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

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Keyword:	base temperature, embryo growth, endosperm rupture, Ranunculaceae, thermal thresholds

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

5

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Abstract

- Threshold-based thermal time models provide insight to 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 ~~the~~ 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 4- to 6-fold longer in the presence of GA_3 compared to W+C.
- *A. barbaricina* is characterized by a multi-step seed germination. The slow embryo growth during W+C reflects the 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 is only partially mimicked by 250 mg·L^{-1} GA_3. The thermal time approach could be a valid tool to model thermal kinetics of embryo growth and radicle protrusion.

Keywords: base temperature, embryo growth, endosperm rupture, Ranunculaceae, thermal thresholds.

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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 (Baskin and Baskin 2014; Finch-Savage and Leubner-Metzger 2006). 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). Intra-specific variation in the germination requirements~~, as well in the~~ 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 ~~the~~ testa and endosperm rupture are sequential events controlled by phyto-hormone balance, is widespread over the phylogenetic tree and has been described for many families, including Ranunculaceae (Hepher and 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 both (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 and Leubner-Metzger 2006; Müller et al. 2006). ABA induces dormancy during maturation, and GA plays a key role on 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 stimulate totally germination (Baskin and Baskin 2014; Frattaroli et al. 2013).

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1 Temperature is one of the most important environmental conditions which control germination
2 (Garcia-Huidobro et al. 1982; Probert 2000); it determines also the fraction of germinated seeds in a
3 population and the rate at which they emerge (Heydecker 1977). In non-dormant seeds, the
4 germination response to accumulated temperature has been modelled by a thermal time (θ)
5 approach (Covell et al. 1986; Ellis et al. 1986; Hardegree 2006; Pritchard and Manger 1990). In this
6 model, seeds accumulate units of thermal time (°Cd) to germinate for each percentile *g,* of the
7 whole population. When the seeds are subjected to temperatures (T) above the base temperature for
8 germination (T_b), the germination rate increases linearly with temperature to an optimum
9 temperature (T_o), above which germination rate starts to decrease (Garcia-Huidobro et al. 1982).
10 Thus, in this sub-optimal range ($T_o - T_b$), germination occurs in the time t_g, when the thermal time
11 accumulated has reached the critical value (θ_g) for percentile g of the population, which can be
12 described as $\theta_g = (T - T_b)t_g$. Intra-specific variation in T_b among populations may be due to different
13 pre-harvest environmental conditions, and related to seed developmental heat sum (Daws et al.
14 2004). The thermal time approach has been used also to predict seed germination in the field (i.e.,
15 Hardegree and Van Vactor 2000; Steadman et al. 2003; Chantre et al. 2009). Recently, the impact
16 of different simulated climate change scenarios on seed dormancy release and germination timing
17 was investigated in *Vitis vinifera* subsp. *sylvestris* (Orrù et al. 2012), and used to model the *in situ*
18 natural regeneration patterns of *Rhamnus persicifolia* (Porceddu et al. 2013). However, to date there
19 are no specific studies on threshold temperatures and thermal time requirements for embryo growth
20 in morphophysiologically dormant (MPD) seeds. MPD is one of the least understood dormancy
21 classes, but was also proposed to be the ancient class of dormancy (Willis et al. 2014).
22 Seeds of Ranunculaceae species can exhibit morphological (MD) and MPD (Baskin and Baskin
23 1994, 2014; Cho et al. 2016; Walck et al. 1999;). *Aquilegia* sp. pl. seeds have linear underdeveloped
24 embryos (*sensu* Baskin and Baskin 2007) and stratification of the seeds at 3 – 5°C for two – four
25 weeks is recommended before sowing for germination (Ellis et al. 1985). Mattana et al. (2012b)

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1 reported MPD for *Aquilegia barbaricina* and *A. nugorensis*, which could be broken more efficiently
2 by a combination of warm and cold stratifications.

3 The aims of our investigations on *Aquilegia barbaricina* seeds were to: (1) identify the phases
4 of germination in MPD seeds; (2) individually characterise the thermal requirements for embryo
5 growth, dormancy release, and germination in MPD seeds; and (3) assess the intra-specific
6 variability on embryo growth and seed germination based on two distinct populations.
7

8 **Materials and Methods**

9 *Study species*

10 *Aquilegia barbaricina* Arrigoni & E.Nardi (Ranunculaceae) is a rhizomatous perennial herb that
11 branches underground and has stems 30-60 cm high (Arrigoni and Nardi 1977). The fruits are erect
12 capsules which produce dark trigonal seeds, each with a linear underdeveloped embryo. This
13 species is endemic to the Gennargentu and Supramontes regions of Central-Eastern Sardinia where
14 the plant grows from 800 to 1,400 m a.s.l. in wet woodlands, meadows and stream margins, mainly
15 occurring on siliceous substrates and secondarily on limestone ones (Garrido et al. 2012). The
16 plants flower and fruit centred on May and July, respectively. The species is included in the IUCN
17 Red Lists (http://www.iucnredlist.org), and it is classified as "Critically Endangered" (Fenu et al.
18 2011), and also as one of the 50 most endangered plants of the Mediterranean islands (de
19 Montmollin and Strahm 2005).
20

21 *Seed lot details*

22 Seeds of *A. barbaricina* were collected directly from plants in riparian woods of *Alnus glutinosa* at
23 the time of natural dispersal in early summer 2011 in two different populations in Central-Eastern

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1 Sardinia (Italy), specifically in Rio Correboi (RC; Villagrande Strisaili, OG) and in Rio Olai (RO;
2 Orgosolo, NU; see Table 1).
3
4 *Germination tests*

