

## Research Paper

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# A comparative study on temperature and water potential thresholds for the germination of *Betula pendula* and two Mediterranean endemic birches, *Betula aetnensis* and *Betula fontqueri*

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**Abstract**

The influence of temperature and water availability on seed germination can vary across the geographic range of species with a large distribution. *Betula pendula* is a widely spread European tree that has differentiated into two narrowly distributed taxa, endemic to Mediterranean mountains: *Betula aetnensis* in Sicily and *B. fontqueri* in Spain and Morocco. We tested the hypothesis that the regeneration niche, expressed by temperature and water potential thresholds, varies across these species and is influenced by the local climate. Seeds were collected from six populations of *B. pendula*, one of *B. fontqueri* and two of *B. aetnensis*. Germination tests were conducted between 5 and 30°C. The thermal thresholds were calculated before and after cold stratification. The osmotic potential tested ranged from 0 to −1.5 MPa. Time to reach 30 and 50% of germination was calculated by fitting non-linear models. Germination was promoted by high temperatures, but the response to stratification was heterogeneous.  $T_b$  and  $\Psi_b$  differed between and within species.  $T_b$  ranged between 2.22 and 8.94°C for unstratified seeds. Mediterranean species had higher drought tolerance, while *B. pendula* showed contrasting responses to low water potential.  $\Psi_b$  reached a minimum value of −1.15 MPa in *B. fontqueri*. High temperatures influenced the  $T_b$  of unstratified seeds negatively, while, after stratification, the  $T_b$  increased with precipitation in the driest month. The heterogeneity observed could reflect higher genetic variability in marginal populations of silver birch. Knowledge of their germination ecology may be useful to mitigate future impacts of climate change on core populations of *B. pendula*.

**Introduction**

The regeneration niche is an important determinant of plant species richness and is defined by the ecological requirements necessary to reproduce and colonize new spaces (Grubb, 1977). One of the most important aspects of the regeneration niche is seed germination, which is primarily controlled by two environmental factors: temperature and water potential (Dürr et al., 2015). In species with a broad distribution, the requirements for seed germination may vary across areas in response to geographical and environmental gradients (Khera and Singh, 2005). For example, a study held by Midmore et al. (2015) shows that, in five different provenances of *Betula pendula*, a widespread European boreal species, seed germination was influenced by latitude. Also, the response to water stress may vary across populations, as demonstrated by Boydak et al. (2003), for six collections of *Pinus brutia* originating from different climatic environments.

Populations that grow at the outer edges of the species distribution area may face extreme temperature or drought conditions. Consequently, they might display genetic adaptation to these environments (Levin, 1993; Garcia-Ramos and Kirkpatrick, 1997; Sexton et al., 2009; Abeli et al., 2014; de Dato et al., 2020) and may be different, even in the germination requirements, from populations living at the core of the species distribution area. In the present context of environmental and climatic changes, studying these adaptations may be a useful instrument for planning adequate conservation measures (Walck et al., 2011).

In the Mediterranean region, mountainous areas may represent the southernmost suitable habitat for species with boreal distribution. For example, *Conopodium majus*, widespread in woodlands and oligotrophic meadows in northern Europe, presents a distribution restricted to mountain areas in the Iberian Peninsula (Tutin et al., 1964) and is differentiated in two subspecies, *C. majus* subsp. *majus* and *C. majus* subsp. *marizianum*, which represent the southernmost populations (Tutin et al., 1964). A similar pattern can be recognized for the widespread boreal tree *B. pendula* Roth. (Ashburner et al., 2013), which is present in the

Southern Mediterranean mountains with two closely related species, *B. fontqueri* Rothm. in the Iberian peninsula and in the Moroccan Atlas and *B. aetnensis* Rafin in Sicily.

*B. aetnensis* is a narrow endemic species of Mount Etna (Sicily, Italy). The Etna birch is a tree morphologically similar to *B. pendula*, of which it is often considered as a subspecies (Tutin et al., 1964; Walters, 1964; Meusel et al., 1965; Pignatti, 1982). However, in this study, we will consider it as a species because of its history of long isolation and poor genetic exchange with other populations of *B. pendula*. The Etnean birch is a neoendemism and remained segregated on Mt. Etna since the end of the last glacial period. The genetic isolation, together with the selective pressure caused by the volcanic territory and the Mediterranean climate, has brought a new endemic taxon to the differentiation. Nowadays, there are only two populations of *B. aetnensis* on Mt. Etna located between the 1000- and 2000-m altitude areas (Strano, 2010). *B. fontqueri* has a comparable genesis to *B. aetnensis* and, as is the latter, is considered by most authors to be a subspecies of *B. pendula*; but in this study, we will consider it as a species for the same reasons as for *B. aetnensis*. This Iberian birch has a scattered distribution on the mountains of Central (Sistema Central) and Southern (Sierra Nevada, Sierras de Cazorla, Segura and Las Villas) Spain (Martín et al., 2008) and is present with some relict populations in the north of Morocco (Peinado and Moreno, 1989). It is included in the red list of plants of the IUCN, classified as endangered due to its reduced distribution and the low density of the existing populations (Martín et al., 2008). These two birches possess higher genetic variability than congeneric species from boreal latitudes (Palmé, 2003) and could be considered a genetic reservoir for the entire genus. Unfortunately, both species have a narrow distribution and are threatened by climate and land-use changes. In addition, *B. aetnensis* has been also subjected, in the last 30 years, to increased volcanic ash deposition, which could prevent its natural regeneration by seed. In this context, it is important to understand the regeneration ecology of these species in order to plan appropriate conservation measures. The adaptation of *B. aetnensis* and *B. fontqueri* to a Mediterranean climate could be reflected in their requirements for seed germination and seedling establishment. Currently, there is no published literature on the seed germination ecology of these Mediterranean birches and is fundamental to fill this gap in order to better understand their regeneration strategies. Given the presence of some relict populations of *B. pendula* in the Apennine range (Italy) (Plini and Tondi, 1989), which might present a certain degree of adaptation to Mediterranean climatic conditions, it is of interest to compare the response to temperature and water availability between these three species and within populations of *B. pendula* originating from the core and the margins of the European species' distribution.

Temperature influences germination and the thermal germination niche can be described by the cardinal temperatures. In fact, there is a minimum ('base temperature',  $T_b$ ) and a maximum temperature ('ceiling temperature',  $T_c$ ) between which germination occurs and an optimal temperature ( $T_o$ ) at which it occurs at the highest speed (Dürr et al., 2015). Many models of the response to temperature in crops (García-Huidobro et al., 1982; Covell et al., 1986; Ellis et al., 1986; Dürr et al., 2015) and wild species (Pritchard and Manger, 1990; Seal et al., 2017; Tudela-Isanta et al., 2018) have been developed using the cardinal temperatures.  $T_b$ , in particular, is a distinctive species trait (Dürr et al., 2015). Nevertheless, other studies show that there is an intraspecific variation in  $T_b$  among seed populations according

to different environmental conditions during seed development (Daws et al., 2004).

The variation in soil water potential, which is the amount of pressure needed by the roots to absorb water from the soil, is another determinant of seed germination (Hegarty, 1978), especially in environments that are characterized by a dry season, such as the Mediterranean region (Chamorro et al., 2017). Its effect can be simulated by using artificial solutions (Sharma, 1973; Falusi et al., 1983; Chamorro et al., 2017) in order to calculate the base water potential ( $\Psi_b$ ), that is, the condition below which no germination occurs and can be distinctive for a species or population (Kaufmann, 1969; Evans and Etherington, 1990; Choinski and Tuohy, 1991; Daws et al., 2004).

In this study, we estimated the base temperature and water potential thresholds for germination and compared them among the three species of *Betula*. Cold stratification decreases  $T_b$  in *B. pendula* (Midmore et al., 2015) populations from Central and Northern Europe. Here, we compared its effect on the two Mediterranean *Betula* species and on marginal populations of silver birch. Since seedling establishment and not germination is the most critical stage for plant survival, we also calculated the thermal and water potential thresholds up to the stage of cotyledon emergence.

