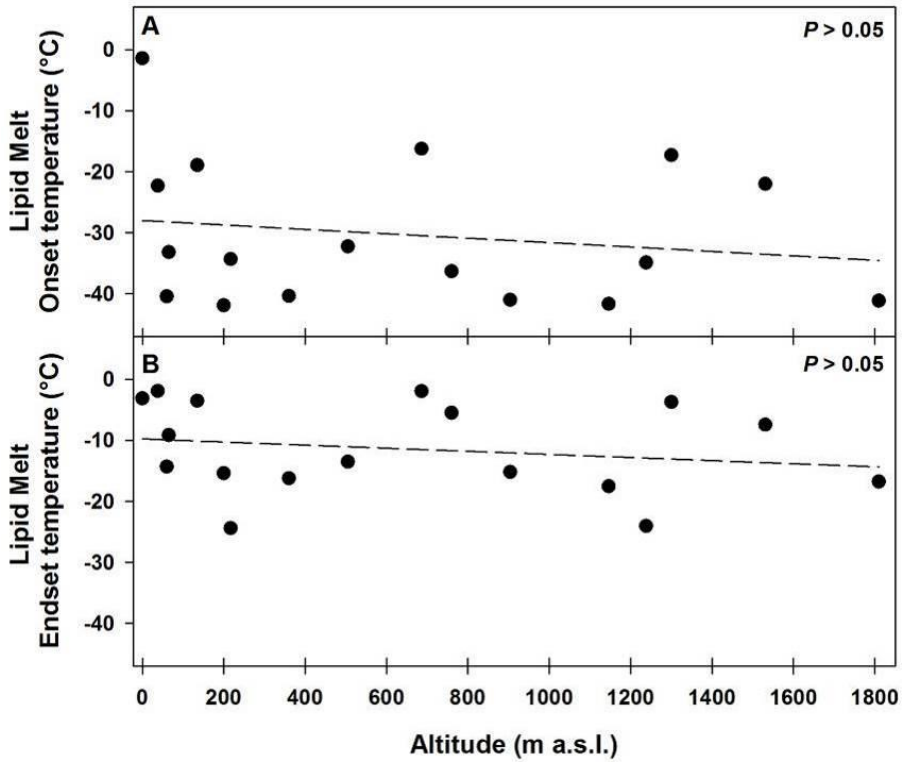

Figure 2 - Relationship between lipid melt onset temperatures and altitude (A), and between lipid melting end temperatures and altitude of the investigated species (B).

Comparison of the lipid thermal fingerprints of seeds from the lowest and highest altitudes and their soil temperature

At the lowest altitude, the minimum daily temperature recorded in winter by data loggers was in the range of ca. 1°C and 4°C. While, the mean daily temperature varied from ca. 8°C to ca. 12°C and the winter period ranged from 0 to 8 days. Whereas, in the summer, the maximum daily temperature varied from approx. 42°C to approx. 60°C, the mean daily temperature ranged from ca. 26°C to ca. 31°C, and the summer period ranged from 70 to 114 days (Fig. 3). Figure 3 highlighted that the lipid crystallization of seeds collected from the lowest altitude ranged from t ca. -22°C to ca. -34°C; while the lipid melt ranged from -30°C to ca. -8°C. However, no significant correlation was found between the soil temperatures and lipid crystallization in winter, as well as between the soil temperatures and the lipid melting in summer ($P > 0.05$; Fig. 3).

At the highest altitude, in winter, the minimum daily temperature ranged between ca. -2°C and 0°C. Whereas, the mean daily temperature varied from ca. 0°C to ca. 4°C and the winter period lasted from 78 to 147 days. Whereas, in summer, the maximum daily temperature ranged approx. from 27°C to approx. 59°C, the mean daily temperatures from ca. 16°C to ca. 23°C, and the summer period lasted from 0 to 52 days (Fig. 4). The lipid crystallization of seeds collected at the highest altitude ranged from ca. -27 °C to t ca. -41°C; while the lipid melting ranged from -32°C to ca. -9°C. However, the linear regression revealed no statistical trend among the soil temperatures and lipid crystallization in winter, as well as between the soil temperatures and the lipid melting in summer ($P > 0.05$; Fig. 4).

This shows that the window of the temperature for lipid crystallization at the lowest altitude is in the range of 12°C and very similar to the 14°C range found in the highest altitude species / seed lots. Similarly, the window of the temperature for lipid melt at the lowest altitude is in the range of 22°C compared to 23°C for the highest altitude.