5 Three replicates of 20 seeds each per condition, were sown in July 2011, on the surface of 1% agar
6 water in 60-mm diameter plastic Petri dishes. Dishes were incubated in the light (12 h of irradiance)
7 at a range of different germination temperatures (10, 15, 20, 25 and 25⁄10°C). In the alternating
8 temperature regime, the light period coincided with the elevated temperature. Further replicates
9 were given a dormancy-breaking treatment consisting of warm (W = 25°C for three months)
10 followed by a cold stratification (C = 5°C for three months), before being incubated at the range of
11 germination temperatures (Table 2). This pre-treatment was chosen on the basis of the findings of
12 Mattana et al. (2012b). Three extra replicates of 20 seeds each were also sown on the surface of 1%
13 agar water with 250 $mg \cdot L^{-1}$ GA_3 and incubated in the light (12 h light) at the range of germination
14 temperatures.
15 Germination was defined as visible radicle emergence. Germinated seeds were scored three
16 times a week. During the germination tests, seeds with a split seed coat were scored, and the time
17 from seed coat splitting to endosperm rupture was monitored daily in 15 seeds for each treatment
18 and investigated population. Germination tests lasted for one to four months. When no additional
19 germination had occurred for two weeks, a cut-test was carried out to estimate the viability of the
20 remaining seeds; soft seed being considered to be non-viable. The final germination percentage was
21 calculated as the mean of three replicates (± 1 SD), on the basis of the total number of filled
22 potentially competent seeds.
23

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1 *Embryo measurements*

2 Embryo growth was assessed at different times, during the above described conditions and
3 germination temperatures by measuring 10 seeds at each sample interval (see Table 2). Seeds were
4 cut in half under a dissecting microscope and images of embryos acquired using a Zeiss SteREO
5 Discovery.V8, with an objective Achromat S 0.63x, FWD 107 mm (Carl Zeiss MicroImaging
6 GmbH) at 6.3× magnification, coupled to a Canon (Power shot G11) digital camera. Embryo and
7 seed lengths were measured using the image analysis software ImageJ 1.41o (National Institutes of
8 Health, Bethesda, MA, USA). Seed length was measured excluding the seed coat. The initial
9 embryo length was calculated by measuring 20 randomly selected seeds before the start of the
10 experiments. The embryo length of seeds with a split seed coat but no radicle protrusion (i.e.,
11 critical embryo length) was determined for 20 randomly selected seeds and used as a surrogate for
12 embryo length for seeds that had germinated before measurements (Vandelook et al. 2007).
13
14 *Thermal time analyses*

15 Thermal time studies were carried out for non-dormant seeds of both populations, germinating at
16 constant temperatures after W+C pre-treatment and with GA_3 treatment. Estimates of time (t_g, days)
17 taken for cumulative germination to reach different percentiles (g) for successive increments of
18 10% germination were interpolated from the germination progress curves (Covell et al. 1986).
19 Germination rate ($1/t_g$) was regressed, using a linear model, as a function of temperature according
20 to the following equation (Garcia-Huidobro et al. 1982):
21
22 $$1/t_g \text{ (days}^{-1}) = (T - T_{bg}) / \theta_g \qquad \text{(Eq. 1).}$$
23

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

10 Germination percentages were transformed to probits using tabular data from Finney (1971).
11 Linear regression was used to express probit(g) as a function of both θ_g and log θ_g for the
12 suboptimal temperature range for each seed lot and the best model evaluated on the basis of the r^2
13 values (Hardegree 2006). The following equation was used to describe the form of cumulative
14 germination response of seeds (Pritchard and Manger 1990):
15

16 $$\text{probit}(g) = \text{K} + \log \theta_g / \sigma \qquad \text{(Eq. 2)},$$

17

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

22 Thermal time modelling was also used to analyse embryo growth rate after W+C or GA_3
23 treatment (Figs 2, 3), to separate out growth rates pre- and post-dormancy release (Fig. 1).
24 Estimates of time (t_e, days) taken for different percentiles of seeds (e) to reach the critical embryo

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1 length were interpolated from the embryo growth progress curves. Embryo growth rate ($1/t_e$) was
2 regressed, using a linear model, as a function of temperature according to the modified equation 1:
3
4 for the sub-optimal range, $1/t_e$ (days^{-1}) = $(T - T_{be}) / \theta_{e1}$ (Eq. 3),
5 while for the supra-optimal range, $1/t_e$ (days^{-1}) = $(T_{ce} - T) / \theta_{e2}$ (Eq. 4).
6
7 An average (± 1 SD) of the *x*-intercept among percentiles was calculated for both sub-optimal and
8 supra-optimal temperature ranges, to establish the base temperature (T_{be}) and, when possible, the
9 ceiling temperature (T_{ce}) for embryo growth, respectively. The optimum temperature for embryo
10 growth (T_{oe}) was calculated as the intercept of sub- and supra-optimal temperatures response
11 functions. Thermal time (θ_e, °Cd) estimates were calculated separately as the inverse of the
12 regression equations. Linear regression equations were recalculated for each percentile, but
13 constrained to pass through T_{be}. Linear regression was used to express probit cumulative percentiles
14 of embryo growth (*e*) as a function of both θ_e and log θ_e and the best model evaluated on the basis
15 of the r^2. Equation 5 was used to describe the form of cumulative percentiles response of seeds to
16 reach the critical, internal embryo length for the sub-optimal temperature range:
17
18 $$\text{probit}(e) = K_1 + \log \theta_{e1} / \sigma_1 \quad \text{(Eq. 5)},$$
19
20 where K_1 is an intercept constant when thermal time (θ_{e1}) is zero and σ_1 is the standard deviation of
21 the response to log θ_{e1} (i.e., the reciprocal of the slope), and represents the sensitivity of the
22 population to θ_{e1}.
23

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Statistical analysis

Generalized 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* pairwise comparisons *t*-test (with Bonferroni adjustment). GLMs with a log link function and quasipoisson 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 quasibinomial error structure was used for analysing germination percentages. Quasipoisson and quasibinomial 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 overdispersion (Crawley 2007). All statistical analyses were carried out with R v. 2.14.0 (R Development Core Team 2011).

