Thermal thresholds for seed germination in Mediterranean species are higher in mountain compared with lowland areas

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Abstract

Each taxon is characterized by a temperature range over which seed germination is possible and this may vary in space and time in relation to climate and ecological conditions. We used thermal modelling to test the hypothesis that thermal thresholds for seed germination can predict germination timing of Mediterranean species along an altitudinal and environmental gradient. Seeds of 18 species were collected in Sardinia from sea level to 1810 m above sea level, and germination tests were carried out at a range of constant (5 to 25°C) and alternating (25/10°C) temperatures. Different dormancy-breaking treatments [gibberellic acid (GA_3), cold (C) and warm (W) stratifications and dry after ripening (DAR)] were applied. The annual pattern of soil temperatures was recorded using 24 data-loggers buried close to the study species. The logged soil temperatures distinguished 'Mediterranean lowland' from 'Mediterranean mountain' species. Although germination was >50% in untreated seeds of most species, GA_3 had a positive effect in all species. C either inhibited or had a neutral effect on germination, W did not enhanced seed germination, while DAR had a positive effect only in species from coastal environments. The thermal time constant (*S*) for 50% germination ranged from 22 to 357°Cd (degree days) above base temperatures (T_b) of –9 to 9°C, depending on species and treatments. Mediterranean lowland species had lower T_b values compared with upland species. This study revealed significant differences in germination thresholds of Mediterranean lowland and mountain species in relation to T_b and *S* that probably have an impact on germination timing in the field and niche competitiveness.

Introduction

Temperature is a major environmental factor responsible for the timing of seed germination and for changes in dormancy states (Baskin and Baskin, 2014). Under a Mediterranean climate, characterized by a high seasonality with hot dry summers and cold wet winters (Joffre *et al.*, 1999; Valladares and Sánchez-Gómez, 2006), plant reproduction must occur in a window of favourable conditions that may vary in length, particularly with altitude, and in which environmental cues and constraints play a central role (Thanos *et al.*, 1995; 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 (≤15°C; Thanos *et al.*, 1989) and slow germination rate (Kadis, 1995; Doussi and Thanos, 2002). It represents an advantageous ecological adaptation of species, as it ensures that germination occurs well into the wet season, in late autumn, and maximizes the length of the growing season before the onset of summer drought (Doussi and Thanos, 2002). Within this ecological context, dry after-ripening, i.e. a period of warm temperatures under dry conditions, may be considered a favourable treatment for seeds of Mediterranean coastal species, for which high temperature pre-treatment might increase the germination response (Schütz, 1999; Pérez-Fernández *et al.*, 2000; Baskin and Baskin, 2014). On the contrary, cold stratification may have a detrimental effect on seed germination of these species, as reported by Skordilis and Thanos (1995) for seeds of *Pinus halepensis*.

However, Mediterranean mountain species are exposed to a different set of environmental conditions and are likely to show different seed germination traits than the typical lowland species. For example, avoiding germination during or immediately before severe winters is important for successful regeneration (Baskin and Baskin, 2003; Fenner and Thompson,

2005; Baskin and Baskin, 2014; Rosbakh and Poschlod, 2015) and most alpine species are 'programmed' to germinate after snow-melt in spring/early summer, preferring high temperatures for germination (Mooney and Billings, 1961; Billings and Mooney, 1968; Körner, 1999; Niederfriniger Schlag and Erschbamer, 2000; Mondoni et al., 2012). Giménez-Benavides et al. (2005) detected that most of the species from high altitude Mediterranean climates readily germinate without treatment, reaching an optimum at relatively high temperatures (20°C). Furthermore, cold-wet stratification increased germination capacity and broke seed dormancy in several species (e.g. García-Fernández et al., 2015; Cuena-Lombraña et al., 2017), as widely reported also for many arctic, boreal and alpine species (Baskin and Baskin, 2014).

Theoretical cardinal temperatures for germination have been used to model responses to temperature in crops (e.g. Garcia-Huidobro et al., 1982; Covell et al., 1986; Ellis et al., 1986) and wild species (e.g. Pritchard and Manger, 1990; Seal et al., 2017; Tudela-Isanta et al., 2018). In these models, seeds accumulate units of thermal time (degree days: °Cd) to germinate above a base temperature for germination (T_b), with germination rate generally increasing linearly with temperature to an optimum temperature (T_o), above which germination rate decreases (Garcia-Huidobro et al., 1982). Pritchard et al. (1999) reported that physiological dormancy release in the seeds of a temperate tree, Aesculus hippocastanum, can be described simply in terms of T_b reduction gradually allowing germination to occur at progressively lower temperatures, and eventually at the temperature for dormancy alleviation. In the same species, local environment across Europe during seed development had an impact on features of the seeds' thermal response (Daws et al., 2004). Similarly, seed dormancy in Centaurium somedanum varied with local climatic gradient and the seed maturation environment, suggesting that dormancy and germination are influenced by both long- and short-term climatic variation (Fernández-Pascual et al., 2013, 2018). Likewise, Rosbakh and Poschlod (2015), studying species with different distributional ranges in a temperate climate zone, found that the initial temperature of seed germination (i.e. a temperature enabling the first 5% of all seeds to germinate) is negatively correlated with habitat temperature.

