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Article *in* Plant Biology · August 2017

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Plant Biology ISSN 1435-8603

RESEARCH PAPER

Dissecting seed dormancy and germination in *Aquilegia barbaricina*, through thermal kinetics of embryo growth

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Keywords

Base temperature; endosperm rupture; morphophysiological dormancy; Ranunculaceae; thermal thresholds.

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Editor

I. Kranner

Received: 21 April 2017; Accepted: 26 July 2017

doi:10.1111/plb.12610

ABSTRACT

- Threshold-based thermal time models provide insight into the physiological switch from the dormant to the non-dormant germinating seed.
- This approach was used to quantify the different growth responses of the embryo of seeds purported to have morphophysiological dormancy (MPD) through the complex phases of dormancy release and germination. *Aquilegia barbaricina* seeds were incubated at constant temperatures (10–25 °C) and 25/10 °C, without pre-treatment, after warm+cold stratification (W+C) and GA_3 treatment. Embryo growth was assessed and the time of testa and endosperm rupture scored. Base temperatures (T_b) and thermal times for 50% (θ_{50}) of embryo growth and seed germination were calculated.
- W+C enabled slow embryo growth. W+C and GA_3 promoted rapid embryo growth and subsequent radicle emergence. The embryo internal growth base temperature (T_{be}) was *ca.* 5 °C for W+C and GA_3-treated seeds. GA_3 treatment also resulted in similar T_b estimates for radicle emergence. The thermal times for embryo growth (θ_{e50}) and germination (θ_{g50}) were four- to six-fold longer in the presence of GA_3 compared to W+C.
- *A. barbaricina* is characterised by a multi-step seed germination. The slow embryo growth during W+C reflects continuation of the maternal programme of development, whilst the thermal kinetics of both embryo and radicle growth after the removal of physiological dormancy are distinctly different. The effects of W+C on the multiphasic germination response in MPD seeds are only partially mimicked by 250 $mg \cdot l^{-1}$ GA_3. The thermal time approach could be a valid tool to model thermal kinetics of embryo growth and radicle protrusion.

INTRODUCTION

Requirements for seed dormancy loss and germination are specific for each species and depend on plant provenance (*i.e.* distribution and habitat) and phylogeny (Finch-Savage & Leubner-Metzger 2006; Baskin & Baskin 2014). Even closely related species, either growing in a variety of habitats (*e.g.* Vandelook *et al.* 2009) or co-occurring in a given habitat, may differ in their germination response to pre-dispersal environmental signals (*e.g.* Daws *et al.* 2002; Karlsson *et al.* 2008). Intraspecific variation in germination and embryo growth, among populations/ecotypes has also been related to differences in post-dispersal environment, mainly due to habitat (Donohue 2005; Giménez-Benavides *et al.* 2005; Mondoni *et al.* 2008).

Multi-step germination, in which testa and endosperm rupture are sequential events controlled by phytohormone balance, is widespread over the phylogenetic tree and has been described for many families, including Ranunculaceae (Hepher & Roberts 1985). In most species the seed-covering layers impose some level of physical constraint to radicle protrusion, which has to be overcome by a decrease in resistance of the surrounding tissue, an increased growth potential of the embryo or a combination of the two (Kucera *et al.* 2005; Müller *et al.* 2006). Abscisic acid (ABA) and gibberellic acid (GA) play an important role in a number of physiological processes in plants, including seed germinative growth. For example, in *Lepidium sativum* and *Arabidopsis thaliana* seeds, endosperm rupture is promoted by 10 μM GA_{4+7} and inhibited by about 10 μM ABA (Finch-Savage & Leubner-Metzger 2006; Müller *et al.* 2006). ABA induces dormancy during maturation, and GA plays a key role in dormancy release and in the promotion of germination, affecting both embryo growth and germination rate (*e.g.* Chen *et al.* 2008; Mattana *et al.* 2012a; Porceddu *et al.* 2016). Nevertheless, the role of gibberellins in dormancy release is controversial (Bewley 1997); although GA is associated with dormancy release and/or germination, in several species this treatment alone does not completely stimulate germination (Frattaroli *et al.* 2013; Baskin & Baskin 2014).

Temperature is one of the most important environmental conditions that control germination (Garcia-Huidobro *et al.* 1982; Probert 2000); it also determines the fraction of germinated seeds in a population and the rate at which they emerge (Heydecker 1977). In non-dormant seeds, the germination response to accumulated temperature has been modelled with a thermal time (θ) approach (Covell *et al.* 1986; Ellis *et al.*

1986; Pritchard & Manger 1990; Hardegree 2006). In this model, seeds accumulate units of thermal time (°Cd) to germinate for each percentile, *g,* of the whole population. When the seeds are subjected to temperatures (T) above the base temperature for germination (T_b), the germination rate increases linearly with temperature to an optimum temperature (T_o), above which germination rate starts to decrease (Garcia-Huidobro *et al.* 1982). Thus, in this suboptimal range (T_o–T_b), germination occurs in the time t_g, when the thermal time accumulated has reached a critical value (θ_g) for percentile g of the population, which can be described as $\theta_g = (T-T_b)t_g$. Intraspecific variation in T_b among populations may be due to different pre-harvest environmental conditions, and related to seed developmental heat sum (Daws *et al.* 2004). The thermal time approach has also been used to predict seed germination in the field (*i.e.* Hardegree & Van Vactor 2000; Steadman *et al.* 2003; Chantre *et al.* 2009). Recently, the impact of different simulated climate change scenarios on seed dormancy release and germination timing was investigated in *Vitis vinifera* subsp. *sylvestris* (Orrù *et al.* 2012), and used to model the *in situ* natural regeneration patterns of *Rhamnus persicifolia* (Porceddu *et al.* 2013). However, to date there are no specific studies on threshold temperatures and thermal time requirements for embryo growth in morphophysiologically dormant (MPD) seeds. MPD is one of the least understood dormancy classes, but was also proposed to be the ancient class of dormancy (Willis *et al.* 2014).

Seeds of Ranunculaceae species can exhibit morphological (MD) and MPD (Baskin & Baskin 1994, 2014; Walck *et al.* 1999; Cho *et al.* 2016). *Aquilegia* sp. pl. seeds have linear underdeveloped embryos (*sensu* Baskin & Baskin 2007) and stratification of the seeds at 3–5 °C for 2–4 weeks is recommended before sowing for germination (Ellis *et al.* 1985). Mattana *et al.* (2012b) reported MPD for *Aquilegia barbaricina* and *A. nugorensis*, which could be broken more efficiently by a combination of warm and cold stratifications.

The aims of our investigations on *A. barbaricina* seeds were to: (i) identify the phases of germination in MPD seeds; (i) individually characterise the thermal requirements for embryo growth, dormancy release and germination in MPD seeds; and (iii) assess the intraspecific variability on embryo growth and seed germination based on two distinct populations.

