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Sequential temperature control of multi-phasic dormancy release and germination of *Paeonia corsica* seeds

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Abstract

Aims
The physiological responses during dormancy removal and multi-phasic germination were investigated in seeds of *Paeonia corsica* (Paeoniaceae).

Methods
Seeds of *P. corsica* were incubated in the light at a range of temperatures (10–25 and 25/10°C), without any pre-treatment, after W (3 months at 25°C), C (3 months at 5°C) and W + C (3 months at 25°C followed by 3 months at 5°C) stratification, and a GA_3 treatment (250 mg·l^{-1} in the germination substrate). Embryo growth, time from testa to endosperm rupture and radicle emergence were assessed as separate phases. Epicotyl–plumule emergence was evaluated incubating the germinated seeds at 15°C for 2 weeks, at 5 and 25°C for 2 months on agar water before transplanting to the soil substrate at 10, 15 and 20°C and at 15°C for 2 months on the surface agar water with GA_3.

Important Findings
Embryo growth, testa rupture, endosperm rupture (radicle emergence) and growth of the epicotyl were identified as four sequential steps in seeds of *P. corsica*. Gibberellic acid alone and warm stratification followed by 15°C promoted embryo growth and subsequent seed germination. Cold stratification induced secondary dormancy, even when applied after warm stratification. After radicle emergence, epicotyl–plumule emergence was delayed for ca. 3 months. Mean time of epicotyl–plumule emergence was positively affected by cold stratification (2 months at 5°C) and GA_3. *P. corsica* seeds exhibited differential temperature sensitivity for the four sequential steps in the removal of dormancy and germination processes that resulted in the precise and optimal timing of seedling emergence.

Keywords: cold/warm stratification, embryo growth, epicotyl dormancy, seed dormancy, Paeoniaceae

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INTRODUCTION

Seed dormancy is an important adaptation to prevent germination when the environment does not remain favourable long enough for seedlings to become established and thus survive (Baskin and Baskin 2014). The process of germination in non-dormant seeds starts when the dry seeds come into contact with water and ends when the radicle has emerged through all the coats enveloping the embryo (Finch-Savage and Leubner-Metzger 2006; Weitbrecht *et al.* 2011). Testa and endosperm rupture have been identified as two sequential steps during germination (Hepher and Roberts 1985; Krock *et al.* 2002; Liu *et al.* 2005; Linkies *et al.* 2009; Müller *et al.* 2006). In many plant species, the seed-covering layers impose a physical constraint to radicle protrusion, which has to be overcome by the growth potential of the embryo (Kucera *et al.* 2005; Ma *et al.* 2010; Muller *et al.* 2006), as, e.g. in *Paeonia ostii* T. Hong & J. X. Zhang var. *lishizhenii* B. A. Shen (Wang and van

Staden 2002). Two-step germination (i.e. visible distinction between testa rupture and endosperm rupture) is widespread over the entire phylogenetic tree and has been described for many plant families, including Ranunculaceae (Hepher and Roberts 1985), Amaranthaceae (Karssen 1976), Solanaceae (Krock *et al.* 2002; Petruzzelli *et al.* 2003) and Brassicaceae (Liu *et al.* 2005; Muller *et al.* 2006). In seeds with underdeveloped embryos, if embryo growth and radicle emergence are completed in about 4 weeks under suitable conditions, seeds are suggested to have morphological dormancy (MD); if germination is delayed and seeds require a dormancy-breaking treatment such as exposure to moist cold (0–10°C) and/or to moist warm (≥15°C) stratification to enable germination, they are reported to have morphophysiological dormancy (MPD; Baskin and Baskin 2014; Nikolaeva 1977). Nine types of MPD have been defined, based on cold and/or warm stratification requirements for germination, temperature requirements (warm vs. cold) for embryo growth, timing of root and shoot emergence and response to gibberellic acid (GA) (Baskin and Baskin 2014).