The application of threshold models to predict seed germination and dormancy release has been reported in several studies (Garcia-Huidobro et al., 1982; Batlla and Benech-Arnold, 2004; Finch-Savage and Leubner-Metzger, 2006; Bradford et al., 2008; Dürr et al., 2015). Moreover, thermal time modelling is a valuable tool to assess and predict seed germination in native species (e.g. Galíndez et al., 2017; Seal et al., 2017), including in the field (e.g. Chantre et al., 2009) and in relation to the environment (Trudgill et al., 2005). Some general principles have emerged of higher T_b values and shorter thermal time requirements for seeds of tropical and temperate species (Tompsett and Kemp, 1996; Trudgill et al., 2000; Finch-Savage, 2004; Dürr et al., 2015). However, little is known about seed thermal thresholds of native Mediterranean species (e.g. Orrù et al., 2012; Porceddu et al., 2013; Mattana et al., 2016). Although the low optimal germination temperatures and slow germination rate which characterize the 'Mediterranean germination syndrome' suggest low values of T_b and longer thermal times for typical Mediterranean lowland species, this has not been quantitatively assessed yet. Therefore, the aim of this study was to evaluate the effect of temperature and various pre-treatments (cold and warm wet stratification, dry after-ripening, gibberellic acid) on the thermal threshold for seed germination of Mediterranean lowland and mountain species in the context of their varying natural environment.

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), from sea level up to 1834 m above sea level (masl) in the highest mountain region (Gennargentu Massif, CE-Sardinia) (Table 1). The experimental sites were chosen to represent a diversity in bioclimatic conditions (Bacchetta et al., 2009) and geological substrata (Carmignani et al., 2001). Species belonging to the most representative families in the Mediterranean area were chosen, with priority given to: (1) exclusive Sardinian endemics; (2) the Sardinian, the Corsican and the Tuscan Archipelago endemics; and (3) Tyrrhenian insular endemics (Table 1).

Seed collecting and soil temperature recording

Seeds of 18 species were collected from 26 provenance localities, considering also different populations of the same species (Table 1). Several collecting trips were carried out each year from late spring (May) to 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 Habitat Directive annexes, 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 temperature logger, Onset Computer Corporation, Cape Cod, MA, USA) were buried at ca 2–3 cm depth, at sites at different altitudes (from ca 25 to ca 1800 masl) and time periods (from April 2009 to September 2012; 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).

Germination experiments

According to seed availability, four replicates of 30 seeds ($n = 120$) or three replicates of 20 seeds ($n = 60$) 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 for a maximum of 4 months 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), with light applied during the warm phase. In addition, depending on the seed availability and according to prior knowledge of the study species, different pre-treatments were also applied: (1) pre-chilling ('C', seeds were incubated for 3 months at 5°C in 1% agar water); (2) pre-warming ('W', seeds were incubated for 3 months at 25°C in 1% agar water); and (3) dry after-ripening ('DAR', seeds were stored for 3 months at 25°C inside a sealed glass container with silica gel in a ratio seed:silica gel of 1:1). At the end of each pre-treatment, seeds were incubated at the above listed temperatures. 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

Table 1. Seed lot 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).

No.	Taxon	Family	Distribution*	Locality	Coordinates (WGS84)	Altitude (masl)	Collection date
1	*Brassica tournefortii* Gouan	Brassicaceae	S-Medit	Poetto – Cagliari (CA)	N 39°12′ E 9°10′	5	07/05/2012
2	*Clematis vitalba* L.	Ranunculaceae	Europ-Caucas.	Monte Padenteddu - Pula (CA)	N 39°02′ E 8°54′	760	12/10/2012
3	*Dianthus morisianus* Vals.	Caryophyllaceae	Endem. SA	Portixeddu – Buggerru (CI)	N 39°26′ E 8°26′	65	24/07/2012
4	*Digitalis purpurea* L. var. *gyspergerae* (Rouy) Fiori	Scrophulariaceae	Endem. SA-CO	Is Cioffus – Capoterra (CA)	N 39°06′ E 8°57′	360	26/06/2012
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
6	*Digitalis purpurea* L. var. *gyspergerae* (Rouy) Fiori	Scrophulariaceae	Endem. SA-CO	Bruncu Spina – Desulo (NU)	N 40°00′ E 9°18′	1810	30/08/2012
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
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
9	*Lamyropsis microcephala* (Moris) Dittrich *et* Greuter	Asteraceae	Endem. SA	Bruncu Spina – Desulo (NU)	N 40°01′ E 9°17′	1757	28/08/2012
10	*Lupinus luteus* L.	Fabaceae	W-Medit.	Buggerru (CI)	N 39°26′ E 8°23′	103	29/05/2012
11	*Nepeta foliosa* Moris	Lamiaceae	Endem. SA	Prados – Oliena (NU)	N 40°15′ E 9°25′	1146	07/08/2012
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
13	*Ptilostemon casabonae* (L.) Greuter	Asteraceae	Endem. SA-CO-H-AT	Mitzaorxia – Laconi (OR)	N 39°52′ E 9°04′	686	22/07/2013
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
15	*Rhamnus alaternus* L. ssp. *alaternus*	Rhamnaceae	Circum-Medit.	S. Barbara – Capoterra (CA)	N 39°08′ E 8°56′	505	12/07/2013
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
17	*Ruta lamarmorae* Bacch., Brullo *et* Giusso	Rutaceae	Endem. SA	Bruncu Spina – Desulo (NU)	N 40°01′ E 9°17′	1675	19/09/2012
18	*Santolina insularis* (Gennari *ex* Fiori) Arrigoni	Asteraceae	Endem. SA	Miniera Luigi – Buggerru (CI)	N 39°22′ E 8°25′	147	11/07/2013
19	*Santolina insularis* (Gennari *ex* Fiori) Arrigoni	Asteraceae	Endem. SA	Sugalaffricu – Laconi (OR)	N 39°52′ E 9°00′	500	21/06/2013
20	*Santolina insularis* (Gennari *ex* Fiori) Arrigoni	Asteraceae	Endem. SA	Separadorgiu – Fonni (NU)	N 40°02′ E 9°17′	1531	12/09/2013
21	*Scrophularia ramosissima* Loisel.	Scrophulariaceae	Endem. SA-CO-BL-GA	Is Arenas – Arbus (VS)	N 39°31′ E 8°25′	25	19/07/2012