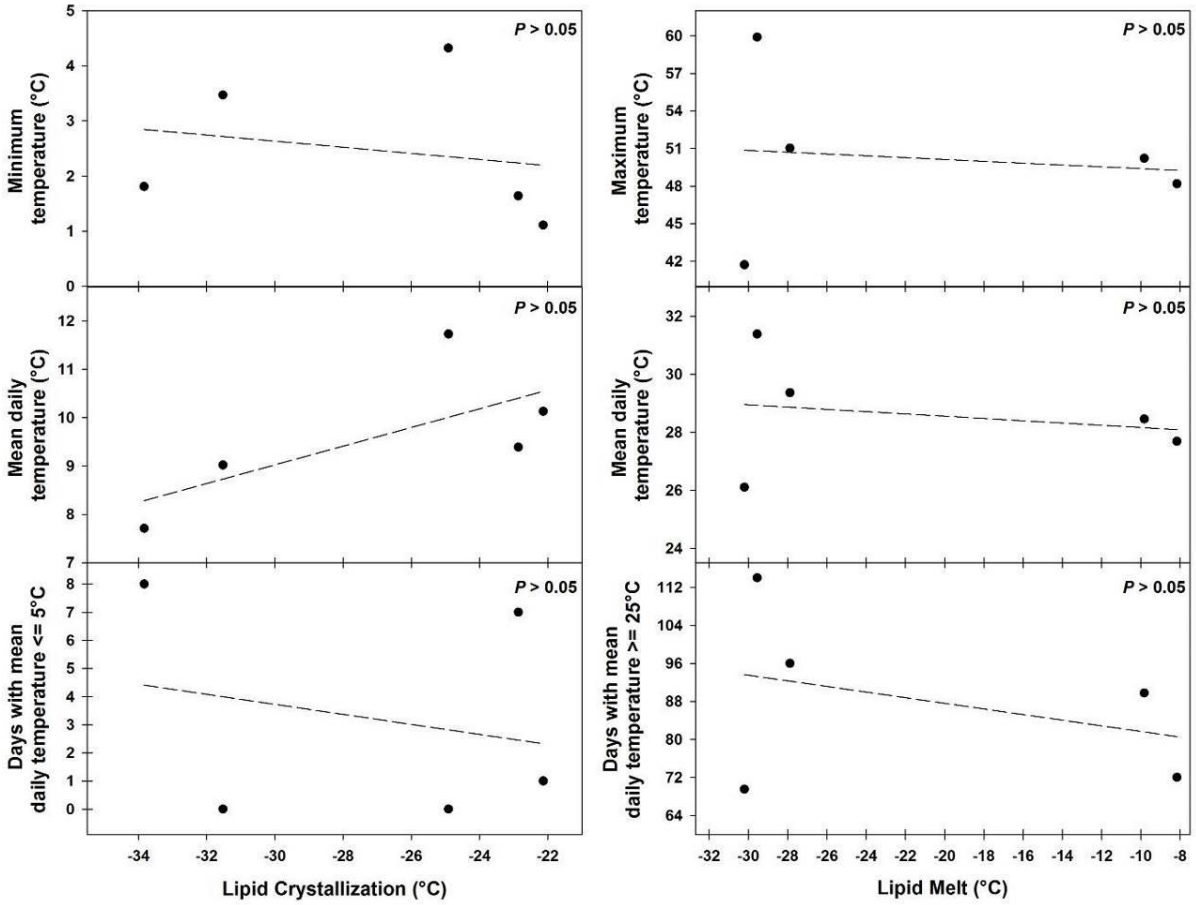

Figure 3 - Relationship between lipid crystallization and melt temperatures of the seeds which are sampled close to the data loggers and the soil temperatures recorded at low altitudes (0-400 m a.s.l.) from December to March for the correlation with lipid crystallization and from June to September for lipid melt. Fitted lines parameters are shown in each plot.

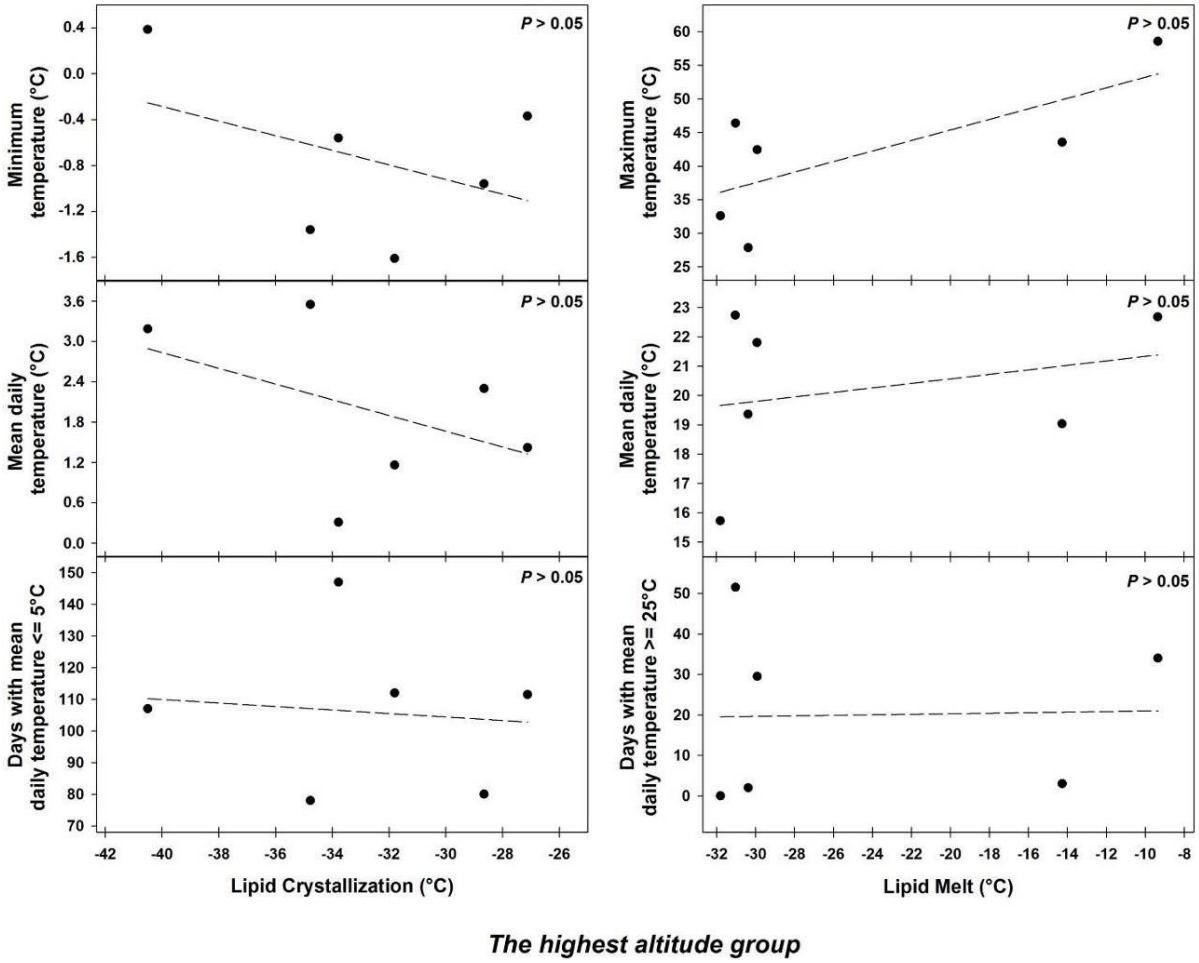

Figure 4 - Relationship between lipid crystallization and melt temperatures of the seeds which were sampled close to the data loggers and the soil temperatures recorded at high altitudes (900-1810 m a.s.l.) from December to March for the correlation with lipid crystallization and from June to September for lipid melt. Fitted lines parameters are shown in each plot.