Abscisic acid (ABA) and GA play an important role in a number of physiological processes during seed germination. ABA induces dormancy while GA plays a key role on dormancy release and germination (Finch-Savage and Leubner-Metzger 2006; Pérez-García *et al.* 2006). High ABA:GA ratio maintains dormancy, while dormancy release involves a net shift to increased GA biosynthesis and ABA degradation resulting in low ABA:GA ratio (Ali-Rachedi *et al.* 2004; Cadman *et al.* 2006). These two hormones may act also in the promotion of testa and endosperm rupture (Finch-Savage and Leubner-Metzger 2006). In *Lepidium sativum* L. and *Arabidopsis thaliana* (L.) Heynh. endosperm rupture is promoted by GA and inhibited by ABA and endosperm weakening is known to be promoted by GA and inhibited by ABA (Müller *et al.* 2006). However, in some species, as in *Leymus chinensis* (Trin.) Tzvel., dormancy release and seed germination appear not significantly affected by hormones (Ma *et al.* 2010).

According to Martin (1946) and Baskin and Baskin (2014), Paeoniaceae have seeds with rudimentary embryos, thus they need to grow before the seed germinates. The seeds of most *Paeonia* spp. are difficult to germinate and may need treatments to promote internal embryo growth, radicle and epicotyl emergence; several studies reported that seeds exhibit embryo dormancy, and a long delay may be observed between the growth of the radicle and subsequent growth of the epicotyl (Barton and Chandler 1957; Jing and Zheng 1999; Wang and van Staden 2002). It's known that dormancy is a major obstacle to the germination of peony. In this way, seed dormancy has been studied in different species of Paeoniaceae, and all of them are assumed to have MPD (Barton 1933; Nikolaeva *et al.* 1985; Wang and van Staden 2002). Saunders (1918) observed that epicotyl growth was delayed until after the radicle emerged from *Paeonia suffruticosa* Andrews seeds, and Barton (1933) found that epicotyl dormancy of seeds of this species could be broken by exposing germinated seeds to temperature of ca. 5°C. Deep simple epicotyl MPD has been found in many *Paeonia* species, e.g. in *P. albiflora* Pall., *P. anomala* L., *P. intermedia* C.A.Mey., *P. spontanea* (Rehder) T.Hong and W.Z.Zhao and in *P. ostii* var. *lishizhenii* (Jing and Zheng 1999; Nikolaeva *et al.* 1985; Wang and van Staden 2002) and non-deep simple MPD in *P. californica* Nutt. (Schlising 1976). More recently, Hao *et al.* (2014) correlated root length and epicotyl–plumule germination in *P. ludlowii* (Stern & G. Taylor) D.Y.Hong seeds, such that root lengths ≥ 6 cm were essential for epicotyl dormancy release by cold stratification.

The taxonomy of the genus *Paeonia* in tyrrhenian islands is extremely controversial and unclear, and currently only *Paeonia corsica* Sieber ex Tausch is reported for Sardinia (Bacchetta *et al.* 2012; Hong 2005; Hong and Wang 2006). *P. corsica* is a geophyte entirely glabrous, very occasionally pubescent on the lower surface of leaves. This species is characterized by lower leaves mostly holosericeous, usually with nine leaflets, and flowers opened wide with slightly concave petals, generally pink, varying from pinkish-white to red (Hong and Wang 2006). These features, made this wild species of potential horticultural interest. To date, there is no information on seed dormancy and germination strategies for *P. corsica*.

The main aims of this study were to: (i) evaluate the effect of warm/cold stratification and GA_3 on the different phases of embryo growth and seed germination, (ii) identify the presence of various sequential steps during germination and (iii) characterize the physiological responses to the germination ecology of *P. corsica*.

MATERIALS AND METHODS

Study species and seedlot details

In Sardinia, *P. corsica* grows from 400 to 1700 m a.s.l. in different geological substrates (sedimentary, volcanic and metamorphic rocks preferably in deep, rich and wet soils). Flowering occurs from late March to early May, and fruiting from about early September to October.

Seeds of *P. corsica* were collected directly from plants near and under riparian woods of *Alnus glutinosa* (L.) Gaertn. with *Taxus baccata* L. and *Rhamnus persicifolia* Moris at the time of natural dispersal in September 2011, at ca. 1200 m a.s.l. along the Rio Correboi (Villagrande Strisaili, OG) in CE Sardinia (Italy). Average seed mass, calculated by weighing 10 replicates of 20 seeds each, was 88.08 ± 14.60 mg.