We wanted to test the following hypotheses:

- (1) There is a difference in seed germination response to temperature and water potential among the Mediterranean species and *B. pendula* due to the long history of isolation of the former ones and to their adaptation to a drier and warmer climate.
- (2) As a consequence of isolation and adaptation, the germination threshold results will be influenced by the local climate.

## Material and methods

### Seed collection

Seeds from eight populations were collected between September and November 2018 across Europe. We sampled five populations of *B. pendula* from Central Italy, the Balkans and Poland; one population of *B. fontqueri* from Central Spain and the two known populations of *B. aetnensis* from Mount Etna (Table 1). All of the populations were natural, except the one in Lazio (Italy), which, according to Plini and Tondi (1989), was planted in the beginning of the 20th century. However, de Dato et al. (2020) consider this population to be natural on the basis of pollen fossil records. From here on, we will refer to these populations by using the following abbreviations: 'CAM' (Campania), 'CRO' (Croatia), 'GAL' (Galvarina), 'LAZ' (Lazio), 'POL' (Poland), 'SAR' (Sartorius), 'SER' (Serbia) and 'SPA' (Spain).

From each collection site, the following bioclimatic parameters, which can represent a limiting factor to plant life, were obtained by interpolation using WorldClim Version2 (Fick and Hijmans, 2017): precipitations of the driest month ('Prec. Driest'), maximum temperature of the hottest month (' $T_{max}$ ') and minimum temperature of the coldest month (' $T_{min}$ ') (Table 1). The interpolations were calculated at a spatial resolution of 1 km<sup>2</sup> and were based on meteorological data collected between 1970 and 2000.

### Germination experiments

#### Temperature

Seed germination was tested at the following constant temperatures: 5, 10, 15, 20, 25 and 30°C with a photoperiod of 12 h of

**Table 1.** Geographical and bioclimatic data of seed collection sites

Species	Location	Latitude	Longitude	Altitude (m)	$T_{\max}$ (°C)	$T_{\min}$ (°C)	Precipitation driest month (mm)
<i>B. aetnensis</i>	Galvarina, Italy	37°44'14" N	014°55'51" E	1700	20.3	-3.9	13
<i>B. aetnensis</i>	Sartorius, Italy	37°46'20" N	015°03'33" E	1670	20.3	-3.6	13
<i>B. fontqueri</i>	Samosierra, Spain	41°07'39,52" N	3°34'55,20"W	1400	22.5	-4.8	22
<i>B. pendula</i>	Campania, Italy	40° 17' 19.4" N	15° 32' 29.6" E	1050	21.4	-1.3	24
<i>B. pendula</i>	Lazio, Italy	42° 05' 23.0" N	12° 05' .997" E	250	27.2	2.5	3
<i>B. pendula</i>	Zaovine, Serbia	43° 51' 47.16" N	19°24'10.97" E	815	21.4	-7.4	64
<i>B. pendula</i>	Plitvička Jezera, Croatia	44° 53' 6.29" N	15°37'29.09" E	619	22.4	-6.2	86
<i>B. pendula</i>	Wracklow, Poland	51° 06' 00.0" N	17° 01' 60.0" E	118	22.7	-5.5	24

$T_{\max}$ , maximum temperature of the hottest month;  $T_{\min}$ , minimum temperature of the coldest month.

light and 12 h of dark. For each temperature, four replicates of 25 seeds each were sown on 1% agar in 9 mm Petri dishes. In addition, to test the effect of cold stratification on germination, following Cabiaux and Devillez (1977), we put four other replicates of 25 seeds each at a temperature of 5°C for 60 d. Light was excluded by wrapping the Petri dishes in a double layer of aluminium foil. After the stratification period, the seeds were moved to the test temperatures and scored every 12 h for the first week and less frequently afterwards. When no further germination or cotyledon emergence was observed for 28 d, the experiments were terminated.

#### Water potential

Osmotic potentials of -0.1, -0.2, -0.4, -0.6, -0.8, -1 and -1.5 MPa were obtained by dissolving polyethylene glycol (PEG 8000, VWR International srl, Milan, Italy) in deionized water. The quantity of PEG needed for each treatment was calculated according to Michel (1983) for a temperature of 20°C. In the control treatment, only deionized water was added to the germination medium. For each population, except LAZ and SER, for which there were not enough seeds, three replicates of 20 seeds each were sown in 6 mm Petri dishes. In each dish, two layers of Whatman N°1 filter paper (Merck Life Science S.r.l., Milan, Italy) were soaked with 2 ml of solution. Each dish was sealed with Parafilm (Bemis Company, Inc., Neenah, WI, USA) and placed in hermetic plastic bags to avoid changes in osmotic potential due to water evaporation. We refilled each Petri dish every 3 d with 1 ml of the correspondent PEG solution. The experiment lasted for 35 d and was conducted at the constant temperature of 20°C with 12 h of light and 12 h of dark. Germination was defined as 1 mm radicle emergence (henceforth referred to as 'germination'). In both experiments, germinated seeds were left in the Petri dishes and scored again when the cotyledon opened (henceforth referred to as 'cotyledon emergence'). At the end of each test, all of the ungerminated seeds were cut and visually inspected to assess their viability by checking the presence or absence of the embryo and its conditions. For each collection, at the end of all of the experiments, the dry weight of 25 seeds was measured, excluding SER, for which there were not enough seeds left.

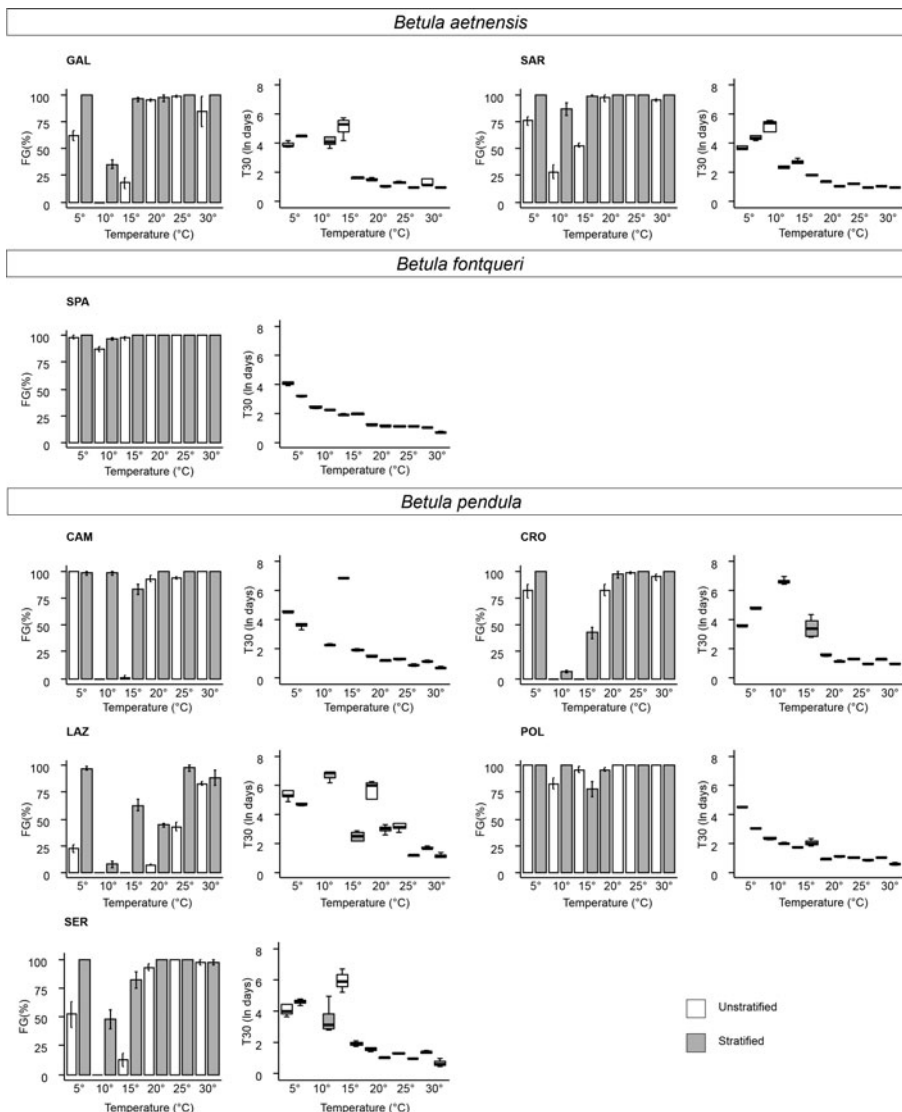
#### Statistical analysis

The effects of the experimental treatments and of each treatment level were assessed on the final germination ('FG') percentage, the