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Results

Embryo growth

GLM analysis detected no statistical significant differences ($P > 0.05$) between initial embryo length and final embryo length measured at the end of W+C, and ~~highlighted also no~~ 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 c. 0.040 mm for RO and c. 0.037 mm for RC, and after 180 days (i.e., at the end of the W+C) these values increased to c. 0.049 and c. 0.044 mm for RO and RC, respectively (Fig. 1). Although final values at the end of W+C were not statistically different ($P > 0.05$; by 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 and P < 0.0001 for RO; r^2 = 0.42 and P < 0.0001 for RC), highlighting a continuous and stable growth through W+C (Fig. 1).

GLM analysis highlighted ~~a~~ statistically significant differences (P < 0.001) on embryo lengths for the "treatment" factor, ~~while~~ 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 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 ~~the~~ 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 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 showed values similar to critical embryo length, while at the end of control test they were similar to initial embryo length (Fig. 2A).

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Testa and endosperm ruptures

Seeds exhibited a multi-step germination which 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 estimate 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 statistical significant differences (P > 0.05)

1 were detected for the interactions "population" × "temperature" and "treatment" × "temperature" ×
2 "population" (Table 3). After W+C treatment, the mean time from testa to endosperm rupture (i.e.,
3 radicle protrusion) decreased with increasing temperature, ranging from just over 6 days at 10°C to
4 just under 2 days at 20°C for RO and RC populations (Fig. 2B). At 25°C and at 25/10°C this time
5 interval was around 4 days and 2 days, respectively, for both RO and RC populations (Fig. 2B). In
6 contrast, the time from testa to endosperm rupture in the GA_3 treatment was slower than after the
7 W+C treatment (Fig. 2B). This was most evident in seeds of RC population, with intervals of 22
8 days at 10°C and c. 5 days at 25°C (Fig. 2B).
9
10 *Seed germination*
11
12 GLM highlighted statistical effects ($P < 0.05$) on seed germination for all applied factors, as
13 well as for all their interactions (Table 3). While no seeds germinated during the control (0) and
14 very low percentages (ca. 1% for RO and ca. 6% in RC) were detected at the end of C during W+C
15 pre-treatment, seeds germinated to > 50% both after W+C and during GA_3 treatments in each
16 population (Fig. 2C). Statistically significant differences ($P < 0.001$) among temperatures were
17 detected within each treatment, except for seeds of RC treated with GA_3 ($P > 0.05$) where the
18 germination range was from ca. 52% (at 10°C) to ca. 80% (at 25°C; Fig. 2C). GA_3 treated seeds of
19 the RO population germinated from c. 12% (at 10°C) to c. 62% (at 20°C; Fig. 2C). After W+C,
20 high germination was observed at 25°C (88 ± 6%) for RO, and at 15°C (81 ± 12%) for RC (Fig.
21 2C).
22
23 *Thermal time approach on embryo growth*
24 GLM analysis (Table 3) did not show statistically significant differences ($P > 0.05$) on embryo
25 growth between populations; therefore a combined population response dataset was used to evaluate

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1 embryo thermal requirements, ascribing this characteristic to the species level. Seeds germinated
2 after W+C and during GA_3 treatments showed differences on both critical embryo length rate ($1/t_e$)
3 and cardinal temperatures (Fig. 3). Based on embryo length rate responses for each 10th percentile
4 (from 10 to 90%) of seeds that reached the critical embryo length, it was possible to estimate the
5 mean base temperature (T_{be}) in the sub-optimal temperature range for W+C and GA_3, and the mean
6 ceiling temperature (T_{ce}) in the supra-optimal temperature range, and subsequently the optimal
7 temperature for embryo growth (T_{oe}) for W+C (Fig. 3). Linear regressions for the different
8 percentiles of sub-optimal temperature range for W+C were calculated passing through 5°C, which
9 corresponds to an embryo growth rate equal to 0, value obtained at the end the W+C pre-treatment
10 (see Fig. 1). The obtained regression lines were then constrained to pass through the common value
11 of T_{be}. For the supra-optimal temperature range, linear regressions were constrained to pass through
12 the common value of T_{ce}. Linear regressions for the different percentiles for GA_3 were constrained
13 to the common value of T_{be}. These models showed higher values of r^2 for all of the linear regression
14 equations than the model where T_{be} and T_{ce} varied for each percentile. Average T_{be} were 5.20 ± 0.60
15 and 5.30 ± 2.56°C for W+C and GA_3 treatments, respectively (Fig. 3), without statistically
16 significant differences among treatments ($P > 0.05$). Average T_{ce} for W+C was 29.52 ± 2.37°C, and
17 the average T_{oe} was 15.00 ± 1.02°C (Fig. 3) whereas in GA_3 treatment T_{oe} may be assumed as ≥
18 25°C (Fig. 3).

19 Figure 4 shows the relationship between log thermal time (θ_e) and percentages of seeds that
20 reached the critical embryo length expressed in probits, calculated according to Eq. 5. The
21 relationship between log θ_e and probit critical embryo length had better residual sums of square
22 (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
23 when expressed on a linear scale (data not shown). Thermal time required for 50% of seeds to reach
24 the critical embryo length (θ_{e50}) was greater for the GA_3 with value of 2.64 log °Cd compared to the

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1 W+C treated seeds with value of 2.10 log °Cd. However, seed of W+C and GA_3 that reach the
2 critical embryo length showed a very similar σ value (0.51 and 0.43°Cd, respectively; Fig. 4).
3

4 *Thermal time approach on seed germination*

5 The T_{bg} for RO population were 6.85 ± 0.26°C for W+C and 8.43 ± 1.53°C for GA_3 treatment,
6 while for RC population were 5.34 ± 1.38 and 5.42 ± 0.26°C for W+C and GA_3 treatment,
7 respectively (Fig. 5). These values were statistically different ($P < 0.01$) by GLM and a *post-hoc*
8 pairwise comparisons *t*-test highlighted that this difference was determined by the T_{bg} value of GA_3
9 treated seeds belonging to RO population (Fig. 5). For each treatment on both populations, the
10 linear regressions were re-calculated for each percentile, constraining them to pass through the
11 mean T_{bg} (Fig. 5). This model led to no differences in residual sum of squares compared with when
12 T_{bg} was allowed to vary for each percentile, and showed highest values of r^2 for all of the linear
13 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 $r^2 >$
14 0.57 for RC GA_3).