(Continued)

Table 1. (Continued).

No.	Taxon	Family	Distribution*	Locality	Coordinates (WGS84)	Altitude (masl)	Collection date
22	*Scrophularia trifoliata* L.	Scrophulariaceae	Endem. SA-CO-AT	Miniera Luigi – Buggerru (CI)	N 39°22′ E 8°25′	217	11/07/2012
23	*Scrophularia trifoliata* L.	Scrophulariaceae	Endem. SA-CO-AT	Laconi (OR)	N 39°51′ E 9°03′	510	24/07/2013
24	*Scrophularia trifoliata* L.	Scrophulariaceae	Endem. SA-CO-AT	Su Thuttureli – Oliena (NU)	N 40°14′ E 9°25′	1238	17/07/2013
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
26	*Verbascum plantagineum* Moris	Scrophulariaceae	Endem. SA	Monte Nieddu – Pula (CA)	N 39°09′ E 8°54′	200	20/06/2012

specified above. Furthermore, GA_3 treatment was applied in the investigated species, with the exception of six taxa (*Dianthus morisianus*, *Helianthemum caput-felis*, *Lamyropsis microcephala*, *Lupinus luteus*, *Rhamnus alaternus* ssp. *alaternus*, *Silene succulenta* ssp. *corsica*; Appendix 1), by sowing seeds on the surface of 1% agar water with 250 mg l^{-1} of GA_3 and incubating them in the light (12 h light/12 h dark), at the previously cited temperatures.

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

Germination, defined as visible radicle emergence (>1 mm), was recorded three times a week. During stratification in the dark, seeds were scored only at the end of the pre-treatment to avoid any exposure to light. At the end of the germination tests, when no additional germination had occurred for 2 weeks, and no less than 1 month from sowing, a cut-test was carried out to determine the viability of the remaining seeds. Firm seeds were considered to be viable.

Data and statistical analyses

Soil temperatures were analysed for winter and summer seasons. The winter season was considered to be from 21 December to 20 March, while summer ran from 21 June to 21 September. The minimum daily temperature, the mean daily temperature and the duration of the cold 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 warm 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 (SD) for each tested condition. Seed germination during pre-treatments (i.e. C and W stratifications) was also recorded and when seeds germinated during pre-treatments before moving to the incubation temperatures, these were not considered in the final germination percentages.

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 sub-optimal temperature range. However, when 50% of final germination was not reached, the T_b value was not established.

The thermal constant (*S*) expressed in degree days (°Cd), 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 seed lots that reached 50% of final germination after each pre-treatment. Regression analyses were carried out using SigmaPlot version 11.0 (Systat Software, Inc., San Jose, CA, USA).

In addition, the dataset of Trudgill *et al.* (2000), composed of 31 temperate species, was employed to compare their findings with the results of this study.

Effect of pre-treatments, temperatures and their combinations on germination percentages were determined by 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 detailed above) of 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). 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 Core Team, 2011).

Results

Climatic data

Soil temperature data highlighted that in winter the minimum daily temperatures decreased with altitude from *ca* 4°C (*ca* 200 masl) to *ca* −1°C (*ca* 1800 masl) and this pattern could be modelled by a linear correlation (Fig. 1a). The mean daily temperatures followed the same pattern decreasing from *ca* 12°C (*ca* 200 masl) to 0°C (*ca* 1800 masl; Fig. 1b). The cold period increased linearly with altitude from 0 to 25 days at 800 masl (Fig. 1c). At altitudes >800 masl, this period increased considerably, reaching a duration of 147 days at 1810 masl (Fig. 1c).

Table 2. Site information and data loggers for soil temperature measurement details.

Locality	Altitude (masl)	Coordinates (WGS84)	Start of the measurement	End of the measurement	Length of the measurement (days)
Is Arenas – Arbus (VS)	25	N 39°31′ E 8°25′	14/02/2011	09/06/2013	846
Sa Mesa Longa – San Vero Milis (OR)	38	N 40°02′ E 8°23′	18/06/2012	27/12/2012	192
Portixeddu – Buggerru (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

Abbreviations on the province: VS, Medio Campidano; OR, Oristano; CI, Carbonia-Iglesias; UN, Nuoro; OG, Ogliastra. *Data deficient from 19/08/2011 to 08/08/2012.

The correlation resulting from fitting data with two straight lines represented a more suitable model, showing a higher proportion of the variance (higher r^2 values) than the continuous line. A cold period of 90 days, corresponding to the duration of the cold stratification at 5°C (C) in the laboratory, was reached at altitudes ≥1180 masl (Fig. 1c). According to this threshold, it was possible to distinguish between 'Mediterranean lowland' (ML) up to altitudes of 1180 masl, and 'Mediterranean mountain' (MM) at altitudes ≥1180 masl (Fig. 1c).