final cotyledon emergence ('CE') percentage and on the time to reach 30% of germination and cotyledon emergence ( $T_{30}$ ). Germination response was compared between and within species. Time-to-event non-linear models were fitted to the germination and cotyledon emergence using the R package 'drc' (Ritz and Streibig, 2005). From the fitted models, it was possible to calculate the time, expressed in days, at which each experimental treatment  $\times$  population combination would have reached the 30 and 50% germination ( $T_{30}$  and  $T_{50}$ ) of the viable seeds.  $T_{30}$  was used, instead of  $T_{50}$ , for modelling the germination response because its value was more realistic for the treatments with the lowest FG (%) and CE (%). Generalized linear models (GLMs) were fitted to FG (%) and CE (%) data using a binomial error distribution with a logit link. For  $T_{30}$ , we assumed a Gamma distribution based on the error structure, and the identity link was used after comparing the Akaike information criteria (AIC) of models fitted with other functions. Both for temperature and water potential experiments, separated analyses were run, first comparing the three species and then the populations within each species, with the exception of *B. fontqueri*, which was represented by a single population (SPA).

In the temperature experiment, we used three factors such as temperature, stratification treatment and species/population. In the water potential experiment, in contrast, there were only two factors including the osmotic potential and the species/population. Full models, including all factors and their interactions, were fitted for both the response variables [FG (%), CE (%) and  $T_{30}$ ]. To assess the significance of main effects and interactions, an ANOVA with the Wald  $\chi^2$  test was performed followed by a *post hoc* pairwise comparison test (Tukey's HSD) to assess the differences between species/populations and treatments.

To obtain the threshold values for temperature and water potential, the germination (or cotyledon emergence) rate was calculated as  $1/T_{30}$  and  $1/T_{50}$ . For each treatment  $\times$  population combination, a linear model was fitted with the germination speed as a response variable and the temperature or water potential as an independent variable. Once the line equation was obtained, its intersection with the  $x$ -axis, which represented the point at which the germination speed is zero, was calculated. The values obtained represent the base temperatures ( $T_{b30}$  and  $T_{b50}$ ) for unstratified or stratified seeds and the base water potentials ( $\Psi_{b30}$  and  $\Psi_{b50}$ ). In the calculation of the  $T_b$ , the regression lines were fitted from the temperature with the higher germination speed for that treatment to the lower temperature tested (5°C). A two-way ANOVA followed by a *post hoc* test (Tukey)



**Fig. 1.** Effects of test temperatures and cold stratification on the final proportion (FG %) and the time to reach 30% of germination ( $T_{30}$ ). White plots represent unstratified seeds, and grey plots represent stratified seeds. Due to the high range of variation of  $T_{30}$ , to make the figure more readable, its natural logarithm was used instead. FG, final germination.

was performed on the  $T_{bs}$  to investigate the effect of cold stratification, species/population and their interaction, while an one-way ANOVA was executed to test the differences in  $\Psi_{bs}$  among species/populations.

### Comparison between environment and seed traits

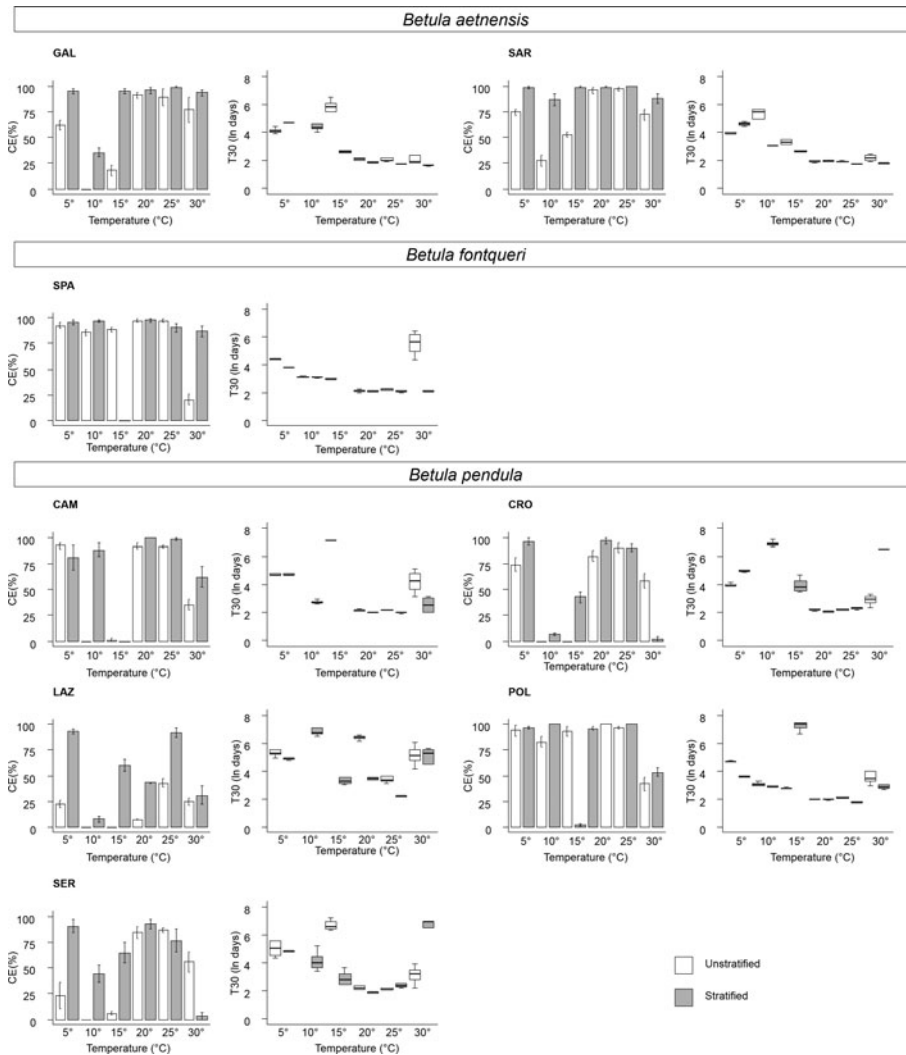
The geographical (latitude and altitude) and bioclimatic variables were then compared with the seed traits. A data matrix was built, including latitude, altitude, Prec. Driest,  $T_{max}$ ,  $T_{min}$  base temperatures and water potential for both germination and seedlings and seed weight. Data were checked for autocorrelation using the Pearson correlation coefficient in order to avoid variables with a strong autocorrelation. Due to the presence of outliers, the population of LAZ was excluded from this part of the analysis. The normal distribution of each variable was checked using the Shapiro–Wilk test, and the data that did not follow a normal distribution were transformed into their natural logarithm. For each seed trait, a linear model was fitted, including all of the environmental variables as predictors. From the full model, with a backward selection, the least significant variable was omitted, and a new model was fitted and compared with the previous one by a

likelihood test. If the two models were not significantly different, the backwards selection proceeded until the model with the lowest AIC was retained. All statistical analyses were performed using R (R Core Team, 2019).