MATERIAL AND METHODS

Study species

Aquilegia barbaricina Arrigoni & E.Nardi (Ranunculaceae) is a rhizomatous perennial herb that branches underground and has stems 30–60-cm long (Arrigoni & Nardi 1977). The fruits are erect capsules that produce dark trigonal seeds, each with a linear underdeveloped embryo. This species is endemic to the Gennargentu and Supramontes regions of central–eastern Sardinia where the plant grows from 800 to 1400 m a.s.l. in wet woodlands, meadows and stream margins, mainly occurring on siliceous substrates and secondarily on limestone ones (Garrido *et al.* 2012). The plants flower and fruit centre on May and July, respectively. The species is included in the IUCN Red Lists (http://www.iucnredlist.org), it is classified as Critically Endangered (Fenu *et al.* 2011) and also as one of the 50 most endangered plants of the Mediterranean islands (de Montmollin & Strahm 2005).

Seed lot details

Seeds of *A. barbaricina* were collected directly from plants in riparian woods of *Alnus glutinosa* at the time of natural dispersal in early summer 2011 in two different populations in central–eastern Sardinia (Italy), specifically in Rio Correboi (RC; Villagrande Strisaili, OG) and in Rio Olai (RO; Orgosolo, NU; see Table 1).

Germination tests

Three replicates of 20 seeds each per condition were sown in July 2011, on the surface of 1% agar water in 60-mm diameter plastic Petri dishes. Dishes were incubated in the light (12 h of irradiance) at a range of germination temperatures (10, 15, 20, 25 and 25/10 °C). In the alternating temperature regime, the light period coincided with elevated temperature. Further replicates were given a dormancy-breaking treatment consisting of a warm (W = 25 °C for 3 months) followed by a cold stratification (C = 5 °C for 3 months), before being incubated at the range of germination temperatures (Table 2). This pre-treatment was chosen on the basis of the findings of Mattana *et al.* (2012b). Three extra replicates of 20 seeds each were also sown on the surface of 1% agar water with 250 $mg \cdot l^{-1}$ GA_3 and incubated in the light (12 h light) at the range of germination temperatures.

Germination was defined as visible radicle emergence. Germinated seeds were scored three times a week. During the germination tests, seeds with a split seed coat were scored, and the time from seed coat splitting to endosperm rupture was monitored daily in 15 seeds for each treatment and investigated population. Germination tests lasted for 1–4 months. When no additional germination had occurred for 2 weeks, a cut-test was carried out to estimate the viability of the remaining seeds; soft seeds being considered non-viable. The final germination

Table 1. Population data and seed lot details.

locality	population code	region	coordinates (UTM–Datum WGS84)	elevation range (m a.s.l.)	aspect	date of collecting	mean seed mass (mg ± SD)
Rio Correboi (Villagrande Strisaili, OG)	RC	Gennargentu	N 40°03′ E 09°20′	1190–1300	E–NE	29/06/2011	1.26 ± 0.06
Rio Olai (Orgosolo, NU)	RO	Supramontes	N 40°07′ E 09°22′	948–970	NE	28/06/2011	1.40 ± 0.05

Table 2. Experimental design.

condition		embryo growth measurements	
code	description	number of measurements	timing
0	Control	5	After 15, 30, 60, 90 and 120 days
W+C	3 months, 25 °C (W) →3 months, 5 °C (C)	13	After 15, 30, 60 and 90 days during warm (W), 15, 30, 60 and 90 days during cold (C), and 15, 30, 60 and 90 and 120 days after sowing for germination
GA_3	GA_3 (250 mg·l^{-1}) in the germination medium	5	After 15, 30, 60, 90 and 120 days

percentage was calculated as the mean of three replicates (±1 SD), on the basis of the total number of filled potentially competent seeds.

Embryo measurements

Embryo growth was assessed at different times, during the above-described conditions and germination temperatures by measuring ten seeds at each sample interval (see Table 2). Seeds were cut in half under a dissecting microscope and images of embryos acquired using a Zeiss SteREO Discovery.V8, with an objective Achromat S 0.63x, FWD 107 mm (Carl Zeiss MicroImaging, Germany) at 6.3× magnification, coupled to a Canon (Power shot G11) digital camera. Embryo and seed lengths were measured using the image analysis software ImageJ 1.41o (National Institutes of Health, Bethesda, MA, USA). Seed length was measured excluding the seed coat. The initial embryo length was calculated through measuring 20 randomly selected seeds before the start of the experiments. The embryo length of seeds with a split seed coat, but no radicle protrusion (*i.e.* critical embryo length), was determined for 20 randomly selected seeds and used as a surrogate for embryo length for seeds that had germinated before measurements (Vandelook *et al.* 2007).

Thermal time analyses

Thermal time studies were carried out for non-dormant seeds of both populations, germinating at constant temperatures after W+C pre-treatment and with GA_3 treatment. Estimates of time (t_g, days) taken for cumulative germination to reach different percentiles (g) for successive increments of 10% germination were interpolated from the germination progress curves (Covell *et al.* 1986). Germination rate ($1/t_g$) was regressed, using a linear model, as a function of temperature according to the following equation (Garcia-Huidobro *et al.* 1982):

$$1/t_g(\text{days}^{-1}) = (T - T_{bg})/\theta_g \quad (1)$$

An average (±1 SD) of the x-intercept among percentiles was calculated for the suboptimal temperature range (10–20 °C) to establish the base temperature for germination (T_{bg}) for each treatment (Ellis *et al.* 1986; Pritchard & Manger 1990). Linear regression equations were recalculated for each percentile, but constrained to pass through T_{bg} (Hardegree 2006). A comparison of regressions was then made between this model and one in which the T_{bg} were allowed to vary for all percentiles, and the best estimate was considered to be that which resulted in the smallest residual variance (Covell *et al.* 1986). Thermal time (θ_g, °Cd) estimates were then calculated separately as the inverse of the suboptimal regression equations (Covell *et al.* 1986; see equation 1).

Germination percentages were transformed to probits using tabular data from Finney (1971). Linear regression was used to express probit(g) as a function of both θ_g and log θ_g for the suboptimal temperature range for each seed lot, and the best model evaluated on the basis of the r^2 values (Hardegree 2006). The following equation was used to describe the form of cumulative germination response of seeds (Pritchard & Manger 1990):

$$\text{probit}(g) = K + \log\theta_g/\sigma \quad (2)$$

where K is an intercept constant when thermal time (θ_g) is zero, and σ is the SD of the response to log θ_g (*i.e.* the reciprocal of the slope) and represents the sensitivity of the population to θ_g (Covell *et al.* 1986). Thus, the flatter the slope of the fitted line the greater the variation in response to thermal time between individual seeds in the population (Daws *et al.* 2004).