The maximum daily temperatures in summer ranged from 19°C (*ca* 1350 masl) to 60°C (*ca* 200 masl), without a significant correlation with altitude ($P = 0.1270$; Fig. 2a). The mean daily temperatures decreased with altitude from 31°C (25 masl) to 16°C (*ca* 1350 masl; Fig. 2b) as well as the duration of the summer period from 113 (25 masl) to 0 days (1810 masl; 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 laboratory, was estimated to be achieved only at altitudes ≤4 masl (Fig. 2c).

Seed germination

Seed germination results of each taxon, including different populations of the same species, are reported in Appendix 1. In the control test, the highest germination percentage was reached by *Lupinus luteus* (100% at all tested temperatures). However, high mean germination percentages >75% were also recorded for eight taxa: *Helianthemum caput-felis*; all three populations of *Ptilostemon casabonae*; the lowest altitude population of *Digitalis purpurea* var. *gyspergerae*; *Helichrysum microphyllum* ssp. *tyrrhenicum*; and populations of *Santolina insularis* at middle and high altitude. Seeds of six other species germinated to
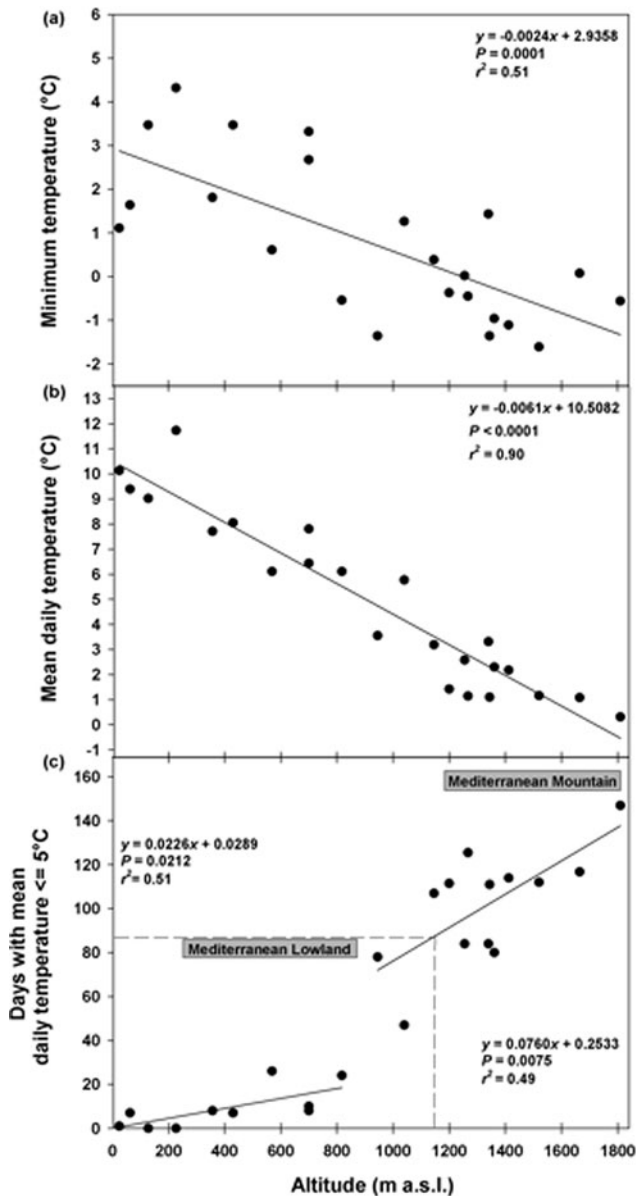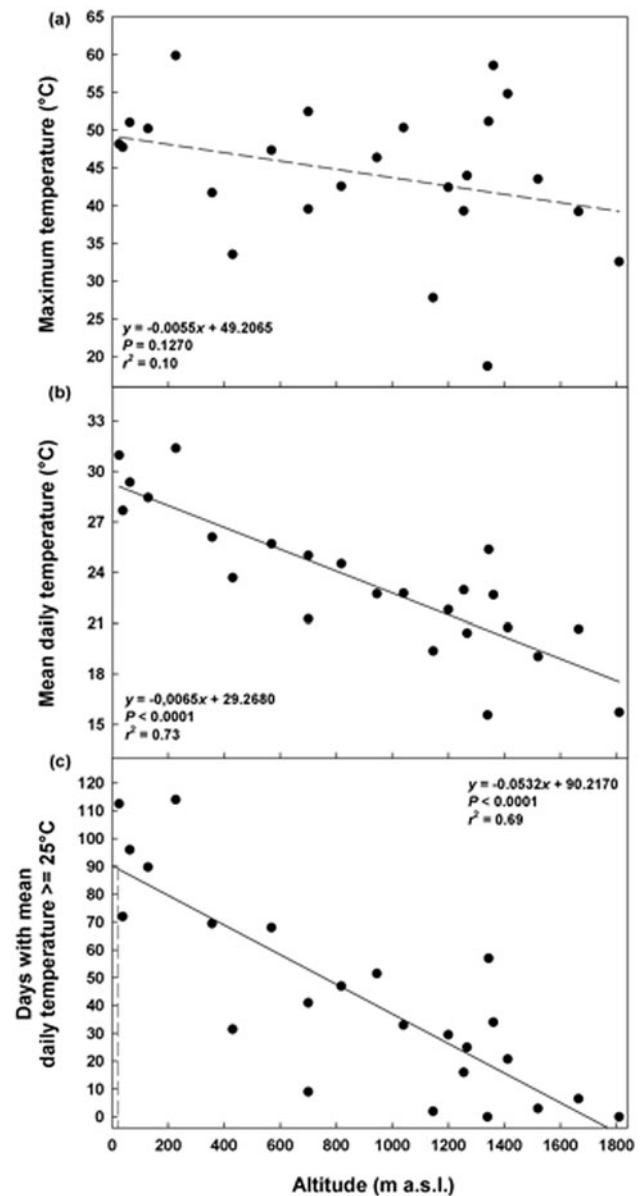
Fig. 1. Soil temperatures recorded in winter at different altitudes: minimum temperature (a), mean daily temperature (b), and days with mean daily temperature ≤5°C (c). Fitted line parameters are shown in each plot. In (c) the dashed lines highlight the value of 90 days which corresponds to the cold stratification (C; 5°C for 3 months) applied in the laboratory. According to this threshold, which was reached at altitudes ≥1180 masl, it was possible to distinguish between Mediterranean lowland and Mediterranean mountain species.