## Results

### Interspecific variation in germination requirements

FG (%) differed significantly ( $P < 0.001$ ) among species with temperature and stratification. It was higher at 25 and 30°C and, generally, lower at 10 and 15°C, with the exception of the populations POL, SAR and SPA (Fig. 1). At 5°C, germination was slower but eventually reached high values (Fig. 1). CE was more sensitive to high temperatures: CE (%) peaked at 25°C and decreased at 30°C (Fig. 2). Cold stratification increased FG (%) in all species and treatments. Germination speed ( $T_{30}$ ) was influenced by temperature and stratification ( $P < 0.001$  and  $P = 0.005$ , respectively, Table 2) but did not differ among species. Stratified seeds germinated faster at all temperatures, except 5°C (Fig. 1). CE was slower at 30°C. It was not recorded for stratified seeds of SPA and CAM due to an experimental error. Base temperatures for FG and CE,



**Fig. 2.** Effects of test temperatures and cold stratification on the final proportion (CE %) and time to reach 30% of cotyledon emergence ( $T_{30}$ ). White plots represent unstratified seeds, and grey plots represent stratified seeds. Due to the high range of variation of  $T_{30}$ , to make the figure more readable, its natural logarithm was used instead. CE, cotyledon emergence.

calculated at 30 ( $T_{b30}$ ) and 50% ( $T_{b50}$ ) of viable seed germination, are significantly different among species (Fig. 3, Table 4). *B. fontqueri* had the lowest base temperatures for the germination of unstratified seeds, while *B. aetnensis* had the highest values (Table 4).  $T_{b30}$  and  $T_{b50}$  for the germination of stratified seeds were similar for the two Mediterranean birches and significantly lower in comparison to *B. pendula*. Stratification alone did not significantly influence the base temperatures, but it did when interacting with the 'species' factor (Fig. 3).

The reduction of water potential influenced FG (%), CE (%) and germination speed in all species, and no seed germinated below  $-1$  MPa (Fig. 4). Drought tolerance differed among species (Fig. 5). The lowest values of  $\Psi_{b30}$  for FG were obtained by *B. fontqueri* ( $-1.15$  MPa  $\pm$  0.03 SE) and *B. aetnensis* ( $-1.05$  MPa  $\pm$  0.01 SE) (Table 4). *B. pendula* had, on average, higher  $\Psi_{b30}$  and  $\Psi_{b50}$  with the exception of POL, whose  $\Psi_{b30}$  was comparable with the Mediterranean species (Table 4). CE followed a similar pattern but was more affected than germination was by water stress.

### Intraspecific variation in germination requirements

#### *Betula aetnensis*

The two existing populations of *B. aetnensis* presented different responses to temperature and drought. In fact, before

stratification, GAL had low or zero FG (%) at medium temperatures ( $18.3\% \pm 5.3$  SE at 15°C and 0% at 10°C), while SAR reached FG (%) >25% at all temperatures (Fig. 1). Germination speed was significantly ( $P < 0.001$ ) influenced by temperature, stratification and their interaction but did not differ significantly among populations. CE closely followed germination in all treatments.  $T_{30}$  decreased after stratification at all temperatures, except 5°C, which significantly slowed germination speed (Fig. 1; Table 4). Unstratified seeds had higher  $T_b$  than other species (Table 4), but stratification decreased their values. *B. aetnensis* had high tolerance to water stress, but the two populations differed ( $P < 0.001$ ) in their response for both FG and CE (Fig. 4), with SAR being the more drought-tolerant (Table 3). Moreover, SAR had the lowest  $\Psi_{b30}$  and  $\Psi_{b50}$  for CE among all of the populations considered (Table 4).

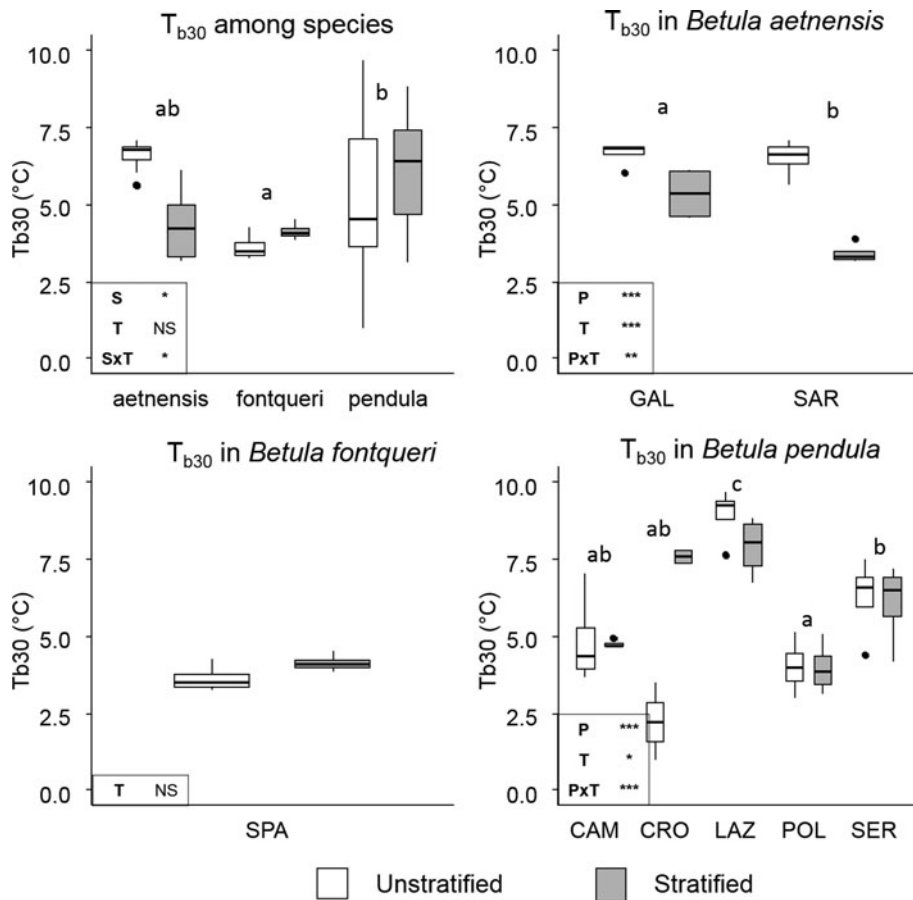
#### *Betula fontqueri*

*B. fontqueri* showed high FG (%), without significant differences among temperatures and stratification treatments (Fig. 1). CE (%) was significantly lower at 30°C for unstratified seeds, and 0 for stratified seeds at 15°C, due to an experimental error. Drought tolerance was comparable to *B. aetnensis*, with FG and CE (%) decreasing significantly only below  $-0.8$  MPa. Germination speed was positively influenced by temperature

**Table 2.** Effects of test temperature, stratification, species/population and their interactions on final percentage (FP) and  $T_{30}$  for germination and cotyledon emergence, based on logit-linked GLM with binomial distribution for FP and gamma distribution with identity link for  $T_{30}$