Correlation between base temperature (T_b) and lipid thermal behaviour of the dry seeds

A negative correlation between T_b and lipid crystallization temperature was highlighted for dry seeds of the studied species (with the exception of *Clematis vitalba* for which was not possible to calculate T_b value). In particular, seeds of *Lupinus luteus* reached the highest lipid crystallization temperature (-6°C) and the lowest T_b (-9°C). By contrast, the lowest lipid crystallization temperature (-40°C) and the highest T_b (10°C) was reached by *Nepeta foliosa* (Fig. 5).

For the warming cycle, the highest of lipid melting temperature (7°C) and the lowest value of T_b (ca. -9°C) were achieved by *Lupinus luteus.* Whereas, seeds of *Digitalis purpurea* had the lowest lipid melting temperature (ca. -32°C) and the highest T_b (10°C; Fig. 6). Nevertheless, the relationship between T_b and lipid melting events of the studied species showed no significant statistical trend ($P > 0.05$; Fig. 2).

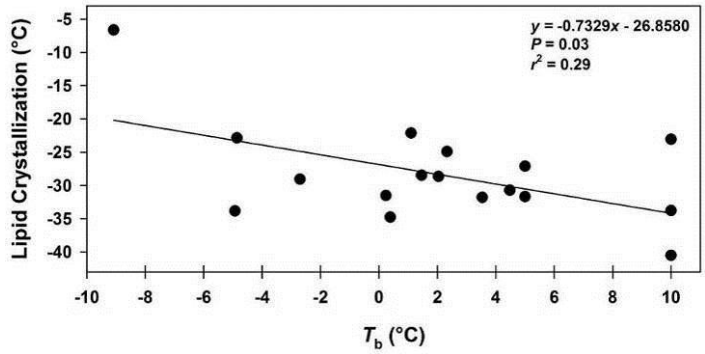

Figure 5 - Relationship between base temperature (T_b) and the main peak of lipid crystallization of the studied species.

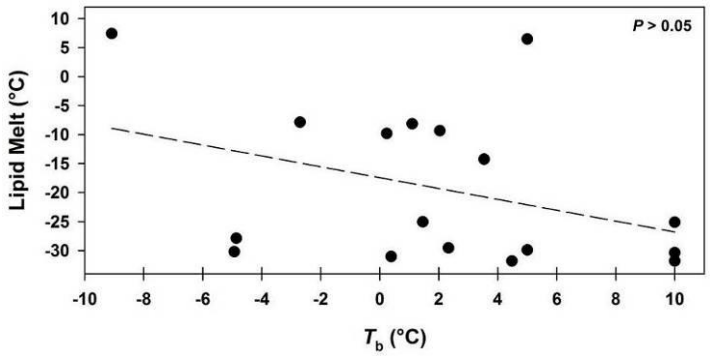

Figure 6 - Relationship between base temperature (T_b) and the main peak of lipid melt of the studied species.

Table 3 - Thermal behaviour of dry lipid-rich seed during the cooling and warming cycle.

Taxon	Altitude (m a.s.l.)	T_b (0 test)	Temperature of transition	
			Cooling Thermogram (Lipid Crystallization)	Warming Thermogram (Lipid Melt)
			Mean peak (°C ± SD)	Mean peak (°C ± S.D.)
Brassica tournefortii	0	*5	-31.68 ± 0.63	6.44 ± 0.35
Helianthemum caput-felis	38	1.10	-22.13 ± 0.52	-8.16 ± 0.66
Rhamnus oleoides	60	-4.86	-22.86 ± 2.82	-27.88 ± 0.67
Dianthus morisianus	65	1.46	-28.46 ± 1.42	-25.06 ± 1.16
Lupinus luteus	103	-9.08	-6.65 ± 2.00	7.40 ± 0.23
Verbascum plantagineum	200	4.48	-30.72 ± 0.64	-31.80 ± 0.56
Scrophularia trifoliata	217	2.33	-24.91 ± 0.29	-29.55 ± 0.53
Rhamnus alaternus	505	*10	-23.06 ± 2.97	-25.11 ± 1.36
Ptilostemon casabonae	686	-2.70	-29.07 ± 1.45	-7.88 ± 1.41
Clematis vitalba	760	ND	-29.96 ± 1.26	-21.66 ± 0.84
Digitalis purpurea	904	0.39	-34.77 ± 0.33	-31.03 ± 0.41
Nepeta foliosa	1146	*10	-40.50 ± 4.58	-30.37 ± 1.12
Scrophularia trifoliata	1238	*5	-27.12 ± 0.26	-29.91 ± 0.39
Santolina insularis	1531	3.54	-31.80 ± 0.68	-14.26 ± 0.17
❖ *Ptilostemon casabonae*	135	0.24	-31.52 ± 1.57	-9.82 ± 1.94
❖ *Ptilostemon casabonae*	1300	2.04	-28.65 ± 1.51	-9.35 ± 2.10
❖ *Digitalis purpurea*	360	-4.93	-33.83 ± 0.25	-30.20 ± 0.37
❖ *Digitalis purpurea*	1810	*10	-33.78 ± 0.89	-31.80 ± 0.20
** Estimated values according to Trudgill et al. (2000)*				
❖ *Target species for water thermal analysis: 2 populations for P. casaboneae and D. purpurea*				

DSC water thermal analysis of hydrated seeds

DSC thermograms for hydrated seeds of the two populations of *Digitalis purpurea* had the same main freezing event at -8°C, followed by a general cluster of freezing events around -20 to -25°C. Overall, freezing events appeared to be more scattered for seeds collected at 1810 m a.s.l. compared to those collected at the lowest altitude (Fig. 7).