15 Figure 6 shows the relationship between log thermal time (θ_g) and germination expressed in
16 probits, calculated according to Eq. 2. The relationship between log θ_g and probit germination had
17 better residual sums of square both in W+C pre-treated seeds (0.1349 and 0.1851 for RO and RC
18 populations, respectively) and in the GA_3 treated seeds (0.0098 and 0.1477 for RO and RC
19 populations, respectively) than when expressed on a linear scale (data not shown). Thermal time
20 required for 50% of germination (θ_{g50}) was greater for the GA_3 treated seeds (2.88 and 2.72 log °Cd
21 for RO and RC, respectively), compared to the W+C pre-treated seeds (2.04 and 2.02 log °Cd for
22 RC and RO, respectively; Fig. 6). In addition, GA_3 treated seeds of RO had a greater σ value (0.45
23 log °Cd) than the seeds belonging to RC population (0.33 log °Cd) and of those W+C pre-treated
24 seeds (0.38 log °Cd and 0.26 log °Cd for RC and RO populations, respectively; Fig. 6).

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1

2 **Discussion**

3 *Seed dormancy and multiple phases to the completion of seed germination*

4 The embryo in seeds of *A. barbaricina* is small at dispersal (0.03 mm long) and must grow before
5 radicle emergence. Therefore, these seeds would be classified as morphologically dormant (MD)
6 following the Baskin and Baskin (2014) dormancy classification system. Generally, if embryos
7 have only MD, growth is completed in a relatively short period, and seeds germinate within about 4
8 weeks (Baskin and Baskin 2014). *A. barbaricina* seeds of each population did not germinate
9 without any treatment, even after 120 days. Nonetheless, embryos in seeds subjected to W+C
10 (25°C, followed by 5°C) grew internally by about 50% to c. 0.04 – 0.05 mm (Fig. 1). The rate of
11 change in embryo length was about 0.00009 mm d^{-1}. After warm followed by cold stratification or
12 GA_3 treatment, seeds started to germinate (radicles emerged) at all tested temperature, due to a
13 rapid increase in embryo growth to c. 0.12 mm. This second phase of internal growth of the embryo
14 is about 800× faster (c. 0.07 mm d^{-1}) than during W+C treatment. This suggests two distinctly
15 different physiological responses of the embryo during treatment.

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

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

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

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

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1

2 *Thermal thresholds for embryo growth and seed germination*

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

13 Constraining the linear regressions of each percentile for germination through the mean T_b
14 resulted in an improvement of the residual sum of squares and r^2 values. Therefore, T_b for embryo
15 growth and for germination can be used to describe the whole population response in *A.*
16 *barbaricina* seeds, as previously reported for other species (e.g., Covell et al. 1986; Ellis et al.
17 1987; Pritchard and Manger 1990; Orrù et al. 2012; Porceddu et al. 2013). The best model was
18 obtained by fitting germination expressed in probit and log-normal (log °Cd) rather than normal
19 distributed thermal times (°Cd), as previously reported for other herbaceous (Covell et al. 1986;
20 Ellis and Butcher 1988) and woody species (Pritchard and Manger 1990; Porceddu et al. 2013).
21 Also, regarding the thermal times of embryo growth rate, the best model was obtained by fitting the
22 values in probit and log-normal (log °Cd) compared to when normal distributed, confirming that
23 this methodology increases the goodness of the model.

24 Seeds of *A. barbaricina* varied in their thermal time estimates to reach θ_{50}, depending on
25 treatment. W+C pre-treated seeds had the lowest θ_{50} values (2.10 log °Cd; i.e., 128 °Cd) for embryo

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1 growth compared to GA_3 treated seeds (2.64 log °Cd; i.e., c. 440 °Cd). The same trend was detected
2 also for germination (radicle emergence) with θ_{50} values of 2.03 log °Cd (110 °Cd) for W+C
3 stratified seeds and c. 2.80 log °Cd (c. 650 °Cd) for GA_3 treated RC and RO seeds. The 4- to 6-fold
4 longer thermal times for embryo growth (θ_{e50}) and germination (θ_{g50}) in GA_3- compared to W+C-
5 treated seeds suggests that GA_3 treatment only partially mimicks / replaces the W+C pre-treatment
6 in seeds of this species.

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

15 The analysis carried out in this study showed that in *A. barbaricina* the thermal requirements
16 for embryo growth did not vary among populations, while for seed germination these were different
17 between populations. Embryo growth could be strictly related to the seeds biology of the species,
18 while germination could be more related to the habitat of provenance of the species. Copete et al.
19 (2014) reported that in seeds of *Narcissus eugeniae* (Amaryllidaceae) belonging to two different
20 populations and tested in both near-natural and laboratory conditions, the embryo growth showed a
21 similar pattern, while radicle emergence did not begin simultaneously. Intra-specific germination
22 differences among populations of a species can arise due several factors, such as light, moisture and
23 temperature (Gutterman 1992; Fenner and Thompson 2005) and can be interpreted as being an
24 adaptation to specific habitat (Meyer et al. 1995, 1997) as detected in this study for *A. barbaricina*.
25