Fig. 2. Soil temperatures recorded in summer at different altitudes: maximum temperature (a), mean daily temperature (b) and days with mean daily temperature ≥25°C (c). Fitted line parameters are shown in each plot. In (c) the dashed lines highlight the value of 90 days which corresponds to the warm stratification (W; 25°C for 3 months) and the dry after-ripening (DAR; 25°C for 3 months on silica gel) applied in the laboratory.

maxima between 50 and 75%: *Silene succulenta* ssp. *corsica*; the low altitude population of *Santolina insularis*; *Rhamnus alaternus* ssp. *alaternus*; *Nepeta foliosa*; *Lamyropsis microcephala*; and the highest altitude population of *Digitalis purpurea* var. *gyspergerae*. Finally, only two species, *Brassica tournefortii* and *Ruta lamarmorae*, failed to achieve 25% seed germination; the cut-test on the non-germinated seeds revealed that they were viable (Appendix 1).

A positive effect of GA$_3$ treatment on seed germination was observed, reaching final germination >50% compared with seeds under control conditions. Overall in relation to the control, GA$_3$ increased germination significantly in eight taxa (Appendix 1).

For example, *Brassica tournefortii* seeds reached final germination close to 100% at all tested temperatures.

Seeds of 14 taxa and 21 seed lots (i.e. *Clematis vitalba*, *Dianthus morisianus*, all three populations of *Digitalis purpurea*, *Helichrysum microphyllum* ssp. *tyrrhenicum*, *Lamyropsis microcephala*, *Nepeta foliosa*, all three populations of *Ptilostemon casabonae*, *Rhamnus alaternus* ssp. *alaternus*, *Rhamnus lycioides* ssp. *oleoides*, *Ruta lamarmorae*, *Santolina insularis* population at low and high altitude, *Scrophularia ramosissima*, all three populations of *Scrophularia trifoliata* and *Verbascum plantagineum*) germinated during C stratification, before moving to the range of incubation temperatures. Differences in seed germination were observed according to altitude, in particular 15 seed lots from
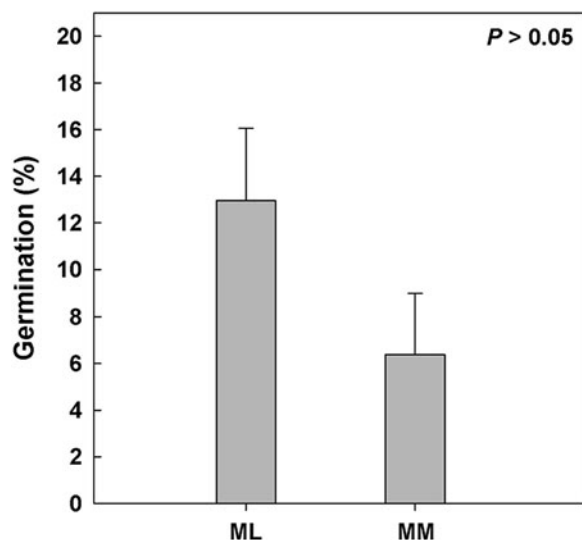
Fig. 3. Seed germination during pre-chilling (C, 5°C for 3 months). Referring to climatic data achieved by data loggers (Fig. 1c), the study species were divided depending on altitude: Mediterranean lowland 'ML' species (0–1180 masl) and Mediterranean mountain 'MM' species (1180–1810 masl). $P > 0.05$ by *post hoc* pairwise comparisons *t*-test.

lowlands germinated during C, with higher values (*ca* 13%) than those of high altitudes (six seed lots with *ca* 6%; Fig. 3).

After C treatment the highest germination was observed for *Clematis vitalba* seeds (*ca* 95%). Nevertheless, high germinations (i.e. >75%) were also recorded for ten species, while seeds of six other taxa reached maxima between 50 and 75%, whereas those of four species only reached 25–50%. In contrast, seed germination was low (<25%) for three species (see details in Appendix 1). C increased germination significantly in only two species (i.e. *Clematis vitalba* and *Nepeta foliosa*) in comparison with the control. C decreased significantly the mean germination of nine species, mostly from lowlands (Appendix 1).