Thermal time modelling was also used to analyse embryo growth rate after W+C or GA_3 treatment (Figs 2 and 3) to separate out growth rates pre- and post-dormancy release (Fig. 1). Estimates of time (t_e, days) taken for different percentiles of seeds (e) to reach the critical embryo length were interpolated from the embryo growth progress curves. Embryo growth rate ($1/t_e$) was regressed, using a linear model, as a function of temperature according to the modified equation (1):
for the suboptimal range,

$$1/t_e\ (\text{days}^{-1}) = (T - T_{be})/\theta_{e1} \quad (3)$$

while for the supra-optimal range,

$$1/t_e\ (\text{days}^{-1}) = (T_{ce} - T)/\theta_{e2} \quad (4)$$

An average (±1 SD) of the x-intercept among percentiles was calculated for both suboptimal and supra-optimal temperature ranges, to establish the base temperature (T_{be}) and, when possible, the ceiling temperature (T_{ce}) for embryo growth, respectively. The optimum temperature for embryo growth (T_{oe}) was calculated as the intercept of sub- and supra-optimal temperature response functions. Thermal time (θ_e, °Cd) estimates were calculated separately as the inverse of the regression equations. Linear regression equations were recalculated for each percentile, but constrained to pass through T_{be}. Linear regression was used to express probit cumulative percentiles of embryo growth (e) as a function of both θ_e and log θ_e and the best model evaluated on the basis of the r^2. Equation (5) was used to describe the form of cumulative percentiles response of seeds to reach the critical internal embryo length for the suboptimal temperature range:

$$\text{probit}(e) = K_1 + \log\theta_{e1}/\sigma_1 \quad (5)$$

where K_1 is an intercept constant when thermal time (θ_{e1}) is zero and σ_1 is the SD of the response to log θ_{e1} (*i.e.* the reciprocal of the slope), and represents the sensitivity of the population to θ_{e1}.

Statistical analysis

Generalised linear models (GLMs) were used to compare embryo length, period of the endosperm rupture phase, final germination percentages and base temperature (T_b). Then, significant differences within each condition were analysed by a *post-hoc* pair-wise comparisons *t*-test (with Bonferroni adjustment). GLMs with a log link function and quasi-Poisson error structure were used for analysing embryo length, rate of endosperm rupture and T_b values, while a GLM with a logit link function and quasi-binomial error structure was used for analysing germination percentages. Quasi-Poisson and quasi-binomial error structures and *F* tests with an empirical scale parameter instead of chi-squared on the subsequent ANOVA were used in order to overcome residual over-dispersion (Crawley 2007). All statistical analyses were carried out with R version 2.14.0 (R Development Core Team 2011).

RESULTS

Embryo growth

The GLM analysis detected no statistically significant differences ($P > 0.05$) between initial embryo length and final embryo length measured at the end of W+C, and did not highlight statistical differences ($P > 0.05$) among populations. The mean initial embryo length was 0.029 ± 0.006 mm for seeds of both populations (Fig. 1). During the W+C pre-treatment the embryo length increased slowly over time (Fig. 1). The mean embryo lengths after 90 days (*i.e.* at the end of the warm treatment) were *ca.* 0.040 mm for RO and *ca.* 0.037 mm for RC, and after 180 days (*i.e.* at the end of the W+C) these values increased to *ca.* 0.049 and *ca.* 0.044 mm for RO and RC, respectively (Fig. 1). Although final values at the end of W+C were not statistically different ($P > 0.05$ with GLM) from the initial embryo length, the linear regressions showed positive and significant relationships between the embryo length and time of the treatment ($r^2 = 0.56$, $P < 0.0001$ for RO; $r^2 = 0.42$, $P < 0.0001$ for RC), highlighting continuous and stable growth through W+C (Fig. 1).

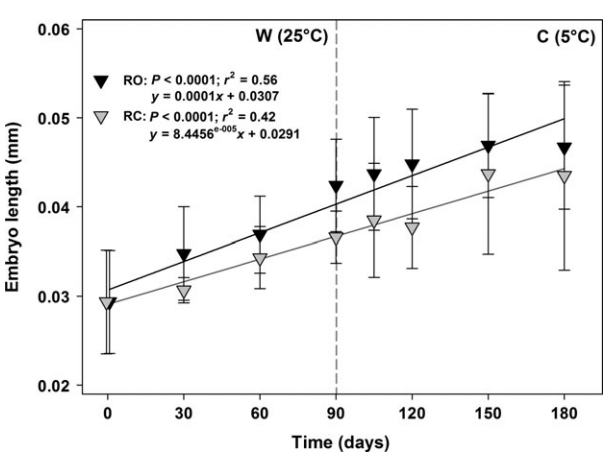

Fig. 1. Embryo growth trend in *A. barbaricina* during stratification at 25 °C for 3 months (W) and then at 5 °C for another 3 months (C) for seeds collected in Rio Correboi (RC) and Rio Olai (RO) populations. Initial and final embryo lengths measured at the end of W and C pre-treatments, are not significantly different at $P > 0.05$ with GLM, or between populations. Data are mean (±SD) of 20 seeds for initial embryo length and of ten seeds for each subsequent measurement.

The GLM analysis highlighted statistically significant differences ($P < 0.001$) on embryo lengths for the 'treatment' factor, whereas no statistical differences were found for one-way analysis of 'population' and 'temperature' factors and for all their interactions (Table 3). Incubation temperatures had a statistically significant effect ($P < 0.001$) on final embryo length with respect to the initial embryo length, or that calculated at the end of W+C pre-treatment (Fig. 2A). Temperatures in the control (0) had no effect on embryo growth, and the small differences between initial embryo lengths were due to the elapsed period from the initial to final (120 days) measurements (Fig. 2A). The mean critical embryo lengths calculated on seeds incubated at different germination conditions after W+C pre-treatment with a split seed coat but without endosperm rupture (as well as no radicle protrusion) were 0.115 ± 0.020 mm and 0.117 ± 0.023 mm for RO and RC populations, respectively (see Fig. 2A). In both populations, values obtained at the end of W+C and during GA_3 treatment showed values similar to critical embryo length, while at the end of the control test they were similar to initial embryo length (Fig. 2A).

Testa and endosperm rupture

Seeds exhibited a multi-step germination that followed embryo growth, such that there was a delay between testa rupture, following embryo elongation and exposure of the endosperm, and endosperm rupture, when the radicle emerged (Fig. 2B). Statistically significant differences ($P < 0.001$) were found on estimated rate of endosperm rupture, for 'treatment' and 'temperature' factors, while no statistical difference ($P > 0.05$) was detected for the 'population' factor. A statistically significant difference ($P < 0.01$) was found for the interactions treatment × population and treatment × temperature, and no statistically significant differences ($P > 0.05$) were detected for the interactions population × temperature and treatment × temperature × population (Table 3). After W+C treatment, the mean time from testa to endosperm rupture (*i.e.* radicle protrusion) decreased with increasing temperature, ranging from just over 6 days at 10 °C to just under 2 days at 20 °C for RO and RC populations (Fig. 2B). At 25 °C and at 25/10 °C this time interval was around 4 days and 2 days, respectively, for both RO and RC populations (Fig. 2B). In contrast, the time from testa to endosperm rupture in the GA_3 treatment was slower than after the W+C treatment (Fig. 2B). This was most evident in seeds of RC population, with intervals of 22 days at 10 °C and *ca.* 5 days at 25 °C (Fig. 2B).