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1 *Conclusions*

2 The results indicate that *A. barbaricina* is characterized by a multi-step seed germination. The slow
3 embryo growth during W+C treatment reflects the continuation of the maternal programme of
4 development that was punctuated by seed dispersal, whilst the thermal kinetics of both embryo
5 growth and radicle protrusion after the removal of physiological dormancy are distinctly different.
6 The thermal time model developed in this work allowed to identify the thermal thresholds (T_b and
7 θ_{50}) requirements of embryo growth and seed germination of this species. The beneficial effects of
8 W+C treatment on the multiphasic germination response in MPD seeds is only partially mimicked
9 by 250 $mg \cdot L^{-1}$ GA_3 treatment, having a similar controlling influence on base temperature for
10 embryo growth and germination but not on rate processes. This attempt to model thermal
11 requirement for embryo growth using a thermal time approach was confirmed by the morphological
12 observations. This model could be applied in those species whose seeds have also a morphological
13 component of dormancy (MD and MPD), could be a valid tool to model thermal kinetics of embryo
14 growth and radicle protrusion, and may be also useful to predict seedling emergence in the field.
15

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1 **Tables:**

2 Table 1 – Population data and seed lot details.

Locality	Population code	Region	Geographical 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

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1 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 $mg \cdot L^{-1}$) in the germination medium	5	After 15, 30, 60, 90 and 120 days

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

Embryo length (mm)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
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)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
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 (%)	**Df**	**Deviance**	**Resid. Df**	**Resid. Dev**	***F***	***P* (>F)**
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 ***

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1 **Figure captions:**

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4 Figure 1 – Embryo growth trend during stratification at 25°C for 3 months (W) and then at 5°C for
5 another 3 months (C) for seeds collected in Rio Correboi (RC) and Rio Olai (RO) populations.
6 Initial and final embryo lengths measured at the end of W and C pre-treatments, are not
7 significantly different at $P > 0.05$ with GLM, as well as between populations. Data are the mean (±
8 SD) of 20 seeds for initial embryo length and of 10 seeds for each subsequent measurement.

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2 Figure 2 – Final embryo length values (A), time from seed coat splitting to endosperm rupture (B)
3 and cumulative germination percentages (C) achieved at the end of germination tests (120 days),
4 after each pre-treatment (0, control; W+C, 25°C for 3 months and then 5°C for another 3 months;
5 GA_3, 250 mg L^{-1} in the germination substrate) for each population (Rio Olai and Rio Correboi).
6 Embryo lengths measured at the start of germination tests (initial embryo length) are reported as a
7 reference for the control and GA_3 while the value assessed at the end of pre-treatment is reported
8 for W+C (black circles; A). The results in the alternating temperature regime (25/10°C) are here
9 highlighted with a grey diamonds (A and B) and grey coarse bar (C). Data are the mean of 10 seeds
10 (± SD) for embryo measurements, 20 (± SD) seeds (when available) for endosperm rupture rate and
11 3 replicates (± SD) of 20 seeds each for germination data. Dash lines (A) correspond to the critical
12 embryo length. General linear models (GLMs) were carried out within each treatment to test the
13 effect of temperature on embryo growth, rate of endosperm rupture and germination. Values with
14 the same letter are not different at $P > 0.05$ by *post hoc* pairwise *t*-test comparisons (with
15 Bonferroni adjustment).

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2 Figure 3 - Cardinal temperatures (T_{be}, base temperature, T_{oe}, optimal temperature and T_{ce}, ceiling
3 temperature) to reach critical embryo length for seeds of *Aquilegia barbaricina*, calculated after
4 W+C (25°C for 3 months and then 5°C for another 3 months) and incubated at a different range of
5 germination temperatures (10, 15, 20 and 25°C), and T_{be} calculated after GA_3 (250 mg L^{-1} in the
6 germination substrate) treatment and incubated at constant temperatures in the suboptimal range (≤
7 25°C). Linear regressions for the different percentiles of sub-optimal temperature range for W+C
8 were calculated passing through 5°C, which corresponds to an embryo growth rate equal to 0, value
9 obtained at the end of the W+C pre-treatment (see figure 1), and after were constrained to pass
10 through the common value of T_{bg}; for the supra-optimal temperature range, linear regressions were
11 constrained to pass through the common value of T_{ce}. Linear regressions for the different percentiles
12 for GA_3 were constrained to the common value of T_{bg}. Percentiles for which regression lines had a
13 $P > 0.05$, T_{bg} and T_{ce} values were not calculated.

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2 Figure 4 - Probit percentages of seeds of *Aquilegia barbaricina* that reached the critical embryo

3 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}

4 in the germination substrate) treatments as a function of log thermal time requirement (log θ_e).

5 Thermal times were calculated from critical embryo length time-courses assuming T_b of 5.20 and

6 5.30°C, for W+C and GA_3, respectively. Thermal times to reach 50% of seeds that reached the

7 critical embryo length (θ_{e50}) are also reported.