	Radicle emergence						Cotyledon emergence					
	FP			$T_{30}$			FP			$T_{30}$		
	d.f.	$\chi^2$	<i>P</i> -value	d.f.	$\chi^2$	<i>P</i> -value	d.f.	$\chi^2$	<i>P</i> -value	d.f.	$\chi^2$	<i>P</i> -value
<i>Betula ssp.</i>												
Temp	5	1150.15	<b>&lt;0.001</b>	5	69.738	<b>&lt;0.001</b>	5	1120.821	<b>&lt;0.001</b>	5	54.452	<b>&lt;0.001</b>
Strat	1	357.432	<b>&lt;0.001</b>	1	7.902	<b>0.005</b>	1	185.575	<b>&lt;0.001</b>	1	3.397	0.065
Species	2	151.239	<b>&lt;0.001</b>	2	3.875	0.144	2	226.972	<b>&lt;0.001</b>	2	11.797	<b>0.003</b>
Temp × Strat	5	65.599	<b>&lt;0.001</b>	5	1.883	0.865	5	58.313	<b>&lt;0.001</b>	5	7.944	0.159
Temp × Species	10	101.798	<b>&lt;0.001</b>	10	28.990	<b>0.001</b>	10	184.166	<b>&lt;0.001</b>	10	57.179	<b>&lt;0.001</b>
Strat × Species	2	26.595	<b>&lt;0.001</b>	2	3.272	0.195	2	57.989	<b>&lt;0.001</b>	2	1.654	0.437
Temp × Strat × Species	10	34.825	<b>&lt;0.001</b>	10	56.597	<b>&lt;0.001</b>	10	177.427	<b>&lt;0.001</b>	9	49.439	<b>&lt;0.001</b>
<i>B. aetnensis</i>												
Temp	5	360.538	<b>&lt;0.001</b>	5	173.010	<b>&lt;0.001</b>	5	319.145	<b>&lt;0.001</b>	5	188.964	<b>&lt;0.001</b>
Strat	1	146.642	<b>&lt;0.001</b>	1	13.559	<b>&lt;0.001</b>	1	172.628	<b>&lt;0.001</b>	1	12.293	<b>&lt;0.001</b>
Pop	1	59.653	<b>&lt;0.001</b>	1	1.283	0.257	1	42.613	<b>&lt;0.001</b>	1	0.455	0.500
Temp × Strat	5	66.875	<b>&lt;0.001</b>	5	59.704	<b>&lt;0.001</b>	5	58.999	<b>&lt;0.001</b>	5	59.321	<b>&lt;0.001</b>
Temp Strat × Pop	5	31.177	<b>&lt;0.001</b>	5	30.753	<b>&lt;0.001</b>	5	47.209	<b>&lt;0.001</b>	5	24.608	<b>&lt;0.001</b>
Strat × Pop	1	1.180	0.277	1	4.439	0.035	1	1.110	0.292	1	3.612	0.057
Temp × Strat × Pop	5	5.258	0.385	4	34.596	<b>&lt;0.001</b>	5	2.453	0.784	4	34.199	<b>&lt;0.001</b>
<i>B. fontqueri</i>												
Temp	5	1.746	0.883	5	5220.145	<b>&lt;0.001</b>	5	92.593	<b>&lt;0.001</b>	5	159.504	<b>&lt;0.001</b>
Strat	1	0.406	0.524	1	53.945	<b>&lt;0.001</b>	1	1.442	0.230	1	1.316	0.251
Temp × Strat	5	2.430	0.787	5	510.238	<b>&lt;0.001</b>	5	108.370	<b>&lt;0.001</b>	4	75.966	<b>&lt;0.001</b>
<i>B. pendula</i>												
Temp	5	565.784	<b>&lt;0.001</b>	5	356.750	<b>&lt;0.001</b>	5	523.616	<b>&lt;0.001</b>	5	214.857	<b>&lt;0.001</b>
Strat	1	146.017	<b>&lt;0.001</b>	1	34.671	<b>&lt;0.001</b>	1	76.284	<b>&lt;0.001</b>	1	3.471	0.062
Pop	4	460.935	<b>&lt;0.001</b>	4	36.004	<b>&lt;0.001</b>	4	320.874	<b>&lt;0.001</b>	4	13.027	<b>0.011</b>
Temp × Strat	5	57.815	<b>&lt;0.001</b>	5	16.195	<b>0.006</b>	5	52.621	<b>&lt;0.001</b>	5	24.005	<b>&lt;0.001</b>
Temp × Pop	20	188.905	<b>&lt;0.001</b>	20	214.456	<b>&lt;0.001</b>	20	306.039	<b>&lt;0.001</b>	20	172.989	<b>&lt;0.001</b>
Strat × Pop	4	67.268	<b>&lt;0.001</b>	4	8.196	0.085	4	59.844	<b>&lt;0.001</b>	4	16.829	<b>0.002</b>
Temp × Strat × Pop	20	118.587	<b>&lt;0.001</b>	14	149.125	<b>&lt;0.001</b>	20	270.229	<b>&lt;0.001</b>	13	113.643	<b>&lt;0.001</b>

Degrees of freedom (d.f.), Wald  $\chi^2$  and *P*-values are reported. Temp, temperature; Pop, population; strat, stratification. In bold are the values where  $p < 0.05$ .



**Fig. 3.** Boxplots showing the variation of the  $T_{b30}$  for the germination of unstratified and stratified seeds between and within species. At the bottom left of each subplot are displayed the results from a two-way factorial ANOVA: P, populations; S, species; T, treatment;  $P \times T$  or  $S \times T$ , interaction between population or species and treatments. The letters on the boxplots indicate differences between the samples (Tukey's HSD *post hoc* test). NS, not significant, \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

and stratification.  $T_{30}$  for FG decreased with warmer temperatures. In contrast, the speed of CE was significantly lower at 30°C. Base temperatures for FG were not influenced by stratification, remaining below 4.50°C, a value comparable with POL, the northernmost population considered (Table 4). Instead, threshold values for stratified seedlings were significantly lower ( $P = 0.016$  and 0.017 for  $T_{b30}$  and  $T_{b50}$ ), falling to 0.57°C ( $\pm 0.97$  SE) and 0.16°C ( $\pm 1.31$  SE) for  $T_{b30}$  and  $T_{b50}$ , respectively (Table 4). These were the lowest base temperatures calculated for stratified CE among all the populations. Lower water potentials reduced the speed of FG and particularly of CE (Fig. 4).  $\Psi_{bs}$  of *B. fontqueri* is among the lowest (Table 4), demonstrating high drought tolerance in this Mediterranean birch.

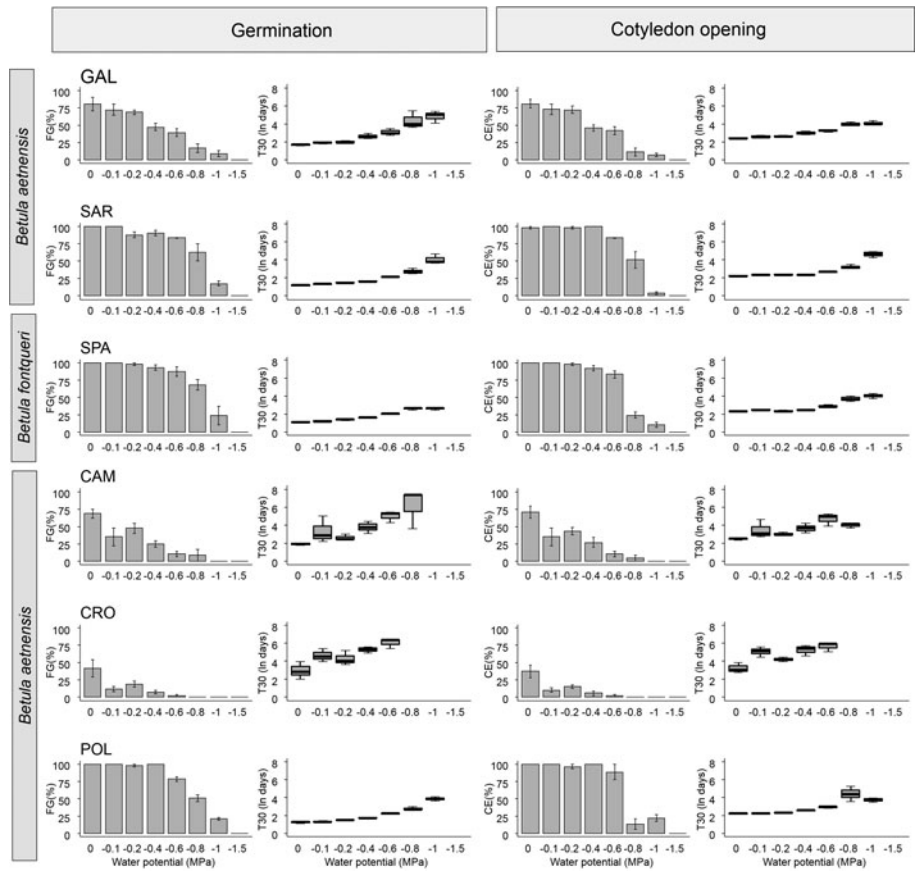
#### *Betula pendula*

The five populations of *B. pendula* displayed heterogeneous responses to temperature, stratification and water potential treatments, which influenced significantly FG (%) and germination speed (Tables 2 and 3). The interaction between stratification and populations did not influence the speed of germination (Table 2). FG (%) was higher at 20, 25 and 30°C, lower at 10 and 15°C and the thermal germination window widened after stratification (Fig. 1). Only POL reached high FG (%) at 10 and 15°C, while LAZ had significantly lower germination across all treatments. Stratification increased FG (%), even though its effect was small for LAZ and CRO at 10°C (Fig. 1). Cold stratification improved germination speed across all populations and temperatures, with the exception of CRO and SER, whose germination was slower at 5°C after stratification, as in