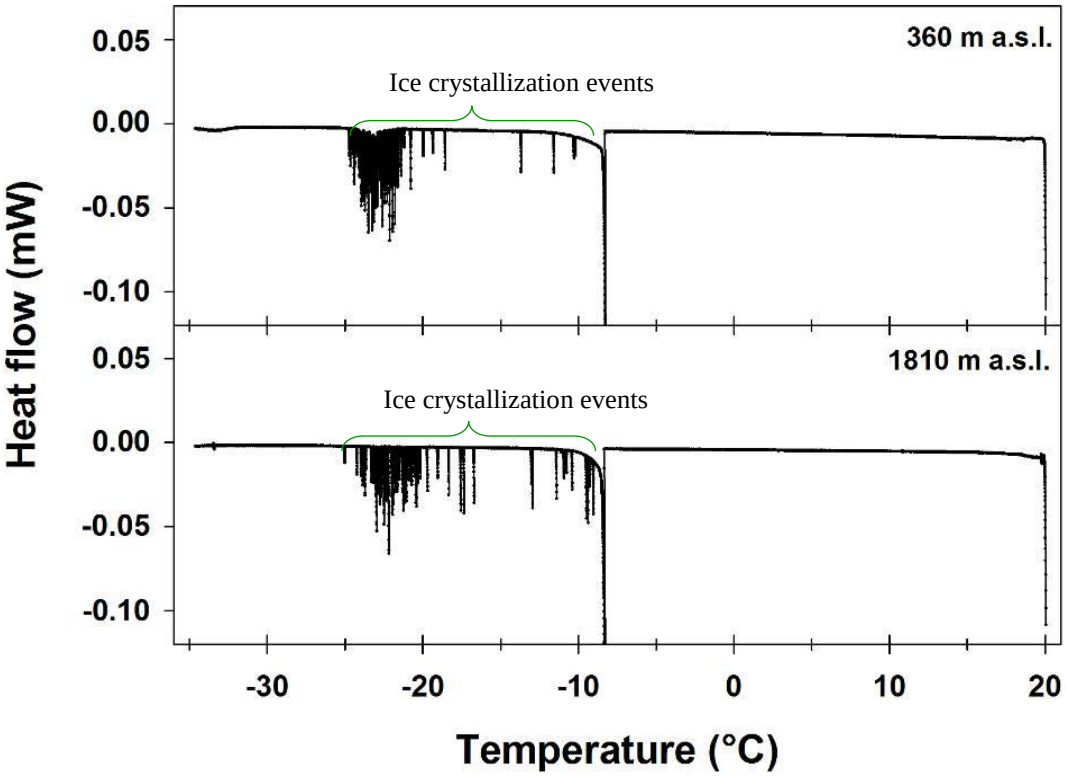

Figure 7 - DSC cooling thermograms of *Digitalis purpurea* for seeds collected at 360 and 1810 m a.s.l. Samples were cooled to -40 at a rate of 10°C per 200 min. Samples with 19.43 mg of average weight of seeds were used for the thermal analysis and the analysis was replicated once due to the length of programme.

For *Ptilostemon casabonae*, slightly different observation was noted between the freezing events of the two populations. Seeds of the both populations started the freezing events approximately at -14°C and finished at ca. -19°C. However, there were only two main freezing events noted for seeds from the highest altitude plants compared to the several freezing events for seeds sampled from the lowest altitude (Fig. 8).

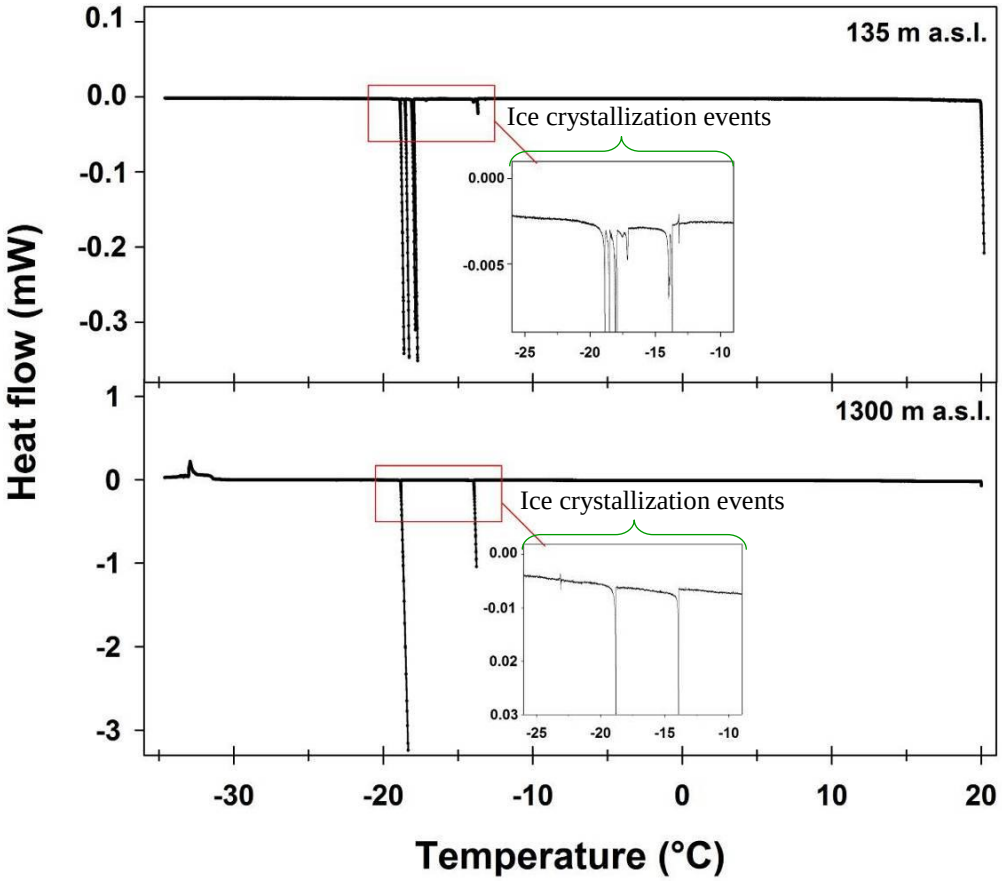

Figure 8 - DSC cooling thermograms of *Ptilostemon casabonae* for seeds collected at 135 and 1300 m a.s.l. Samples were cooled to -40 at a rate of 10°C per 200 min. Samples with 22.97 mg of average weight of seeds were used for the thermal analysis and the analysis was replicated once due to the length of programme.

The average proportion of osmotically active water (OA) as compared to the total water content was ca. 86 and 85% for the lowest and highest altitude population of *D. purpurea* and ca. 86 and 84% for the population at low and high elevation of *P. casabonae*, respectively (Fig. 9). Likewise, the average proportion of osmotically inactive water (OIA) compared to total water content was approximately 16% for both populations of *D. purpurea* and for *P. casabonae* at the low elevation, while the highest altitude population reached ca. 15% (Fig. 9). This results confirms that the seeds from both populations were fully hydrated for this experiment and means that the high moisture freezing limit for both species' seeds is approximately 15%.