8

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

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

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

For Peer Review

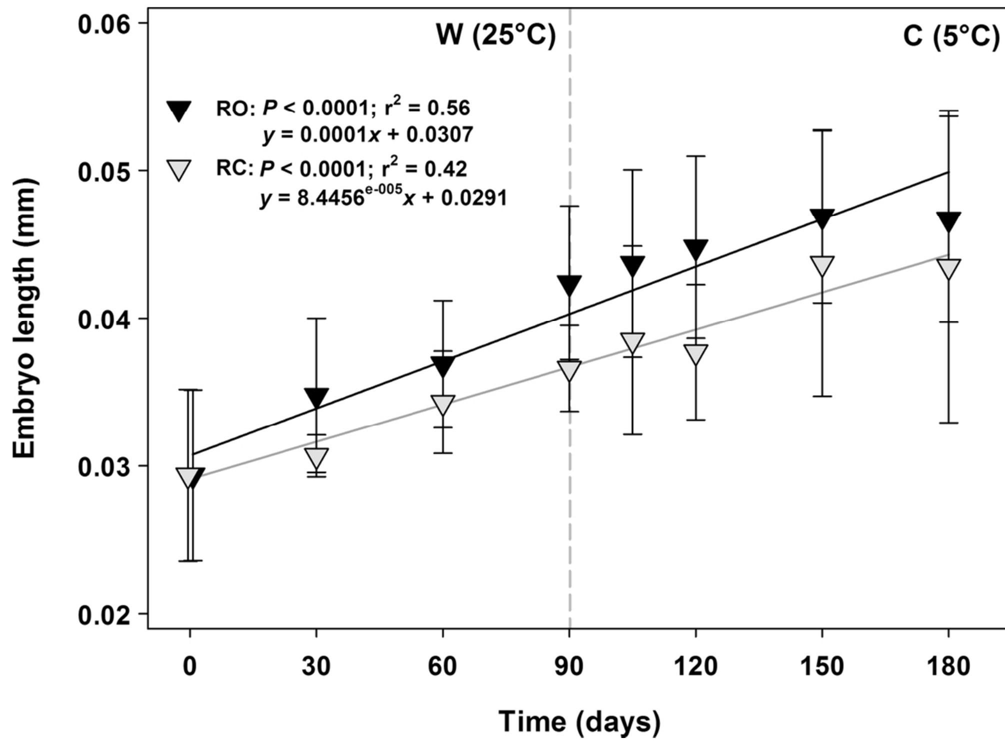

Fig. 1 – Embryo growth trend during stratification at 25°C for 3 months (W) and then at 5°C for another 3 months (C) for seeds collected in Rio Correboi (RC) and Rio Olai (RO) populations. Initial and final embryo lengths measured at the end of W and C pre-treatments, are not significantly different at $P > 0.05$ with GLM, as well as between populations. Data are the mean (± SD) of 20 seeds for initial embryo length and of 10 seeds for each subsequent measurement.

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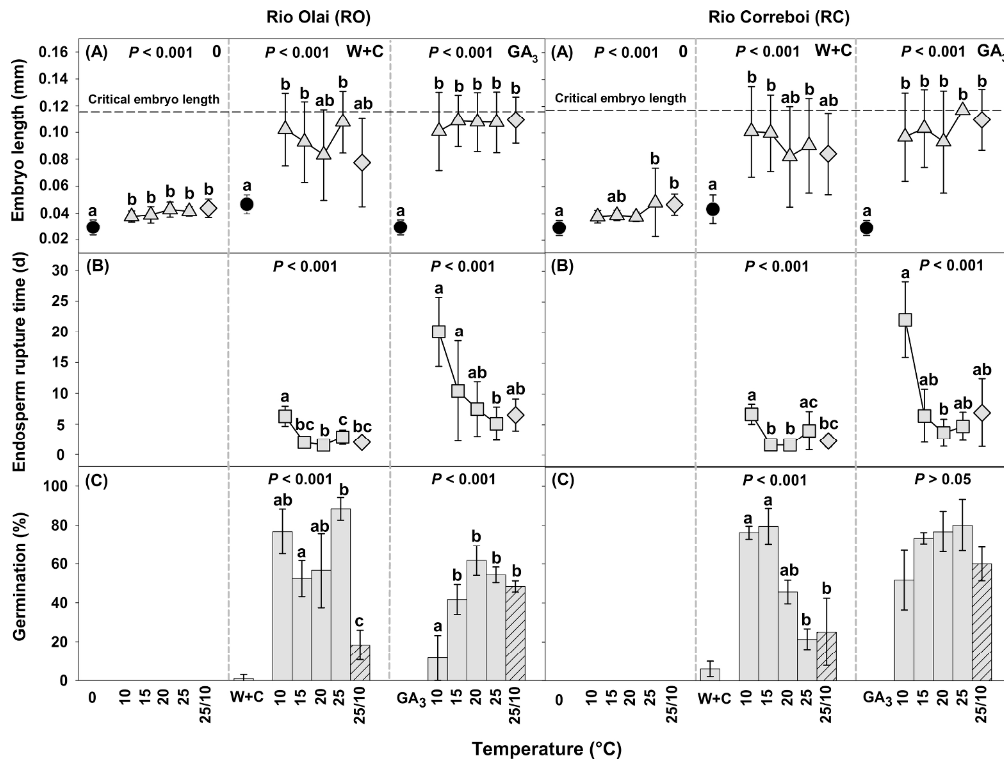

Fig. 2 – Final embryo length values (A), time from seed coat splitting to endosperm rupture (B) and cumulative germination percentages (C) achieved at the end of germination tests (120 days), after each pre-treatment (0, control; W+C, 25°C for 3 months and then 5°C for another 3 months; GA_3, 250 mg 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 a grey diamonds (A and B) and grey coarse bar (C). Data are the mean of 10 seeds (± SD) for embryo measurements, 20 (± SD) seeds (when available) for endosperm rupture rate and 3 replicates (± SD) of 20 seeds each for germination data. Dash lines (A) correspond to the critical embryo length. General linear models (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 pairwise t-test comparisons (with Bonferroni adjustment).

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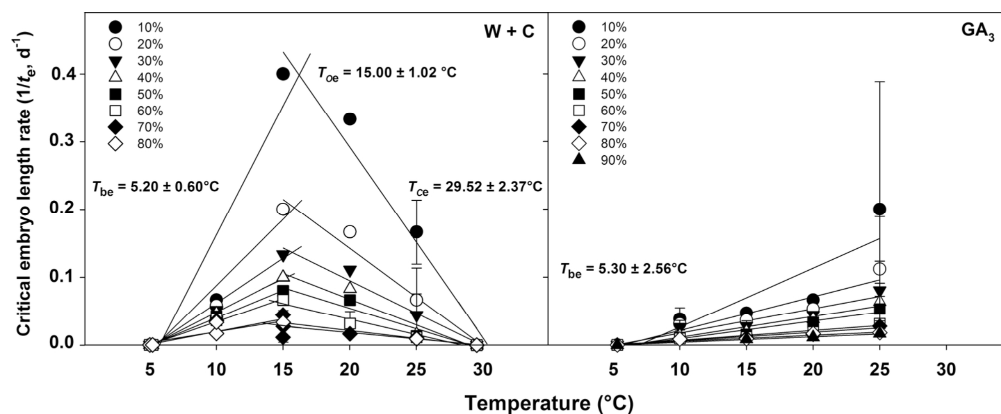

Fig. 3 - Cardinal temperatures (T_{be}, base temperature, T_{oe}, optimal temperature and T_{ce}, ceiling temperature) to reach critical embryo length for seeds of *Aquilegia barbaricina*, calculated after W+C (25°C for 3 months and then 5°C for another 3 months) and incubated at a different range of 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 sub-optimal 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 figure 1), and after were 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.