Seed germination

The GLM highlighted statistical effects ($P < 0.05$) on seed germination for all applied factors, as well as for all their

Table 3. GLM results for the following factors: Treatment (0, control; W+C, 25 °C for 3 months and then 5 °C for another 3 months; GA_3, 250 mg·l^{-1} in the germination substrate), Temperature (10, 15, 20, 25 and 25⁄10 °C),Population (RO, Rio Olai; RC, Rio Correboi) and interaction of these for embryo length (mm), rate of endosperm rupture (days) and seed germination (%).

	df	deviance	resid. df	resid. Dev	*F*	*P* (>F)
Embryo length (mm)						
Null			298	5.2642		
Treatment	2	3.1617	296	2.1025	244.1995	<2e^{-16}***
Population	1	0.0010	295	2.1015	0.1549	0.6942
Temperature	4	0.0445	291	2.0570	1.7179	0.1462
Treatment: Population	2	0.0023	289	2.0548	0.1738	0.8406
Treatment: Temperature	8	0.0824	281	1.9724	1.5905	0.1275
Population: Temperature	4	0.0112	277	1.9612	0.4322	0.7853
Treatment: Population: Temperature	8	0.0315	269	1.9297	0.6088	0.7702
Rate of endosperm rupture (days)						
Null			283	64.390		
Treatment	1	12.1639	282	52.226	104.7452	<2.2e^{-16}***
Population	1	0.3706	281	51.855	3.1910	0.0752
Temperature	4	20.3480	277	31.507	43.8047	<2.2e^{-16}***
Treatment: Population	1	0.9061	276	30.601	7.8028	0.0056**
Treatment: Temperature	4	2.4064	272	28.195	5.1804	0.0005***
Population: Temperature	4	0.6684	268	27.527	1.4388	0.2215
Treatment: Population: Temperature	4	0.4923	264	27.034	1.0599	0.3768
Germination (%)						
Null			89	5098.6		
Treatment	2	3640.4	87	1458.2	445.2532	<2.2e^{-16}***
Population	1	55.4	86	1402.8	13.5411	0.0005***
Temperature	4	206.9	82	1196.0	12.6505	1.584e^{-07}***
Treatment: Population	2	149.2	80	1046.8	18.2441	6.461e^{-07}***
Treatment: Temperature	8	457.8	72	589.0	13.9978	3.024e^{-11}***
Population: Temperature	4	165.5	68	423.5	10.1215	2.501e^{-06}***
Treatment: Population: Temperature	8	167.1	60	256.4	5.1091	7.146e^{-05}***

** $P < 0.01$, *** $P < 0.001$

interactions (Table 3). While no seeds germinated during the control (0) and very low percentages (*ca.* 1% for RO and *ca.* 6% in RC) were detected at the end of C during W+C pre-treatment, seeds germinated to >50% both after W+C and during GA_3 treatments in each population (Fig. 2C). Statistically significant differences ($P < 0.001$) among temperatures were detected within each treatment, except for seeds of RC treated with GA_3 ($P > 0.05$), where the germination range was from *ca.* 52% (at 10 °C) to *ca.* 80% (at 25 °C; Fig. 2C). GA_3-treated seeds of the RO population germinated from *ca.* 12% (at 10 °C) to *ca.* 62% (at 20 °C; Fig. 2C). After W+C, high germination was observed at 25 °C (88 ± 6%) for RO, and at 15 °C (81 ± 12%) for RC (Fig. 2C).

Thermal time approach on embryo growth

The GLM analysis (Table 3) did not show statistically significant differences ($P > 0.05$) in embryo growth between populations. Therefore, a combined population response dataset was used to evaluate embryo thermal requirements, ascribing this characteristic to the species level. Seeds that germinated after W+C and during GA_3 treatments showed differences in both critical embryo length rate ($1/t_e$) and cardinal temperatures (Fig. 3). Based on embryo length rate responses for each 10th percentile (from 10% to 90%) of seeds that reached the critical embryo length, it was possible to estimate the mean base temperature (T_{be}) in the suboptimal temperature range for W+C and GA_3, and the mean ceiling temperature (T_{ce}) in the supra-optimal temperature range, and subsequently the optimal temperature for embryo growth (T_{oe}) for W+C (Fig. 3). Linear regressions for the different percentiles of suboptimal temperature range for W+C were calculated passing through 5 °C, which corresponds to an embryo growth rate equal to 0, the value obtained at the end the W+C pre-treatment (see Fig. 1). The obtained regression lines were then constrained to pass through the common value of T_{be}. For the supra-optimal temperature range, linear regressions were constrained to pass through the common value of T_{ce}. Linear regressions for the different percentiles for GA_3 were constrained to the common value of T_{be}. These models showed higher values of r^2 for all of the linear regression equations than the model where T_{be} and T_{ce} varied for each percentile. Average T_{be} were 5.20 ± 0.60 °C and 5.30 ± 2.56 °C for W+C and GA_3 treatments, respectively (Fig. 3), without statistically significant differences among treatments ($P > 0.05$). Average T_{ce} for W+C was 29.52 ± 2.37 °C, and the average T_{oe} was 15.00 ± 1.02 °C (Fig. 3), whereas in GA_3 treatment T_{oe} may be assumed to be ≥25 °C (Fig. 3).

Figure 4 shows the relationship between log thermal time (θ_e) and percentages of seeds that reached the critical embryo length expressed in probits, calculated according to equation (5). The relationship between log θ_e and probit critical embryo length had better residual sums of square (0.1420 for W+C and 0.1228 for GA_3) and r^2 (0.95 and 0.97 for W+C and GA_3, respectively) than when expressed on a linear scale (data