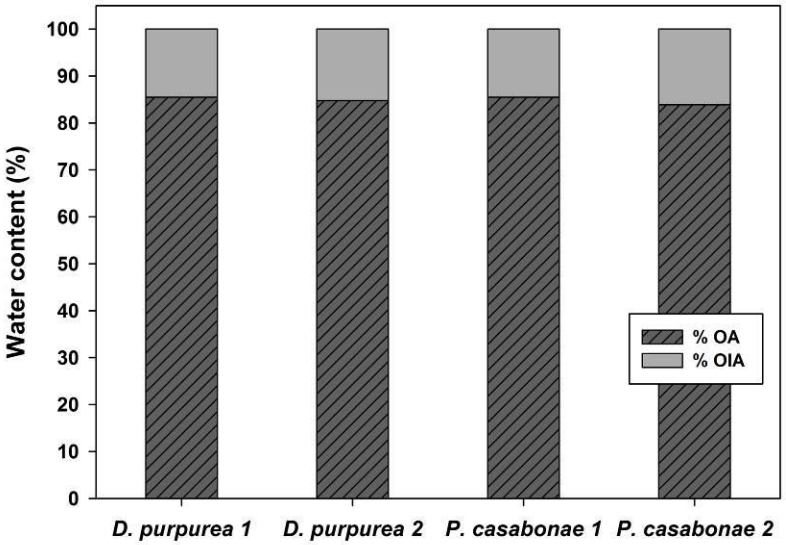

Figure 9 - Summary profiles of osmotically active (OA) and inactive (OIA) water contents calculated as % of total water content for each population of the two selected species (*D. purpurea* 1 = 360 m a.s.l.; *D. purpurea* 2 = 1810 m a.s.l.; *P. casabonae* 1 = 135 m a.s.l. and *P. casabonae* 2 = 1300 m a.s.l.).

Both populations of *D. purpurea* showed the same onset temperatures for ice crystallization (ca. at -8°C) and reached approximately the same value of osmotically active water content. Figure 10 summarizes the scattered freezing events at the lowest and highest altitudes. Highest altitude seeds showed a high number of freezing events but each freezing event with a very low osmotically active water content apart from two freezing events at ca. -22°C (~33 J/g) and at ca. -9°C (~25 J/g). One obvious difference was noted for the lowest altitude seeds where the osmotically active water content was its highest at ca. -23°C (~56 J/g).

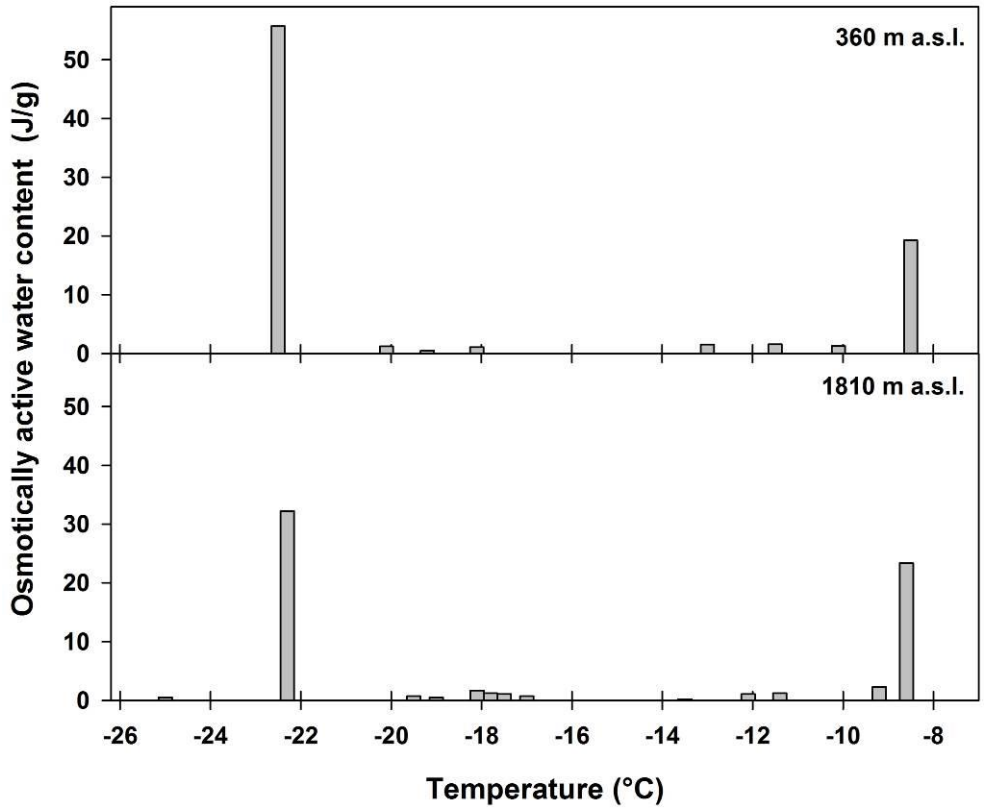

Figure 10 - Osmotically active water during the freezing activity from 0 to -25°C for the two populations of *Digitalis purpurea*.

An interesting observation was noted for *P. casabonae*, such that the lowest altitude seeds showed high a number of freezing events (ca. 6) compared to only two events at the highest altitude seeds (Fig. 11). The low number of freezing events at the highest altitude was compensated with one large freezing event at -19.5°C with ~50 J/g. Differences among the two populations were also observed in the onset and end freezing temperatures. In particular, seeds collected at low altitude showed a larger window of freezing temperatures, around 11°C (from -10 to -21°C), in comparison with those of the highest elevation, ca. 5°C (from -14 to -19°C), (Fig. 11).