169x68mm (300 x 300 DPI)

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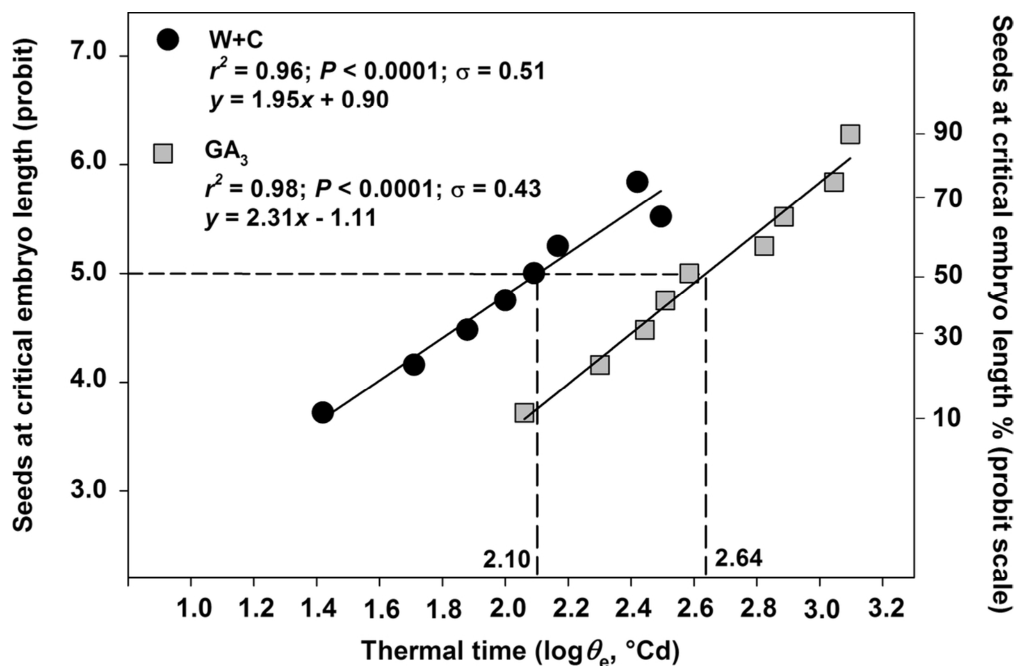

Fig. 4 - Probit percentages of seeds of *Aquilegia barbaricina* that reached the critical embryo length after W+C (25°C for 3 months and then 5°C for another 3 months) and after GA_3 (250 mg L^{-1} in the germination substrate) treatments as a function of log thermal time requirement (log θ_e). Thermal times were calculated from critical embryo length time-courses assuming T_b of 5.20 and 5.30°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.

104x68mm (300 x 300 DPI)

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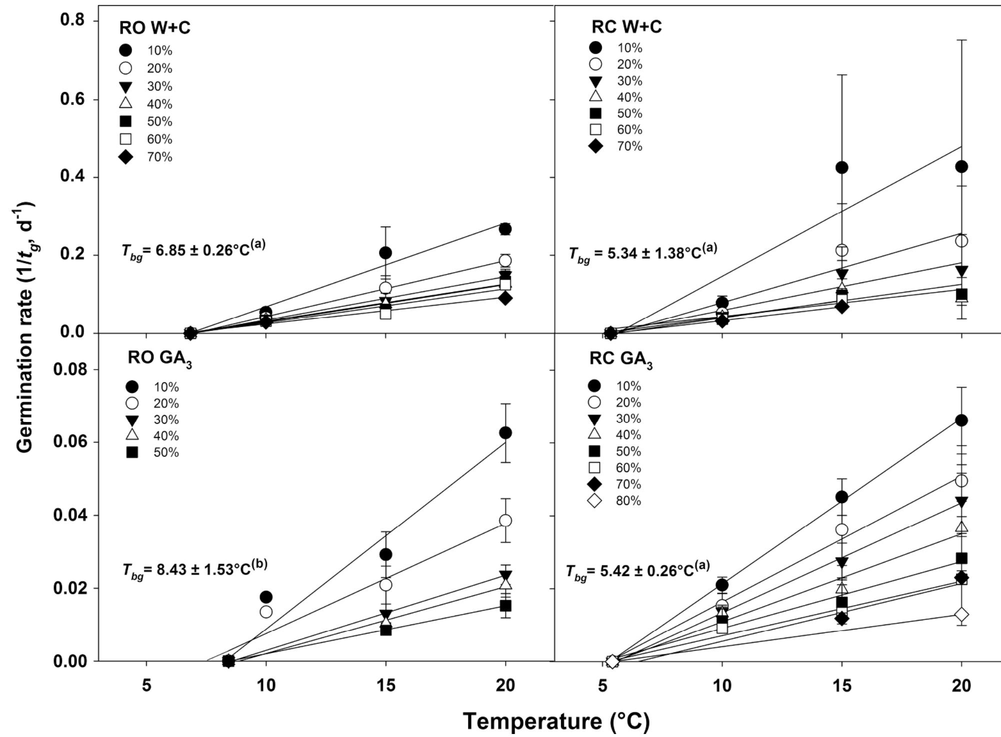

Fig. 5 - Base temperatures for germination (T_{bg}) for the two populations (RO, Rio Olai; RC, Rio Correboi) of *Aquilegia barbaricina*, calculated after W+C (25°C for 3 months and then 5°C for another 3 months) and GA_3 (250 mg L^{-1} in the germination substrate) treatments, and incubated at constant 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.