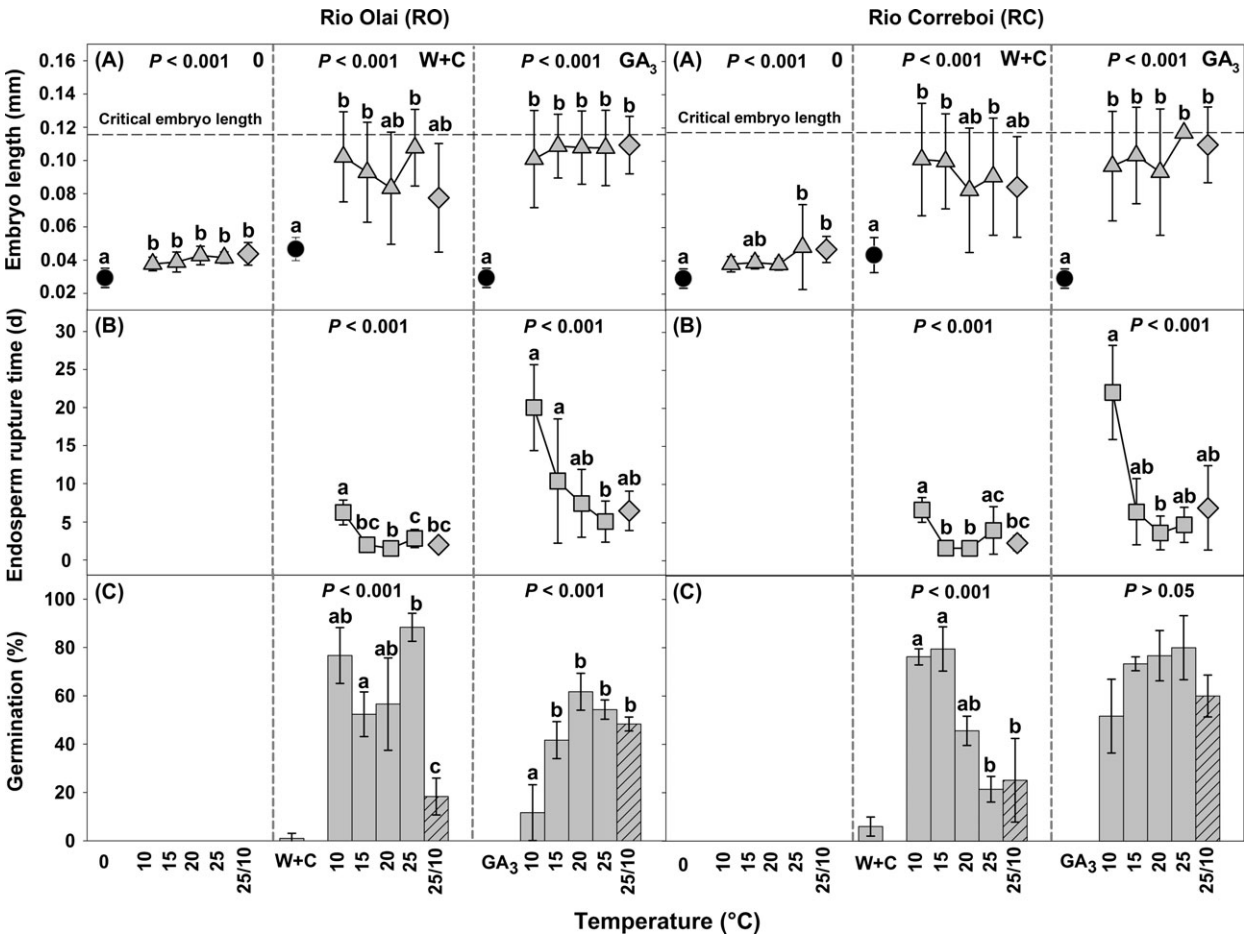

Fig. 2. Final embryo length values for *A. barbaricina* (A), time from seed coat splitting to endosperm rupture (B) and cumulative germination percentages (C) achieved at the end of germination tests (120 days), after each pre-treatment (0, control; W+C, 25 °C for 3 months and then 5 °C for another 3 months; GA_3, 250 mg·l^{-1} in the germination substrate) for each population (Rio Olai and Rio Correboi). Embryo lengths measured at the start of germination tests (initial embryo length) are reported as a reference for the control and GA_3, while the value assessed at the end of pre-treatment is reported for W+C (black circles; A). The results in the alternating temperature regime (25⁄10 °C) are here highlighted with grey diamonds (A and B) and grey coarse bar (C). Data are the mean of ten seeds (±SD) for embryo measurements, 20 (±SD) seeds (when available) for endosperm rupture rate and three replicates (±SD) of 20 seeds each for germination data. Dashed lines (A) correspond to the critical embryo length. GLMs were carried out within each treatment to test the effect of temperature on embryo growth, rate of endosperm rupture and germination. Values with the same letter are not different at $P > 0.05$ by *post-hoc* pair-wise *t*-test comparisons (with Bonferroni adjustment).

not shown). Thermal time required for 50% of seeds to reach the critical embryo length (θ_{e50}) was longer for the GA_3 with a value of 2.64 log °Cd compared to the W+C-treated seeds with a value of 2.10 log °Cd. However, seeds of W+C and GA_3 that reach the critical embryo length showed a very similar σ value (0.51 and 0.43 °Cd, respectively; Fig. 4).

Thermal time approach on seed germination

The T_{bg} for the RO population were 6.85 ± 0.26 °C for W+C and 8.43 ± 1.53 °C for GA_3 treatment, while for the RC population it was 5.34 ± 1.38 °C and 5.42 ± 0.26 °C for W+C and GA_3 treatment, respectively (Fig. 5). These values were statistically different ($P < 0.01$) according to the GLM, and a *post-hoc* pair-wise comparisons *t*-test highlighted that the difference was determined by the T_{bg} value of GA_3-treated seeds belonging to the RO population (Fig. 5). For each treatment on both populations, the linear regressions were re-calculated for each percentile, constraining them to pass through the mean T_{bg} (Fig. 5). This model led to no differences in residual sum of squares compared with when T_{bg} was allowed to vary for each percentile, and showed highest values of r^2 for all of the linear regression equations ($r^2 > 0.91$ for RO W+C, $r^2 > 0.58$ for RC W+C, $r^2 > 0.88$ for RO GA_3 and $r^2 > 0.57$ for RC GA_3).

Figure 6 shows the relationship between log thermal time (θ_g) and germination expressed in probits, calculated according to equation (2). The relationship between log θ_g and probit germination had better residual sums of squares both in W+C pre-treated seeds (0.1349 and 0.1851 for RO and RC populations, respectively) and in the GA_3-treated seeds (0.0098 and 0.1477 for RO and RC populations, respectively) than when expressed on a linear scale (data not shown). Thermal time required for 50% of germination (θ_{g50}) was longer for the GA_3-treated seeds (2.88 and 2.72 log °Cd for RO and RC, respectively) compared to the W+C pre-treated seeds (2.04 and 2.02 log °Cd for RC and RO, respectively; Fig. 6). In addition, GA_3-treated seeds of RO had a larger σ value (0.45 log °Cd) than seeds belonging to the RC population (0.33 log °Cd) and of

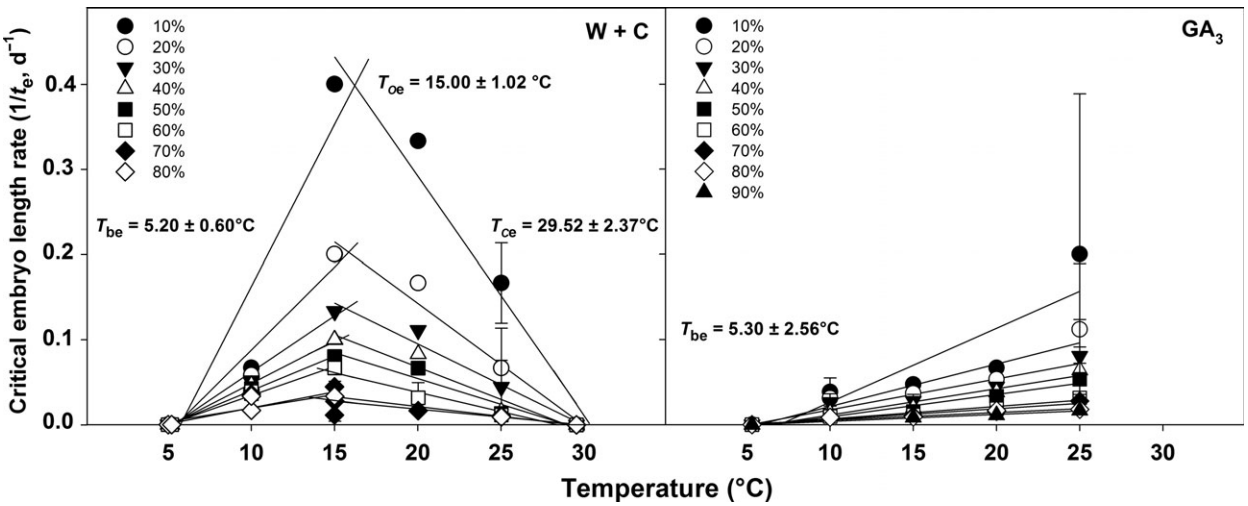

Fig. 3. Cardinal temperatures (T_{be}, base temperature, T_{oe}, optimal temperature, T_{ce}, ceiling temperature) to reach critical embryo length for seeds of *A. barbaricina*, calculated after W+C (25 °C for 3 months and then 5 °C for another 3 months) and incubated at a range of germination temperatures (10, 15, 20 and 25 °C), and T_{be} calculated after GA_3 (250 mg·l^{-1} in the germination substrate) treatment and incubated at constant temperatures in the suboptimal range (≤25 °C). Linear regressions for the different percentiles of suboptimal temperature range for W+C were calculated passing through 5 °C, which corresponds to an embryo growth rate equal to 0, value obtained at the end of the W+C pre-treatment (see Fig. 1), and after constrained to pass through the common value of T_{bg}; for the supra-optimal temperature range, linear regressions were constrained to pass through the common value of T_{ce}. Linear regressions for the different percentiles for GA_3 were constrained to the common value of T_{bg}. Percentiles for which regression lines had a $P > 0.05$, T_{bg} and T_{ce} values were not calculated.