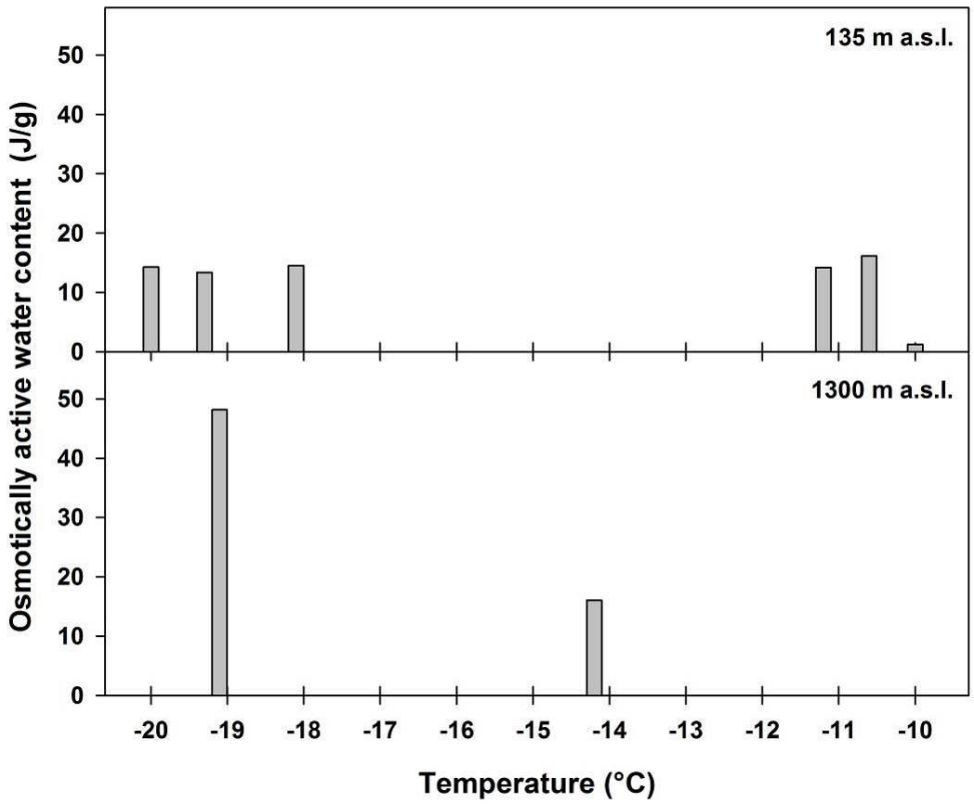

Figure 11 - Osmotically active water during the freezing activity from 0 to -25°C for the two populations of *Ptilostemon casabonae*.

Discussion

The thermal proprieties of seed lipids of each species were observed through DSC and correlated with altitude, as the studied species were selected from different elevation distribution. Different studies have reported that seed germination and dormancy could be influenced by the altitude at which seeds are collected (Holm, 1994; Vera *et al*., 1997; Rosbakh and Poschlod, 2014; Walder and Ershlamer, 2015). However, less is known about the relationship between the thermal proprieties of seeds and the altitude. Although the cooling and the warming thermographs for dry seeds of each species highlighted no statistical trend with altitude, lipids of seeds sampled from the lower altitude showed higher crystallization onset and end temperatures. While, lipids of seeds sampled at the higher altitude showed lower crystallization onset and end temperatures. Less markedly, lipids of seeds sampled from the lower altitudes seem to have higher melting temperatures compared to seeds collected at higher altitudes. Indirectly, the achieved results seem to confirm that the biochemical pathway for seed lipid composition may be affected by the natural environment in which they are located.

Seed lipids of the lowest altitude species, collected close to the data loggers, have not highlighted a significant statistical correlation with the soil temperatures. However, the lipid crystallization temperatures and the mean daily temperatures in winter seem to have a positive correlation. In particular, lower mean daily temperatures were associated with lower temperature of lipid crystallization. Furthermore, although species collected at high altitudes have not revealed a significant statistical trend between lipid crystallization and melt and the soil temperatures, the lipid melt temperatures and the maximum temperatures recorded in summer seem to have a positive correlation. This also suggests that the soil temperatures experienced by seeds along with the altitudinal gradient may have an influence on their lipid thermal fingerprints, therefore data obtained require further close examinations.

Previous studies reported that climate has a strong influence on plant recruitment and temperature and water supply are the major critical drivers for seed dormancy (initiation, break) and germination (Walk *et al*., 2011; Baskin and Baskin, 2014). Indeed, dormancy may be broken by higher or lower temperatures, depending on species, in order for the completion of germination to occur in the correct season (autumn or spring, respectively) and for subsequent growth (Finch-Savage *et al*., 2007). For example, species of temperate habitats started to germinate immediately after snowmelt, because the low temperatures or frost, represent the main hazard for seedling establishment in these regions. While, in other climates (e.g. Mediterranean), germination occurs well into the wet season, in late autumn, ensuring that growing season begin before the onset of the hot dry summer conditions (Thanos *et al*., 1995; Doussi and Thanos, 2002; Rosbakh and Poschlod, 2014). Furthermore, seed germination and emergence are influenced by the position of seeds in the soil bank profile (Traba *et al*., 2004). For example, seeds buried in the substrate at alpine sites are most likely to deteriorate at a slower rate than seeds buried in lowland soils because of the average temperature experienced will be lower (Mondoni *et al*., 2011); and, in the case where seeds are shed during a dry period, desiccation tolerance may enable seeds to accumulate in the soil seed bank and wait until the onset of prolonged rainfall before germination occurs (Pritchard *et al*., 2004). Although the limiting role of local environmental factors (e.g. light, temperature fluctuations, soil surface, etc.) in relationship to seed dormancy and germination has been exhaustively investigated (e.g. Grime *et al.*, 1981; Baskin and Baskin, 2014), less is known of how thermal thresholds (i.e. T_b) for seed germination are related to the biophysical properties of dry seeds collected at different altitudinal gradients. In this study, a variation in T_b values among species is apparent; this is not surprising since thermal thresholds for seed germination may be significantly influenced by the local climatic conditions they experience (Rosbakh and Poschlod, 2014; Dürr *et al*., 2015). Furthermore, the lipid crystallization temperatures of dry

seeds highlighted a positive correlation with T_b; while no significant statistical trend was reported between lipid melting events and the T_b. This suggests that differences in germination strategy between species modulated by T_b may be related also to the lipid thermal fingerprints.