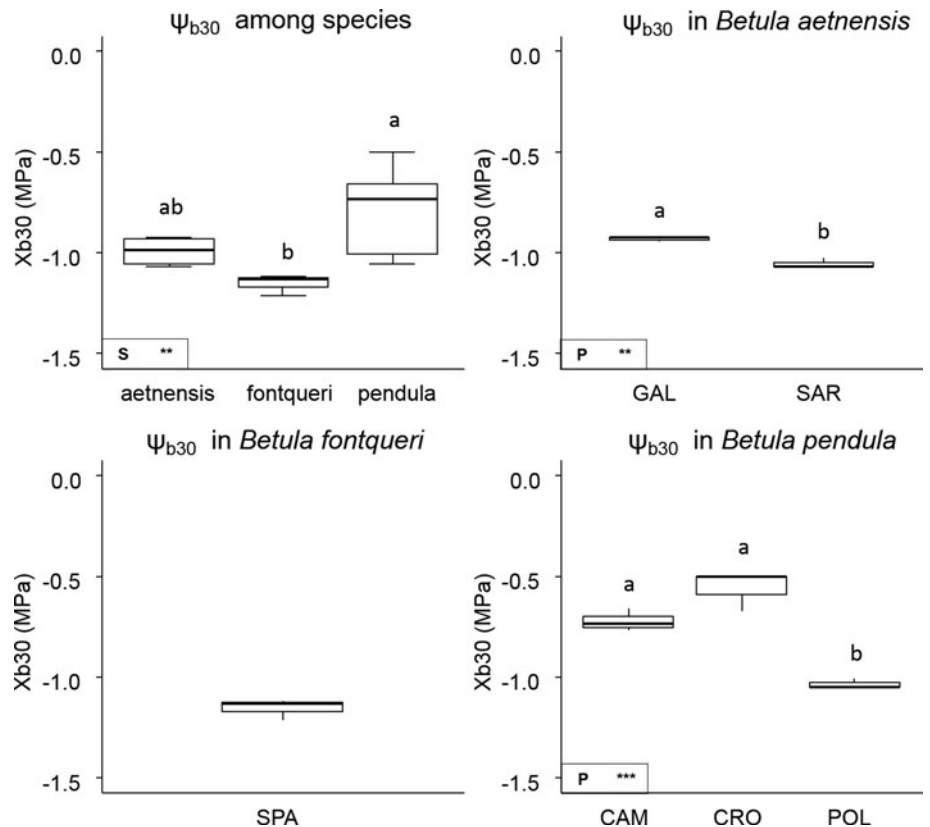
*B. aetnensis* (Fig. 1). CE followed FG closely, except at 30°C, where the final proportion was lower and  $T_{30}$  was higher (Fig. 2).  $T_{b30}$  for germination in unstratified seeds ranged from 2.22°C ( $\pm 0.54$  SE) in CRO to 8.94°C ( $\pm 0.45$  SE) in LAZ. The values increased slightly for  $T_{b50}$ . After stratification, base temperatures increased in CRO, decreased in LAZ and did not change significantly for the other populations (Fig. 3).  $T_{bs}$  were more heterogeneous for CE, and a clear pattern could not be described (Table 4). These results were probably influenced by the low CE speed at high temperatures and should be interpreted carefully. CAM and CRO resulted in drought sensitivity, while POL germinated at 88.9% ( $\pm 11.1$ ) still at  $-0.6$  MPa, a behaviour closer to *B. aetnensis* and *B. fontqueri* (Fig. 4). CRO had the highest threshold values for water potential for both germination stages, while the  $\Psi_{bs}$  of POL were comparable with the Mediterranean birches (Table 4).

#### Influence of the environment on germination thresholds

Prec. Driest did not follow a normal distribution and was transformed in its natural logarithm ('lnPrec.Driest'). All threshold values estimated for 50% FG, and CE were highly correlated (Pearson's correlation coefficient  $> 0.9$ ) to the ones estimated for 30% and were not included in the modelling. The threshold values calculated for CE were correlated with the ones obtained for germination (Pearson's correlation coefficient  $> 0.9$  for  $T_b$  of unstratified seeds and  $\Psi_b$ ; Pearson's correlation coefficient  $> 0.6$  for  $T_b$  of stratified seeds) and were, as well, excluded from further analysis. A strong negative correlation (Pearson's



**Fig. 4.** Effects of water potential on the final proportions (FG% and CE%) and the time to reach 30% for germination and cotyledon emergence ( $T_{30}$ ). Due to the high range of variation of  $T_{30}$ , to make the figure more readable, its natural logarithm was used instead. CE, cotyledon emergence; FG, final germination.



**Fig. 5.** Boxplots showing the variation of the  $\psi_b$  (30) for germination between and within species. At the bottom left of each subplot are displayed the results from one-way factorial ANOVA: P, populations; S, species. The letters on the boxplots indicate differences between the samples (Tukey's HSD *post hoc* test). NS, not significant, \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



**Table 3.** Effects of water potential and its interaction with species or populations on FP and  $T_{30}$  for germination and cotyledon emergence based on logit-linked GLM with binomial distribution for FP and gamma distribution with identity link for  $T_{30}$ 

	Radicle emergence						Cotyledon emergence					
	FP			$T_{30}$			FP			$T_{30}$		
	d.f.	$\chi^2$	P-value	d.f.	$\chi^2$	P-value	d.f.	$\chi^2$	P-value	d.f.	$\chi^2$	P-value
<i>Betula ssp.</i>												
Treat	7	449.938	<b>&lt;0.001</b>	6	13.631	<b>0.034</b>	7	496.436	<b>&lt;0.001</b>	6	32.387	<b>&lt;0.001</b>
Species	2	166.534	<b>&lt;0.001</b>	2	17.807	<b>&lt;0.001</b>	2	154.284	<b>&lt;0.001</b>	2	17.497	<b>&lt;0.001</b>
Treat × Species	14	29.923	<b>0.008</b>	12	34.676	<b>&lt;0.001</b>	14	49.732	<b>&lt;0.001</b>	12	35.548	<b>&lt;0.001</b>
<i>B. aetnensis</i>												
Treat	7	219.605	<b>&lt;0.001</b>	6	60.508	<b>&lt;0.001</b>	7	229.573	<b>&lt;0.001</b>	6	267.411	<b>&lt;0.001</b>
Pop	1	74.169	<b>&lt;0.001</b>	1	14.311	<b>&lt;0.001</b>	1	64.036	<b>&lt;0.001</b>	1	34.342	<b>&lt;0.001</b>
Treat × Pop	7	10.498	0.162	6	25.590	<b>&lt;0.001</b>	7	14.397	<b>0.045</b>	6	56.828	<b>&lt;0.001</b>
<i>B. fontqueri</i>												
Treat	7	100.720	<b>&lt;0.001</b>	6	670.920	<b>&lt;0.001</b>	7	129.190	<b>&lt;0.001</b>	6	137.600	<b>&lt;0.001</b>
<i>B. pendula</i>												
Treat	7	184.191	<b>&lt;0.001</b>	6	19.860	<b>&lt;0.001</b>	7	196.063	<b>&lt;0.001</b>	6	37.309	<b>&lt;0.001</b>
Species	2	200.528	<b>&lt;0.001</b>	2	26.494	<b>&lt;0.001</b>	2	173.929	<b>&lt;0.001</b>	2	32.428	<b>&lt;0.001</b>
Treat × Pop	14	42.269	<b>&lt;0.001</b>	9	52.576	<b>&lt;0.001</b>	14	56.725	<b>&lt;0.001</b>	9	56.726	<b>&lt;0.001</b>

Degrees of freedom (d.f.), Wald  $\chi^2$  and P-values are reported. Pop, population; treat, treatment. In bold are the values where  $p < 0.05$ .