After W treatment, seeds of four species (all three populations of *Ptilostemon casabonae*, the middle altitude population of *Scrophularia trifoliata*, *Helichrysum microphyllum* ssp. *tyrrhenicum*, and the highest altitude population of *Santolina insularis*) achieved mean germination >75%. Seeds of two species germinated to maxima of 50–75%, while only one species reached 25–50% (Appendix 1). W treatment did not significantly increase germination of all the tested species in comparison with the control. In contrast, W decreased significantly the mean seed germination of five lowland species (Appendix 1).

The highest seed germination percentages following DAR were observed in two species (*Helianthemum caput-felis* and *Lupinus luteus*). However, high mean germination percentages >75% were also recorded for five taxa. Furthermore, seeds of seven species reached maxima between 56 and 75%, including *Ruta lamarmorae*, with 56% (Appendix 1). DAR only increased germination significantly for the study species compared with the control in *Brassica tournefortii*, *Helianthemum caput-felis* and *Ruta lamarmorae*, while it decreased germination in two taxa.

Amongst the investigated species, generalized linear models highlighted a statistically significant effect ($P < 0.001$) on germination of treatment and temperature factors as well as of their interactions ($P < 0.001$, $P < 0.01$ and $P < 0.05$); with the exception of four taxa (see details in Appendix 2).

Thermal thresholds for seed germination

Thermal threshold estimates of each seed lot are reported in Appendix 3. T_b of seeds under control conditions (0) varied by 18°C, from −9 to *ca* 9°C; while GA$_3$-treated seeds had T_b values varying by 11°C, from −5 to 6°C. C treatment reduced the interspecies range of values to 6°C, from *ca* 1 to *ca* 7°C. In DAR-treated seeds, T_b varied by 14°C, from −5 to 9°C. Finally, T_b of seeds treated with W varied from 0 to 9°C (see details in Appendix 3).

Control seeds had S values that varied more than *ca* 15-fold, between 22 to 357°Cd. With GA$_3$, the S value ranged less than fourfold, from 53 to 196°Cd. Cold (C) had a similar effect on S to GA$_3$ with values recorded from 33 to 204°Cd. In contrast, in DAR treatment, S values of 41 to 313°Cd were similar to the control. Finally, for seeds treated with W, S varied from 56 to 238°Cd (Appendix 3).

Amongst species and all treatments there was a general tendency for T_b and S to be negatively correlated, but this was statistically significant for seeds under control conditions only ($P = 0.0190$, $r^2 = 0.88$).
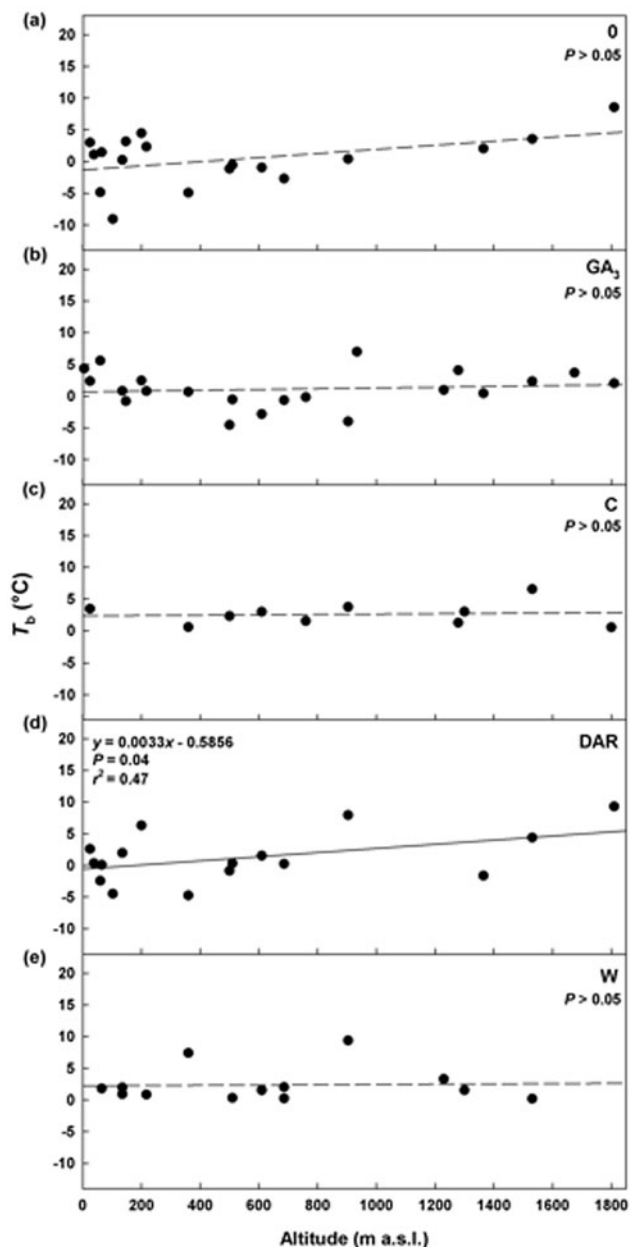
Correlation between base temperature and altitude

A positive correlation between base temperature (T_b) and altitude was highlighted only for DAR-treated seeds (Fig. 4d), while a positive trend between T_b and altitude for control seeds (0) just failed statistical significance ($P = 0.0651$; Fig. 4a). No correlation was found for seeds treated with GA$_3$, C and W, with T_b estimated to be around 2°C for all species, regardless of altitude (Fig. 4b, c and e). To explore further these apparent differences in T_b with altitude, species were divided between ML and 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. 5). 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 3).