227x165mm (300 x 300 DPI)

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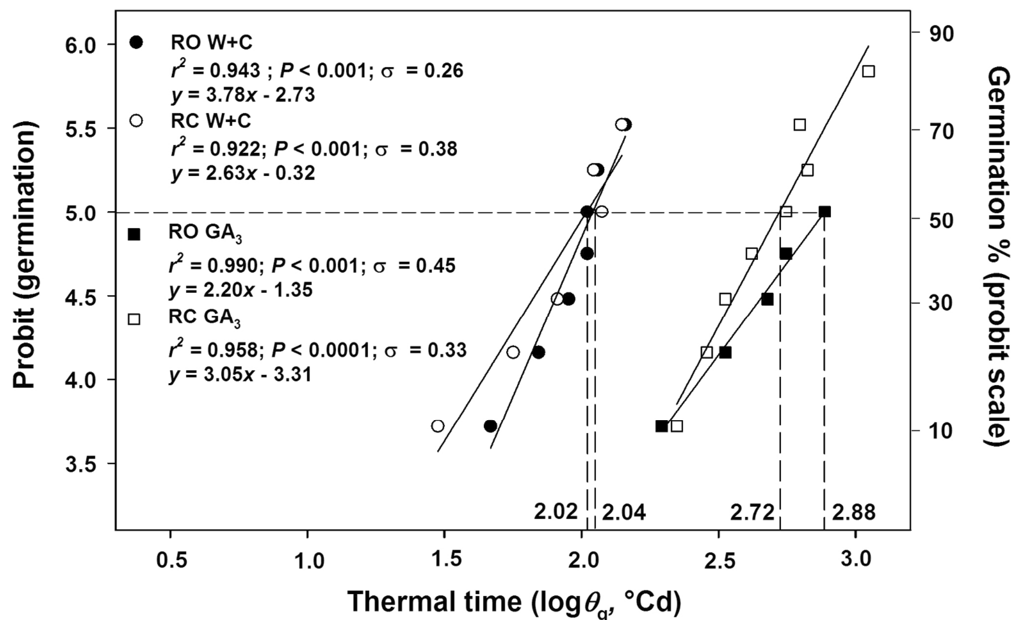

Fig. 6 – Probit germination after W+C (25°C for 3 months and then 5°C for another 3 months) and after GA_3 (250 mg L^{-1} in the germination substrate) treatments for each population (RO, Rio Olai; RC, Rio Correboi) as a function of log thermal time requirement (log θ_q). Thermal times were calculated 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% of germination (θ_{q50}) 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_{q50}) are also reported.

172x107mm (300 x 300 DPI)

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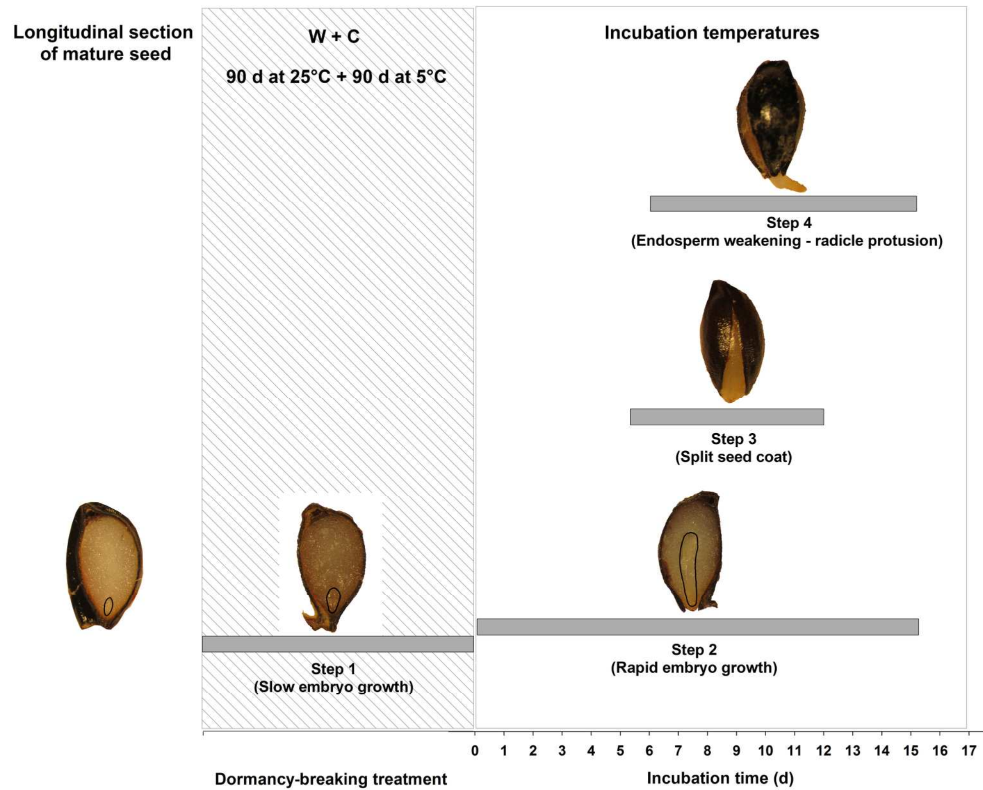

Fig. 7 – Growth phases in *Aquilegia barbaricina* seeds: slow embryo growth (step 1) during W+C treatment (25°C for 3 months and then 5°C for another 3 months), and interval of time (in days) to complete the rapid embryo growth (step 2), split seed coat (step 3) and endosperm weakening – radicle protrusion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

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