**Table 4.** Base temperature ( $T_b$ ) for fresh and stratified seeds and base water potential ( $\Psi_b$ ) calculated from germination and cotyledon opening speed at 30 and 50%

Germination						
Population	Fresh seeds		Cold stratification		$\Psi_{b30}$ (MPa)	$\Psi_{b50}$ (MPa)
	$T_{b30}$ (°C)	$T_{b50}$ (°C)	$T_{b30}$ (°C)	$T_{b50}$ (°C)		
<i>B. aetnensis</i>						
GAL	6.64 (0.20)	7.31 (0.15)	5.35 (0.43)	5.44 (0.11)	-0.93 (0.01)	-0.88 (0.02)
SAR	6.52 (0.32)	7.45 (0.16)	3.42 (0.16)	4.13 (0.25)	-1.05 (0.01)	-1.01 (0.02)
<i>B. fontqueri</i>						
SPA	3.63 (0.23)	4.19 (0.16)	4.14 (0.14)	4.31 (0.14)	-1.15 (0.03)	-1.09 (0.03)
<i>B. pendula</i>						
CAM	4.87 (0.75)	5.48 (0.59)	4.75 (0.07)	5.23 (0.12)	-0.72 (0.03)	-0.67 (0.02)
LAZ	8.94 (0.45)	10.56 (0.19)	7.91 (0.50)	9.04 (0.46)		
CRO	2.22 (0.54)	3.66 (0.43)	7.58 (0.12)	8.06 (0.10)	-0.55 (0.06)	-0.57 (0.09)
SER	6.26 (0.66)	6.83 (0.65)	6.08 (0.67)	7.06 (0.36)		
POL	4.02 (0.45)	4.56 (0.37)	3.97 (0.43)	4.19 (0.47)	-1.03 (0.01)	-0.98 (0.01)
Cotyledon opening						
<i>B. aetnensis</i>						
GAL	5.68 (0.30)	6.29 (0.26)	5.40 (0.09)	6.19 (0.33)	-1.12 (0.00)	-1.05 (0.02)
SAR	6.05 (0.18)	6.93 (0.16)	4.25 (0.23)	4.65 (0.40)	-1.23 (0.02)	-1.15 (0.02)
<i>B. fontqueri</i>						
SPA	3.83 (.21)	4.46 (0.14)	0.57 (0.97)	0.16 (1.31)	-1.22 (0.03)	-1.12 (0.01)
<i>B. pendula</i>						
CAM	4.01 (0.88)	3.89 (0.92)	1.85 (0.46)	3.32 (0.61)	-0.88 (0.04)	-0.86 (0.04)
LAZ	4.04 (0.72)	-0.32 (3.22)	7.04 (0.22)	7.83 (0.39)		
CRO	1.79 (0.48)	1.14 (0.72)	7.36 (0.14)	7.62 (0.11)	-0.59 (0.04)	-0.55 (0.04)
SER	6.75 (0.32)	7.11 (0.34)	2.79 (3.42)	6.72 (0.25)		
POL	4.50 (0.11)	5.10 (0.22)	4.36 (0.13)	4.45 (0.14)	-1.13 (0.05)	-1.05 (0.04)

GAL, Galvarina; SAR, Sartorius; SPA, Spain; CAM, Campania; LAZ, Lazio; CRO, Croatia; SER, Serbia; POL, Poland.

correlation coefficient = -0.78) was found between altitude and  $T_{max}$ : in fact, despite the lower latitude, the more elevated locations were cooler in summer. A positive correlation (Pearson's correlation coefficient = 0.84) was found between base water potential and base temperature of stratified seeds. Both variables were left in the analysis to investigate the nature of this relationship.

$T_{b30}$  of unstratified seeds was influenced by  $T_{max}$  ( $P = 0.010$ ): for every 1°C increase in  $T_{max}$ ,  $T_{b30}$  decreased by 1.45°C. The model that better described the influence of environment on the  $T_{b30}$  of stratified seeds also retained  $T_{max}$ , but in this case, its effect was not significant ( $P = 0.296$ ). However, their relationship was still negative, with a reduction of -0.49°C of  $T_{b30}$  for each degree of increase in  $T_{max}$ .  $T_{b30}$  of stratified seeds increased significantly ( $P = 0.026$ ) with the precipitation of the driest month.  $\Psi_{b30}$  was described by the full model, in which altitude,  $T_{max}$ ,  $T_{min}$  and  $\ln Prec$ . Driest all concurred in explaining the dependent variable, but none of them had a significant effect (Table 5). Finally, seed weight was explained, but not significantly, by altitude ( $P = 0.091$ ) and  $T_{max}$  ( $P = 0.254$ ).

## Discussion

### Interspecific differences in germination requirements

*B. fontqueri* and *B. aetnensis*, although derived from *B. pendula*, are species with a mountain Mediterranean distribution and have adapted to a seasonally drier and warmer climate. For all three species, germination was maximum at 30°C, and, for most of the populations of *B. pendula* and *B. aetnensis*, it was reduced or even inhibited at 15 and 10°C in the absence of cold stratification. This behaviour could prevent germination in autumn and postpone it to spring. In fact, even though under natural conditions some seeds may germinate in autumn, they are unlikely to survive winter conditions (Vanhatalo et al., 1996). Moist and chilling winter conditions represent the natural way of cold stratification and widen the range of temperatures suitable for germination in *B. pendula* (Midmore et al., 2015). Vanhatalo et al. (1996) observed dormancy release in *B. pendula* at cold stratification temperatures (2.4 and 5.5°C) and its induction at 12.4°C. A positive effect of cold stratification was reported also for *B. aetnensis* by Strano and Poli Marchese (2011). They found that unstratified

**Table 5.** Summary of GLMs of the influence of environmental variables on the threshold values for germination and seed weight

Models	Estimate	SE	t-value	P-value	Adjusted $R^2$	d.f.
$T_{b30}$ unstratified						
Intercept	36.255	7.941	4.566	0.006	0.709	5
$T_{max}$	-1.455	0.368	-3.955	<b>0.011</b>		
$T_{b30}$ stratified						
Intercept	9.287	8.095	1.147	0.315	0.633	4
$T_{max}$	-0.498	0.414	-1.202	0.296		
ln Prec. Driest	1.961	0.571	3.437	<b>0.026</b>		
$\Psi_{b30}$						
Intercept	1.636	1.535	1.066	0.480	0.814	1
Altitude	0.000	0.000	-1.005	0.498		
$T_{max}$	-0.163	0.071	-2.287	0.262		
$T_{min}$	0.049	0.031	1.550	0.365		
ln Prec. Driest	0.412	0.089	4.609	0.136		
Seed weight						
Intercept	-0.007	0.012	-0.638	0.569	0.487	3
Altitude	0.000	0.000	2.460	0.091		
$T_{max}$	0.001	0.000	1.407	0.254		

$T_{b30}$ , base temperature to 30% FG;  $\Psi_{b30}$ , base water potential to 30% FG;  $T_{max}$ , maximum temperature of the hottest month;  $T_{min}$ , minimum temperature of the coldest month; ln Prec. Driest, natural logarithm of the minimum precipitation of the driest month; SE, standard error; d.f., degrees of freedom. In bold are the values where  $p < 0.05$ .

seeds reached the maximum FG at 30°C and the lowest at 15 and 35°C. However, after cold stratification, high germination occurred even at these temperatures. Instead, the single population tested of *B. fontqueri* showed high germination at all temperatures, without need for stratification. A germination temperature of 16°C in the presence of light was reported for this species by Blanca et al. (1999), as well as the absence of seed dormancy. However, the authors affirmed that the germination response varies among individuals.