Thermal thresholds of Mediterranean vs temperate species

ML showed a minimum T_b of −9°C and MM a minimum of 2°C, while in temperate species (T) T_b values were not below −2°C (Fig. 6a). Concerning S, Mediterranean species (*sensu lato*) were different from temperate species, with a relatively slower germination (longer thermal times), while among MM and ML there was no difference (Fig. 6b).
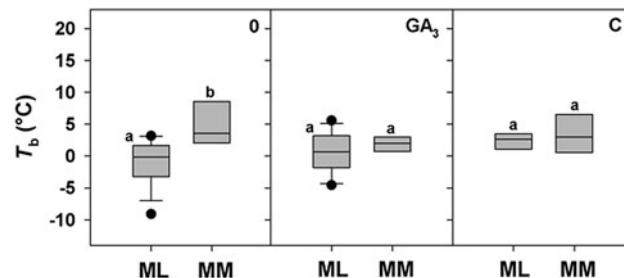
Discussion

The soil temperature data recorded in Sardinia during this study confirmed a typical pattern of the Mediterranean climate with high temperatures in summer and cool winters (Joffre *et al.*, 1999; Medrano *et al.*, 2009; Kadis and Georghiou, 2010). In particular, colder winters occur at high altitudes than in the lowlands (until 800 masl). Conversely, much warmer summers are detected at lower elevations (up to 200 masl). However, the high temperatures recorded in summer along the whole altitudinal gradient, highlight the fact that Mediterranean mountains have the potential for high evaporation and imposition of water stress, which can

Fig. 4. Relationships between base temperatures for germination (T_b) of the investigated species, for which it was possible to calculate T_b, and altitude after each pre-treatment: 0; control (a); GA₃, 250 mg l⁻¹ of GA₃ in the germination substrate (b); C, 5°C for 3 months (c); DAR, 25°C for 3 months on silica gel (d); W, 25°C for 3 months (e).

Fig. 5. Relationships between base temperatures for germination (T_b) and the Mediterranean lowland 'ML' species (0–1180 masl) and Mediterranean mountain 'MM' species (1180–1810 masl) for 0 (control), GA₃ (250 mg l⁻¹ of GA₃ in the germination substrate) and C (5°C for 3 months) treatments. Box plots with the same letter are not different at $P > 0.05$ by *post hoc* pairwise comparisons *t*-test.

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). Indeed, it has recently been shown that the base water potential for germination in alpine species can vary with niche preference for siliceous and calcareous soils (Tudela-Isanta *et al.*, 2018). However, such determinations were beyond the scope of the study reported here.

An inter-specific variation in the sensitivity of seed germination to the applied treatments was identified among species and altitudes. GA₃-treated species responded positively to the treatment. Furthermore, GA₃ widened the temperature range for germination in eight species, triggering germination at 5°C compared with the control test. This indicates that a certain degree of dormancy is found in the study species' seed lots. 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 dormancy (PD) or morphophysiological (MPD) dormancy (Finch-Savage and Leubner-Metzger, 2006; Baskin and Baskin, 2014). Seeds collected from low altitude sites can germinate during cold stratification before moving to higher temperatures, confirming the general benefits of cold temperature to seed germination in coastal Mediterranean species ('Mediterranean germination syndrome'; Thanos *et al.*, 1991; Skordilis and Thanos, 1995; Thanos *et al.*, 1995; Doussi and Thanos, 2002). This temperature sensitivity provides a considerable ecological advantage to seeds by ensuring that germination is completed at the most appropriate season (mid to late autumn), thus allowing seedlings to avoid arid conditions during summer (Luna *et al.*, 2008; Kadis and Georghiou, 2010). Furthermore, with the exception of three species, cold stratification either inhibits or has a neutral effect on seed germination compared with the control. This is especially the case for lowland species, which, on the basis of logged data, are not naturally exposed to 90 days continually at 5°C. Nonetheless, this behaviour is in agreement with previous studies on Mediterranean species, where relatively long-term chilling may not enhance seed germination and may even be detrimental to seed germination, e.g. *Pinus halepensis* (Skordilis and Thanos, 1995).

The investigated species did not benefit from the W treatment, especially at constant temperatures. This is not surprising as the climatic data detected in this study showed that 90 days continually at 25°C does not naturally occur at middle–high altitudes in Sardinia. Such W treatment is known to overcome dormancy in endospermic seeds with an under-developed embryo, e.g. *Paeonia corsica* (Porceddu *et al.*, 2016) or with larger embryos, such as in *Arum maculatum* (Pritchard *et al.*, 1993). Consequently, we expected a similar response in *Clematis vitalba*, as seeds of Ranunculaceae species have been reported to exhibit MD and MPD (e.g. Baskin and Baskin, 1994, 2014; Porceddu *et al.*, 2017). However, W treatment did not enhance seed germination of *C. vitalba*. Interestingly, the alternating temperature regime triggered seed germination in *C. vitalba* under control conditions, while cold stratification and GA₃ treatments promoted seed germination at all tested temperatures (Picciau *et al.*, 2017).