Different studies reported that the inter-population variability in seed germination represents an adaptive strategy in unpredictable environments (Gutterman, 1994; Kigel, 1995; Cruz *et al*., 2003; Zhou and Bao, 2014). However, little is known on the relationship between the biophysical adaptation of populations of the same species which grow at different extreme altitudes, their freezing behaviour and water activity. In this study, the water thermal analysis of hydrated seeds of *D. purpurea* showed similar freezing events for both populations, not exhibiting therefore a clear inter-populational difference. However, a clear variation was noted between water activity and the number freezing events among the two populations of *P. casabonae*. Indeed, seeds of the lowest altitude population showed a greater number of freezing events and a larger window of freezing temperature compared to those of the highest elevation. Since an evident difference was not observed for the two populations of *D. purpurea*, this may suggest that the variation found could be an adaptation strategy of *P. casabonae* alone.

Conclusion

These investigation have provided new information to the understanding of the different biophysical characteristics of seeds of Mediterranean species buried in soil seed bank along an altitudinal gradient, especially the novel lipid phase change study which provided new insight on the survival mechanism. Further knowledge is provided on the interactions between seeds and the natural environment in which they are located and, consequently, contribute to the understanding of the physiological responses of seeds in the germination processes in their natural habitats. However, wider implications of these relationships need further research, both on the thermal profile and the water activity of the seeds along the altitudinal gradient and on their interactions with the local environmental conditions (i.e. climate, soil surface) to which seeds are exposed.

References

Arroyo M.T.K., Chacon P., Cavieres L.A., 2006. Relationship between seed bank expression, adult longevity and aridity in species of *Chaetanthera* (Asteraceae) in Central Chile. Annals of Botany, 98: 591–600.

Bacchetta G., 2006. La flora del Sulcis (Sardegna sud-occidentale). Guineana, 12: 1–369.

Bacchetta G., Fenu G., Mattana E., 2012. A checklist of the exclusive vascular flora of Sardinia with priority rankings for conservation. Anales del Jardín Botánico de Madrid, 69(1): 81–89.

Baskin C.C., Baskin J.M., 2003. When breaking seed dormancy is a problem. Try a move-along experiment. Native Plants|Spring, 4: 17–21.

Baskin C.C., Baskin J.M., 2014. Seeds: ecology, biogeography, and evolution of dormancy and germination. San Diego: Academic Press.

Block W., 2003. Water status and thermal analysis of alginate beads used in cryopreservation of plant germplasm. Cryobiology, 47: 59–72.

Benson E., Johnston J., Muthusamy J., Harding K., 2006. Physical and engineering perspectives of *in vitro* plant cryopreservation. In: Dutta Gupta S., Ibaraki Y. (eds) *Plant Tissue Culture Engineering*. Springer, Dordrecht, Netherlands, 441–473.

Canvin D.T., 1965. The effect of temperature on the oil content and fatty acid composition of the oils from several oil seed crops. Canadian Journal of Botany 43, 6–69.

Cochrane A., Daws M.I., Hay F.R., 2011. Seed-based approach for identifying flora at risk from climate warming. Austral Ecology, 36: 923–935.

Cruz A., Pérez B., Velasco A., Moreno J.M., 2003. Variability in seed germination at the interpopulation and intraindividual levels of shrub *Erica australis* in response to fire-related cues. Plant Ecology, 169: 93–103.

Doussi M.A., Thanos C.A., 2002. Ecophysiology of seed germination in Mediterranean geophytes. 1. *Muscari* spp. Seed Science Research, 12: 193–201.

Dumet D., Block W., Worland R., Reed B.M.; Benson E.E., 2000. Profiling cryopreservation protocols for *Ribes ciliatum* using differential scanning calorimetry. CryoLetters, 21: 367–378.

Dürr C., Dickie J.B., Yang X.-Y., Pritchard H.W., 2015. Ranges of critical temperature and water potential values for the germination of species worldwide: Contribution to a seed trait database. Agricultural and Forest Meteorology, 200: 222–232.

Eckstein R.L., Hölzel N., Danihelka J., 2006. Biological flora of central Europe: *Viola elatior*, *V. pumila* and *V. stagnina*. Perspectives in Plant Ecology Evolution and Systematics, 8: 45–66.

Ellis R.H., Covell S., Roberts E.H., Summerfield R.J., 1986. The influence of temperature on seed germination rate in grain legumes. II. Intraspecific variation in chickpea (*Cicer arietinum* L.) at constant temperatures. Journal of Experimental Botany, 37: 1503–1515.

Fenner M., Thompson K., 2005. The ecology of seeds. Cambridge, UK: Cambridge University Press.

Fenu G., Fois M., Cañadas E.M., Bacchetta G., 2014. Using endemic-plant distribution, geology and geomorphology in biogeography: the case of Sardinia (Mediterranean Basin). Systematics and Biodiversity, 12(2): 181–193.

Finch-Savage W.E., Cadman C.S.C., Toorop P.E., Lynn J.R. Hilhorst H.W.M., 2007. Seed dormancy release in *Arabidopsis* Cvi by dry after ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing. The Plant Journal, 51: 60–78.

Gresta F., Cristaudo A., Onofri A., Restuccia A., Avola G., 2010. Germination response of four pasture species to temperature, light, and post-harvest period. Plant Biosystems, 144(4): 849–856.

Grime J.P., Mason G., Curtis A.V., Rodman J., Band S.R., 1981. A comparative study of germination characteristics in a local flora. Journal of Ecology, 69: 1017–1059.

Gutterman Y., 1994. Strategies of seed dispersal and germination in plants inhabiting deserts. Botanical Review, 60: 373–425.

Harris H.C., McWilliam J.R., Mason W.K., 1978. Influence of temperature on oil content and composition of sunflower seed. Australian Journal of Agricultural Research 29: 1203–1212.

Holm S.O., 1994. Reproductive patterns of *Betula pendula* and *B. pubescens* coll. along a regional altitudinal gradient in northern Sweden. Ecography, 17: 60–72.

Keddy P.A., Reznicek A.A., 1982. The role of seed banks in the persistence of Ontario's coastal plain flora. American Journal of Botany, 69: 13–22.

Kigel J., 1995. Seed germination in arid and semiarid regions. In: Kigel, J., Galili, G., eds. Seed development and germination. Marcel Dekker, New York, pp. 645–699.