Average base temperatures and water potential were lower for the two Mediterranean birches, compared to *B. pendula*. In Picciau et al. (2019), Mediterranean mountain species had higher  $T_b$  than lowland species to avoid germination when there is still a risk of freezing. Cold stratification decreases  $T_b$  (Porceddu et al., 2013; Dürr et al., 2015; Midmore et al., 2015), but in our study, this only occurred for *B. aetnensis* and some populations of *B. pendula*. Cold stratification contributed to widening the germination window of *B. aetnensis*, lowering its  $T_b$  but had no effect on *B. fontqueri*, which already possesses a low base temperature for the germination of unstratified seeds. However, stratification reduces, in this species, the  $T_b$  for CE, producing cold-tolerant seedlings, capable of establishing when temperatures are still cold.

Low germination temperature and water potential in *B. aetnensis* and *B. fontqueri* represent an adaptive advantage for mountain environments with a seasonally dry and warm climate. For example, summer drought, on Mount Etna, is pronounced for at least 2 months per year (Bagnato et al., 2014), and the anticipation of germination to early spring may increase the possibility, for seedlings, to reach a developmental stage that allows them to tolerate it. Heat tolerance, pronounced in seedlings of *B. aetnensis*, and a low  $\Psi_b$  could also increase their survival chances through summer. Even though  $T_{max}$  on Etna's sites was the lowest

(Table 1), the real value could be higher. In fact, our climatic data come from the interpolation (Fick and Hijmans, 2017) of air temperature data. But summer temperatures of dark, volcanic soil can easily exceed 30°C even at high altitudes (Blandino, unpublished data). The ability to regenerate at low water potential increased the fitness of *B. aetnensis* and *B. fontqueri* in a Mediterranean precipitation pattern.

#### Intraspecific differences in germination requirements

Intraspecific variation in germination requirements and threshold values was observed in *B. aetnensis* and, especially, in *B. pendula*. This has been reported for *B. fontqueri* (Blanca et al., 1999). It appears to be a combination of genetic variability and adaptation to the growing site.

Even though the general trend in *B. pendula* was of germination inhibition at low temperatures, the response, among the populations tested, was heterogeneous, with POL outstanding for high germination at all treatments, comparable with *B. fontqueri*. In contrast, LAZ, which grows in the hottest site out of the climatic envelope of the species, on a lowland sulphuric bog of volcanic origin in Central Italy, germinated poorly even at 20°C and presented the highest base temperatures. The divergent behaviour of this azonal population is mirrored by its genetic distinctiveness (de Dato et al., 2020). The same authors found a high degree of genetic diversity among Mediterranean populations of silver birch, including *B. aetnensis*. A similar result was found by Martín et al. (2008) for the extant Spanish populations of *B. fontqueri*.

The populations of *Betula* in this study had heterogeneous levels of dormancy, ranging from non-deep physiological dormancy to, apparently, no dormancy. According to Thompson and Ooi (2010), dormancy is a seed-specific characteristic, and its breakage

requires slow changes in the seed itself. However, these physiological changes may not ensure germination, meaning that non-dormant seeds still cannot germinate. In fact, germination occurs only when the environmental conditions required by the seed are met. The intraspecific variation in germination requirements and threshold values observed in our populations does not allow for the definition of 'dormant' of any of the species studied. The variation of  $T_b$  with stratification in *B. pendula* is heterogeneous and may be ascribable to local microclimate adaptations, which must be further investigated. The population subject to lower water stress, CRO, presented the higher  $\Psi_b$ , while POL, our northernmost population, had a  $\Psi_b$  comparable with the Mediterranean species, explainable with its low precipitations in the driest, winter months. In *B. aetnensis*, the inhibition of germination at 10 and 15°C was more pronounced for GAL than for SAR. These populations grow on different sides of Mount Etna, and genetic exchange is prevented by topography, leading to a certain degree of isolation. The two populations differed also in their  $\Psi_b$ , higher in GAL than in SAR, probably as adaptations to local edaphic conditions. In fact, the eastern side of Mount Etna, where SAR is located, is exposed to a considerable deposition of volcanic ashes, subject to the direction of the prevalent winds (Calabrese et al., 2011). Here, the soil is mainly constituted of volcanic sand with a low content of organic matter. Water from precipitation is not retained by this kind of soil, creating the conditions for edaphic drought.

### Relationship among seed and environmental traits

$T_b$  is a phylogenetically conserved trait, suggesting that adaptation to temperature may be a relatively slow process (Arène et al., 2017). We found a negative relationship between the  $T_b$  of unstratified seeds and the  $T_{max}$ . However, higher summer temperatures were experienced mainly by the populations of *B. pendula* (Table 1), situated at lower altitudes. Nevertheless, cold stratification changed this relationship, broadening the germination window of *B. aetnensis* by lowering its  $T_b$ .

The base temperature of stratified seeds was influenced by the intensity of annual drought, being lower where the minimum precipitation of the driest months was less.

Depending on the population, this relationship may have different interpretations. In fact, Mediterranean mountain populations do experience drought in summer months, after cold stratification occurs, while in the south of Poland, there is less precipitation in winter. If, in the first case, a low  $T_b$  can anticipate germination before the onset of the dry season, the selective pressure on seeds from POL is direct, to avoid germination in the coldest months. However, the base temperature of POL did not change significantly with stratification and is high enough to avoid germination in the winter months.

None of the environmental parameters considered significantly influenced  $\Psi_b$ , although the best predictive model included all four variables. However, the differences in its value among the three species were significant. Base water potential is a more variable trait than  $T_b$  and can be easily modified by adaptation to local conditions (Arène et al., 2017). A stronger correlation with edaphic drought may have been demonstrated by using *in situ* collected data.

### Conclusion

*B. aetnensis* and *B. fontqueri* survive as relict populations in glacial refugia at low latitudes and developed an adaptation to the

mountain Mediterranean climate. Morphological, histological and ecophysiological adaptations to the Mediterranean climate were already described for the Etna birch (Pavari, 1956; Biondi and Baldoni, 1984; Leonardi et al., 1994). We provided evidence for an adaptation of the regeneration niche of these two species to a warmer and drier climate. Both Mediterranean birches are today endangered by land-use change, herbivory, fungal infection (Blanca et al., 1999; Bagnato et al., 2014; Morales-Molino et al., 2019) and, in the case of *B. aetnensis*, increased deposition of volcanic ashes (Calabrese et al., 2011).

Cotyledon emergence always followed germination at low temperatures, but seedlings do not tolerate excessive warmth and drought. The stage of seedling establishment is more critical than germination, especially in light of increasing average temperatures and drought. Protocols for *ex situ* cultivation and *in situ* interventions (e.g. translocations) should be developed to preserve all of the populations of the rare *B. aetnensis* and *B. fontqueri* and the highly diverse marginal populations of *B. pendula*.

In a scenario of climatic change, the *Betula* species adapted to the Mediterranean mountains appears to be greater prepared to face increasing summer temperatures and drought. Therefore, their conservation is important because they represent a genetic reservoir for the affine *B. pendula* and could prove to be a useful resource for the mitigation of climate change effects in the Northern European populations of silver birch.

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