DAR is an important treatment to break seed dormancy in species growing in dry environments (Bewley, 1997; Probert, 2000; Kucera *et al.*, 2005). In our study, the application of DAR improved the fit of the linear regression in the relationship

Table 3. GLMs results for base temperature of germination (T_b) of the following factors: 'Treatment' (0, control; GA₃, 250 mg l⁻¹ in the germination substrate; C, 5°C for 3 months) and 'Altitude' (Mediterranean lowland 0–1180 masl; Mediterranean mountain 1180–1810 masl), 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

Fig. 6. Relationships between base temperatures for germination (T_b) and thermal time constant S (°Cd) without any pre-treatment for 50% of germination between the study species divided on the basis of altitude: Mediterranean lowland 'ML' species (0–1180 masl) and Mediterranean mountain 'MM' species (1180–1810 masl). A comparable dataset for temperate species (Trudgill *et al.*, 2000) is also presented. Box plots with the same letter are not different at $P > 0.05$ by *post hoc* pairwise comparisons *t*-test.
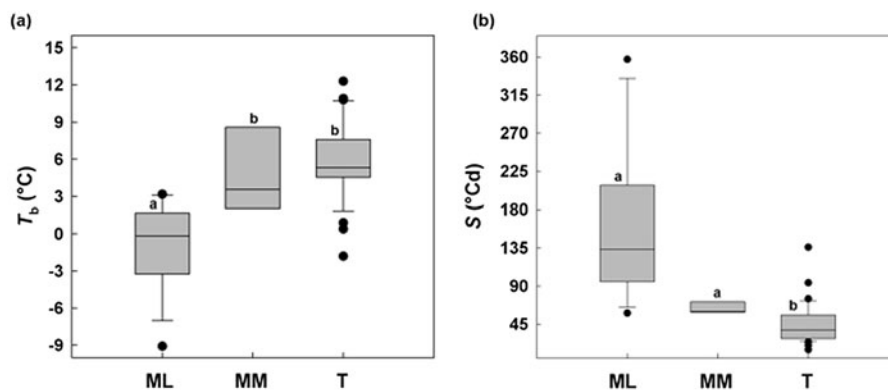
between T_b and altitude compared with the other pre-treatments and had a positive effect only in few cases; in particular, in seeds of species from coastal environments that faced a long dry summer and dispersed their seeds before the wet season, as for example *Brassica tournefortii* and *Helianthemum caput-felis*. Similarly, storage <18 months at high temperatures improved germination in seeds of three Australian Asteraceae (Peishi *et al.*, 1999). Moreover, dryland seeds of *Arabidopsis thaliana*, i.e. Cape Verde Island accessions, respond to DAR dormancy release (Finch-Savage *et al.*, 2007).

Thermal thresholds can vary and change significantly among species as well as populations of the same species (e.g. Ellis *et al.*, 1987; Daws *et al.*, 2004). To explore how much this is the case in Mediterranean species we chose to harvest seed from species along an altitudinal gradient of 1800 m, as the seeds are likely to receive widely differing environmental cues during development and post-dispersal, during autumn to spring. Control seeds of species from higher altitudes were found to have higher T_b estimates, suggesting an avoidance of premature germination under cool conditions when the risk of freezing may still persist. Interestingly, the dormancy breaking treatments of GA₃ and C are effective at lowering T_b of Mediterranean mountain species, indicating the widening of the temperature range over which germination can occur post-treatment (e.g. Pritchard *et al.*, 1999; Daws *et al.*, 2004; Steadman and Pritchard, 2004). In contrast, C increased T_b values in seeds of Mediterranean lowland species, similarly to W, to *ca* 2°C, suggesting a narrowing of the temperature range for germination through an uplift in the low temperature end of the response. Overall, the differential responsiveness to pre-treatments, specifically C in the lowland species, DAR in the mountain species and W in most of the species, reflect a climate-adapted strategy for the timing of seedling emergence (Skordilis and Thanos, 1995) that is modulated through thermal thresholds and times. Indeed, T_b and S values provide insight into the adaptation and the ecological strategies of plants in relation to their thermal environment (Trudgill *et al.*, 2005; Fernández-Pascual *et al.*, 2018), in our study between Mediterranean lowland and mountain species. Mountain species show quantifiable thermal characteristics between Mediterranean lowland (coastal) species and temperate species. This distinctive germination response enables relatively slow germination typical of Mediterranean species (e.g. Doussi and Thanos, 2002), which reduces seed cohort loss as a result of erratic rainfall, and a relatively high T_b, typical of the temperate species, so as to reduce the risk of a seed cohort germinating in winter. These results are consistent with previous studies investigating the seed germination of three mountain species of Sardinia, *Lamyropsis microcephala* (Mattana *et al.*, 2009a), *Rhamnus persicifolia* (Mattana *et al.*, 2009b; Porceddu *et al.*, 2013) and *Gentiana lutea* ssp. *lutea* (Cuena-Lombraña *et al.*, 2017) for which an early spring germination prevails due to a requirement for cold stratification over winter.

The existence of adaptively significant variation in seed germination responses (thresholds and thermal time) amongst groups of species or populations has recently been highlighted in relation to the timing of germination in the field and thus niche competitiveness (Meyer *et al.*, 1997; Daws *et al.*, 2002; Honek *et al.*, 2014; Dürr *et al.*, 2015; Picciau *et al.*, 2018; Tudela-Isanta *et al.*, 2018). Here we have extended such an approach to model the characteristics of Mediterranean species and, in so doing, revealed a shift in germination syndromes between lowland and upland species. We propose that similar studies are undertaken on species that are representative of the world's floras, thereby underpinning studies in plant ecology and vegetation science.

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