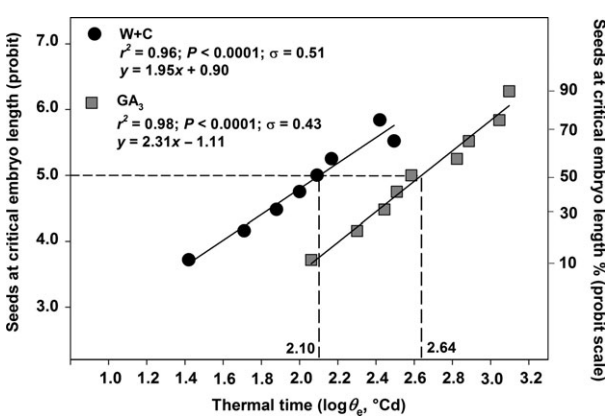

Fig. 4. Probit percentages of seeds of *A. barbaricina* that reached the critical embryo length after W+C (25 °C for 3 months and then 5 °C for another 3 months) and after GA_3 (250 mg·l^{-1} in the germination substrate) treatments as a function of log thermal time requirement (log θ_e). Thermal times were calculated from critical embryo length time courses assuming T_b of 5.2 and 5.3 °C, for W+C and GA_3, respectively. Thermal times to reach 50% of seeds that reached the critical embryo length (θ_{e50}) are also reported.

those W+C pre-treated seeds (0.38 log °Cd and 0.26 log °Cd for RC and RO populations, respectively; Fig. 6).

DISCUSSION

Seed dormancy and multiple phases to the completion of seed germination

The embryo in seeds of *A. barbaricina* is small at dispersal (0.03-mm long) and must grow before radicle emergence. Therefore, these seeds would be classified as morphologically dormant (MD) following the Baskin & Baskin (2014) dormancy classification system. Generally, if embryos have only MD, growth is completed in a relatively short period, and seeds germinate within about 4 weeks (Baskin & Baskin 2014). *A. barbaricina* seeds of each population did not germinate without any treatment, even after 120 days. Nonetheless, embryos in seeds subjected to W+C (25 °C, followed by 5 °C) grew internally by about 50% to *ca.* 0.04–0.05 mm (Fig. 1). The rate of change in embryo length was about 0.00009 mm·day^{-1}. After warm followed by cold stratification or GA_3 treatment, seeds started to germinate (radicles emerged) at all tested temperatures, due to a rapid increase in embryo growth to *ca.* 0.12 mm. This second phase of internal growth of the embryo is about 800× faster (*ca.* 0.07 mm·day^{-1}) than during W+C treatment. This suggests two distinctly different physiological responses of the embryo during treatment.

This study confirmed the presence of multi-step seed germination in the Ranunculaceae, involving the need for embryo growth within the seed before emergence, as previously reported by Hepher & Roberts (1985) for *Trollius ledebouri.* The classical conceptual model of the imbibition process distinguished three principal phases: phase I, marking a rapid uptake of water due to the low water potential of the seed; phase II, in which the water content does not change substantially in the intact seed prior to radicle protrusion; and phase III, when rupture of the covering layers (testa and endosperm) allows growth of the collet and the protruded radicle becomes visible (Bewley 1997). Recently, Toorop (2015) proposed a new dormancy-dependent conceptual model for multiphasic imbibition of seeds in which the classical phase II is split into three sub-classes: phase IIA is identical to the classical phase II; phase IIB is associated with testa rupture; and the transition between