Médail F., Quézel P., 1997. Hot-spots analysis for conservation of plant biodiversity in the Mediterranean Basin. Annals of the Missouri Botanical Garden, 84: 112–127.

Medrano H., Flexas J., Galmés J., 2009. Variability in water use efficiency at the leaf level among Mediterranean plants with different growth forms. Plant Soil, 317: 17–29.

Mondoni A., Probert R.J., Rossi G., Vegini E., Hay F.R., 2011. Seeds of alpine plants are short lived: implications for long-term conservation. Annals of Botany, 107(1): 171–179.

Nadarajan J., Mansor M., Krishnapillay B., Staines H.J., Benson E.E., Harding K., 2008. Applications of differential scanning calorimetry in developing cryopreservation strategies for *Parkia speciosa*, a tropical tree producing recalcitrant seeds. CryoLetters, 29(2): 95–110.

Nadarajan J., Pritchard H.W., 2014. Biophysical Characteristics of Successful Oilseed Embryo Cryoprotection and Cryopreservation Using Vacuum Infiltration Vitrification: An Innovation in Plant Cell Preservation. PLoS ONE 9(5): e96169. doi: 10.1371/journal.pone.0096169.

Pritchard H.W., Daws M.I., Fletcher B.J., Gaméné C.S., Msanga H.P., Omondi W., 2004. Ecological correlates of seed desiccation tolerance in tropical African dryland trees. American Journal of Botany, 91(6): 863–870.

Quilichini A., Debussche M., 2000. Seed dispersal and germination patterns in a rare Mediterranean island endemic (*Anchusa crispa* Viv., Boraginaceae). Acta Oecologica, 21(6): 303–313.

Rosbakh S., Poschlod P., 2014. Initial temperature of seed germination as related to species occurrence along a temperature gradient. Functional Ecology, doi: 10.1111/1365-2435.12304.

Rowell T.A., Walters S.M., Harvey H.S., 1982. The rediscovery of the fen violet, *Viola persicifolia* Schreber, at Wicken Fen. Watsonia, 14: 183–184.

Thanos C.A., Costas C.K., Skarou F., 1995. Ecophysiology of germination in the aromatic plants thyme, savory and oregano (Labiatae). Seed Science Research, 5: 161–170.

Thompson K., Band S.R., Hodgson J.G., 1993. Seed size and shape predict persistence in soil. Functional Ecology, 7: 236–241.

Thompson K., 2000. The functional ecology of seed banks. In: Fenner M., es. Seeds the ecology of regeneration in plant communities. Wallingford, UK: CAB International, 215–235.

Traba J., Azcárate F.M., Peco B., 2004. From what depth do seeds emerge? A soil seed bank experiment with Mediterranean grassland species. Seed Science Research, 14: 297–303.

Trudgill D.L., Squire G.R., Thompson K., 2000. A thermal time basis for comparing the germination requirements of some British herbaceous plants. New Phytologist, 145: 107–114.

Yu S., Sternberg M., Kutiel P., Chen H., 2007. Seed mass, shape, and persistence in the soil seed bank of Israeli coastal sand dune flora. Evolutionary Ecology Research, 9: 325–340.

Vera M.L., 1997. Effects of altitude and seed size on germination and seedling survival of heathland plants in north Spain. Plant Ecology, 133: 101–106.

Walck J.L., Hidayati S.N., Dixon K.W., Thompson K., Poschlod P., 2011. Climate change and plant regeneration from seed. Global Change Biology, 17: 2145–2161.

Walder T., Erschbamer B., 2015. Temperature and drought drive differences in germination responses between congeneric species along altitudinal gradients. Plant ecology, 216(9): 1297–1309.

Zhou Z., Bao W., 2014. Changes in seed dormancy of *Rosa multibracteata* Hemsl. & E. H. Wilson with increasing elevation in an arid valley in the eastern Tibetan Plateau. Ecological Research, 29: 693–700.

General conclusions

In this study, through the data recorded by the soil temperatures, the Mediterranean climate along an altitudinal gradient in Sardinia was characterized, such that a longer and colder winter at high altitudes, and a long dry season, especially in lowlands, was recorded.

The seed germination responses of the study species highlighted a considerable inter-specific variation in the sensitivity to the applied treatments. All species responded positively to hormones; in most cases, cold stratification inhibited or had a neutral effect on seed germination; it was also detected that seeds were insensitive to constant warm stratification temperature while dry after ripening only had a positive effect on germination in a limited number of species. Furthermore, intra-specific differences in germination behaviour have been observed, which may reflect a local adaptation to particular environments.

The thermal thresholds (the base temperature and thermal constant for 50% germination) for seed germination of the study species highlighted a Mediterranean germination pattern for lowland species, suggesting germination in autumn-winter, when water availability, soil moisture and rainfalls are high, and temperatures are not excessively prohibitive for germination and consequent seedlings establishment. While, a thermal temperate behaviour, with a spring germination, was detected for the high Mediterranean mountain species.

The thermal time for 50% germination (θ_{50}), calculated and compared for the three *Rhamnus* species as well as for three different populations of *Digitalis purpurea*, *Scrophularia trifoliata*, *Santolina insularis* and *Ptilostemon casabonae*, revealed that these species / populations have different sensitivities in the accumulation of thermal units (°Cd) according their provenance and on the basis of applied treatments.

In addition, following the identification of lipid thermal profile and water activity of target *taxa*, further knowledge is provided on the interactions between seeds and the natural environment in which they are located.

In conclusion, the results of this study supply a better knowledge on the ecophysiology of the investigated species, which have shown both a inter and intra-specific variability in germination patterns and treatments applied had an important role in the seed germination responses. The analysis carried out in this research highlighted that the base temperature for germination may slightly or significantly change between different species as well as among populations of the same species and seed lot provenance may influence the sensitivity of the seed germination response to thermal time. Therefore, the origin of seed samples should always be taken into account when defining models of germination behaviour, especially in wild species with high degree of physiological variability. In addition, studying the water and lipid phase changes of seeds from different altitudes and the low temperature threshold for germination (T_b), this research has provided fundamental understanding on the seeds ability to adapt to different mechanisms for survival in the extreme conditions.

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