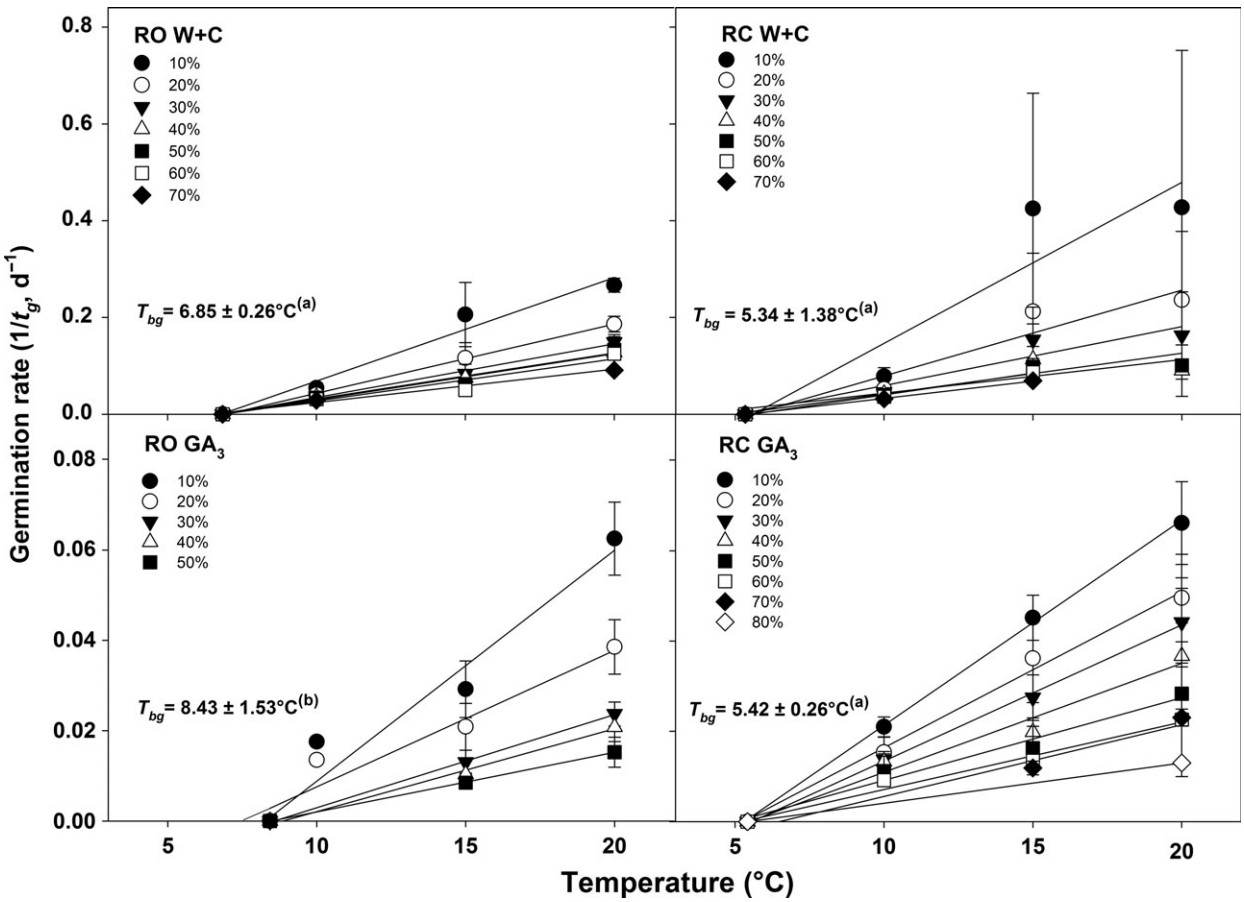

Fig. 5. Base temperatures for germination (T_{bg}) for the two populations (RO, Rio Olai; RC, Rio Correboi) of *A. barbaricina*, calculated after W+C (25 °C for 3 months and then 5 °C for another 3 months) and GA_3 (250 mg·l^{-1} in the germination substrate) treatments, and incubated at constant temperatures (10–20 °C). Within each population, the linear regressions for the different percentiles were constrained to the common value of T_{bg}. Percentiles for which regression lines had a $P > 0.05$, T_{bg} values were not calculated.

phase IIC and phase III indicates the endosperm rupture and radicle protusion. However, for seeds with underdeveloped embryos such as those of *A. barbaricina*, a multi-step seed germination can be described, with at least four well recognisable phases after imbibition: (I) the embryo grows slowly inside the seed; (II) the embryo grows rapidly inside the seed; until the (III) seed coat splits and (IV) the endosperm weakens allowing the radicle protrusion (Fig. 7). Accordingly to the conceptual model for multiphasic imbibition proposed by Toorop (2015), the phases III and IV detected in this work for *A. barbaricina* correspond to the phases from IIB to III. More recently, multiphasic sequential germination steps (*i.e.* embryo growth, testa rupture, endosperm rupture – radicle emergence) including the epicotyl–plumule emergence event, were also identified for *P. corsica* seeds (Porceddu *et al.* 2016).

It is known that the inhibitory effect of ABA is counteracted by gibberellin and that endosperm rupture is under the control of an ABA–gibberellin antagonism (Koornneef *et al.* 2002; Leubner-Metzger 2003; Kucera *et al.* 2005; Weitbrecht *et al.* 2011). In *A. barbaricina*, GA_3-treated seeds had longer mean time courses for the transition from testa rupture to endosperm rupture, compared to W+C stratified seeds. This suggests that GA_3 does not substitute completely for the beneficial

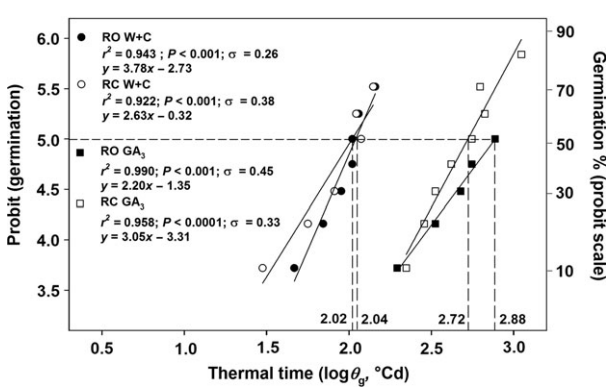

Fig 6. Probit germination after W+C (25 °C for 3 months and then 5 °C for another 3 months) and after GA_3 (250 mg·l^{-1} in the germination substrate) treatments for each population (RO, Rio Olai; RC, Rio Correboi) as a function of log thermal time requirement (log θ_g). Thermal times were calculated from germination time courses assuming T_b of 6.85 °C and 5.34 °C for W+C, and 8.43 °C and 5.42 °C for GA_3, for RO and RC, respectively. Thermal times to reach 50% germination (θ_{g50}) are also reported. Linear regression of W+C for RC was calculated without the value obtained for $g = 40$. Thermal times to reach 50% of germination (log θ_{g50}) are also reported.

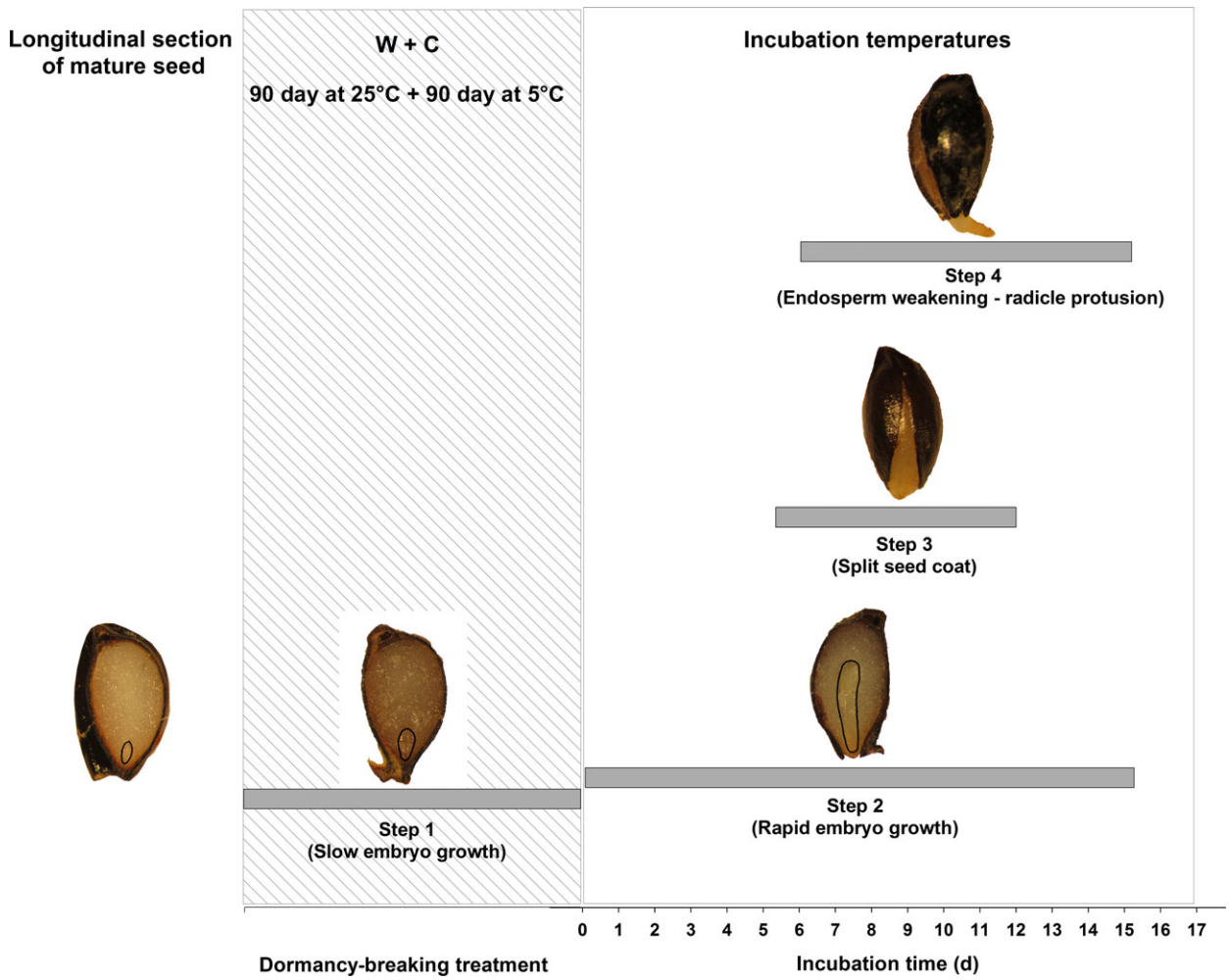

Fig. 7. Growth phases in *A. barbaricina* seeds: slow embryo growth (step 1) during W+C treatment (25 °C for 3 months and then 5 °C for another 3 months), and interval of time (in days) to complete the rapid embryo growth (step 2), split seed coat (step 3) and endosperm weakening – radicle protrusion (step 4) events.

effects of temperature pretreatment when considering kinetics of the germination process.

Overall, germination in *A. barbaricina* is a complex, multi-step process that involves the phased completion of embryo development and phased emergence. In non-dormant seeds (*i.e.* after warm + cold treatment), the critical embryo length is reached <2 days after a shift in temperature, the seed coat splits and the radicle protrudes by *ca.* 6 days, with significant overlap among all the phases during a period of *ca.* 16 days (Fig. 7). This overlap suggests that the seed coat starts to split when the embryos are still growing within the seed, before they reach their 'critical length' for germination and that radical protrusion immediately follows the split of the seed coat (Fig. 7). We next attempted to quantify and compare some key steps in this complex germination response.

Thermal thresholds for embryo growth and seed germination

The base temperature for embryo growth (T_{be}) of non-dormant seeds of *A. barbaricina* was approximately 5 °C both in W+C-stratified and GA_3-treated seeds. For W+C pre-treated seeds, it was possible to calculate all cardinal temperatures, with optimal temperature for embryo growth of *ca.* 15 °C and a ceiling temperature of *ca.* 29 °C. Base temperature for germination (T_{bg}) varied from *ca.* 5 to 7 °C in W+C stratified seeds, and from 5 to 8 °C for GA_3-treated seeds, depending on the provenance. Considering that no seeds of *A. barbaricina* germinated without treatment at the tested constant temperatures, a $T_b \geq 25$ °C (*i.e.* the highest temperature tested) may be hypothesised for dormant seeds of the two investigated populations. However, this should be confirmed through incubating seeds without pre-treatments at higher temperatures. A similar trend was detected in seeds of *V. vinifera* subsp. *sylvestris* (Orrù *et al.* 2012).

Constraining the linear regressions of each percentile for germination through the mean T_b resulted in an improvement of the residual sum of squares and r^2 values. Therefore, T_b for embryo growth and for germination can be used to describe the whole population response in *A. barbaricina* seeds, as previously reported for other species (*e.g.* Covell *et al.* 1986; Ellis *et al.* 1987; Pritchard & Manger 1990; Orrù *et al.* 2012; Porceddu *et al.* 2013). The best model was obtained by fitting

germination expressed in probit and log-normal (log °Cd) rather than normal distributed thermal times (°Cd), as previously reported for other herbaceous (Covell *et al.* 1986; Ellis & Butcher 1988) and woody (Pritchard & Manger 1990; Porceddu *et al.* 2013) species. Also, regarding the thermal times of embryo growth rate, the best model was obtained by fitting the values in probit and log-normal (log °Cd) compared to when normal-distributed, confirming that this methodology increases the goodness of fit of the model.

Seeds of *A. barbaricina* varied in their thermal time estimates to reach θ_{50}, depending on treatment. W+C pre-treated seeds had the lowest θ_{50} values (2.10 log °Cd; *i.e.* 128 °Cd) for embryo growth compared to GA_3-treated seeds (2.64 log °Cd; *i.e. ca.* 440 °Cd). The same trend was detected also for germination (radicle emergence), with θ_{50} values of 2.03 log °Cd (110 °Cd) for W+C stratified seeds and *ca.* 2.80 log °Cd (*ca.* 650 °Cd) for GA_3-treated RC and RO seeds. The four- to six-fold longer thermal times for embryo growth (θ_{e50}) and germination (θ_{g50}) in GA_3- compared to W+C-treated seeds suggests that GA_3 treatment only partially mimicks/replaces the W+C pre-treatment in seeds of this species.

There is now considerable evidence for negative linear relations between association θ_{g50} and T_{bg} in a broad range of species within life forms (Dürr *et al.* 2015). Yet in *A. barbaricina* such a relationship appears to vary with pretreatment, as removal of seed dormancy by GA_3 and W+C results in the same threshold temperature for germination (T_{bg}) but different germination thermal times (θ_{g50}), with GA_3-treated seeds being much slower to grow. Whilst long-standing dormancy classification systems rely heavily on fixed time intervals for the germination process (see Baskin & Baskin 2014), our work emphasises the importance of using thermal time kinetics to dissect the various stages of the germination process.

The analysis carried out in this study showed that in *A. barbaricina* the thermal requirements for embryo growth did not vary among populations, while for seed germination these were different between populations. Embryo growth could be strictly related to the seed biology of the species, whereas germination could be more related to the habitat of provenance of the species. Copete *et al.* (2014) reported that in seeds of *Narcissus eugeniae* (Amaryllidaceae) belonging to two different populations and tested in both near-natural and laboratory conditions, the embryo growth showed a similar pattern, while radicle emergence did not begin simultaneously. Intraspecific germination differences among populations of a species can arise due several factors, such as light, moisture and temperature (Gutterman 1992; Fenner & Thompson 2005), and can be interpreted as being an adaptation to specific habitat (Meyer *et al.* 1995, 1997), as detected in this study for *A. barbaricina*.

CONCLUSIONS

The results indicate that *A. barbaricina* is characterized by multi-step seed germination. The slow embryo growth during W+C treatment reflects the continuation of the maternal programme of development that was punctuated by seed dispersal, while the thermal kinetics of both embryo growth and radicle protrusion after the removal of physiological dormancy are distinctly different. The thermal time model developed in this work allowed us to identify the thermal threshold (T_b and θ_{50}) requirements of embryo growth and seed germination of this species. The beneficial effects of W+C treatment on the multiphasic germination response in MPD seeds is only partially mimicked by 250 $mg \cdot l^{-1}$ GA_3 treatment, having a similar controlling influence on base temperature for embryo growth and germination but not on rate processes. This attempt to model thermal requirement for embryo growth using a thermal time approach was confirmed through the morphological observations. This model could be applied in those species whose seeds also have a morphological component of dormancy (MD and MPD), could be a valid tool to model thermal kinetics of embryo growth and radicle protrusion, and may also be useful to predict seedling emergence in the field.

ACKNOWLEDGEMENTS

We gratefully acknowledge Sardinia Regional Government for financial support to Marco Porceddu PhD scholarship (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007-2013 – Axis IV Human Resources, Objective l.3, Line of Activity l.3.1.). CCB is supported by the 'Provincia di Cagliari – Assessorato Tutela Ambiente'. The Royal Botanic Gardens, Kew, receives grant in-aid from Defra, UK.

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