Experimental trials

As different developmental steps on the seed germination of this species were identified during this study, embryo growth, time from testa to endosperm rupture, radicle and epicotyl–plumule emergence were recorded and scored as separate phases.

Embryo measurements

During and simultaneously with the below described conditions and germination temperatures (see 'Germination test'

section) started in September 2011, embryo growth was assessed at different times (Table 1) by measuring 10 seeds for each sample interval. The seeds were sown on the surface of 1% agar water in 90-mm diameter plastic Petri dishes, and the replicates for each condition correspond to the number of measurements reported in Table 1. 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 107mm (Carl Zeiss MicroImaging GmbH) at 5.0× magnification, coupled to a Canon (Power shot G11) digital camera. Embryo (E) and seed (S) lengths were measured using the image analysis software ImageJ 1.41o (National Institutes of Health, Bethesda, MA, USA). Seed length was measured excluding the thickness of the seed coat and the embryo to seed length (E:S) ratio calculated for each seed. The initial E:S ratio was calculated by measuring 20 randomly selected fresh seeds before the start of the experiments. The critical E:S ratio of seeds with a split seed coat but no radicle protrusion (i.e. when the endosperm was exposed) was determined for 20 randomly selected seeds and used for seeds that had germinated before measurements (Vandelook *et al.* 2007).

Germination test

Three replicates of 20 seeds each per condition (Table 1), were sown on the surface of 1% agar water in 90-mm diameter plastic Petri dishes and incubated in the light (12 h light/12 h dark) at a range of germination temperatures (10, 15, 20, 25 and 25/10°C) for a maximum of 120 days. In the alternating temperature regime, the 12-h light period coincided with the elevated temperature period. Light was provided by white fluorescent lamps (FL40SS.W/37 70–10 µmol m^{-2} s^{-1}). Further replicates were given a warm (W = 25°C for 3 months) and a cold stratification (C = 5°C for 3 months) and a combination of them (W + C), before being incubated at the range of germination temperatures (Table 1). Three extra replicates of 20 seeds each were sown on the surface of 1% agar water with 250 mg·l^{-1} GA_3 and incubated at the range of germination temperatures (Table 1).

Endosperm rupture by radicle emergence

During germination tests, seeds with a split seed coat were scored, and the time from seed coat splitting to endosperm rupture (i.e. when the radicle emerges) was monitored in 15 seeds for each condition. Germination was defined as visible radicle emergence. Germinated seeds were scored three times a week. 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. The final germination percentage was calculated as the mean of three replicates (± 1 SD), on the basis of the total number of firm seeds.

Epicotyl dormancy release

To evaluate the delay of the epicotyl–plumule germination (i.e. when the epicotyl or the first true leaf was emerged) after radicle protrusion in seeds of *P. corsica*, a warm pretreatment (i.e. W = 3 months at 25°C on the surface of 1% agar water, see Table 1), was applied in March 2012 to 200 seeds before incubation for germination at 15°C. Germinated seeds were then: (i) kept at 15°C on agar water for an additional 2 weeks period in order to allow root growth, before transplanting to a sterilized soil substrate of sand/soil/peat (1:1:1) at 10, 15 and 20°C; (ii) moved to 5°C for 2 months on agar water, before transplanting to the soil substrate at 10, 15 and 20°C; (iii) moved to 25°C for 2 months on agar water, before transplanting to the soil substrate at 10, 15 and 20°C and (iv) kept at 15°C for 2 months on the surface of 1% agar water with GA_3 (250 mg l^{-1} in the germination substrate). For each condition, 15 germinated seeds were used and seedlings planted in separate pots. Epicotyl–plumule germination was scored twice per week. The mean time to epicotyl–plumule emergence for each condition was calculated on the basis of the total number of seedlings with the epicotyl–plumule emerged. When no additional radicle or epicotyl–plumule germination occurred for 2 weeks, after a minimum of 4 months, both experiments were stopped.

Table 1: experimental design

Condition		Embryo growth measurements	
Code	Description	Number of measurements	Timing
0	Control	5	After 15, 30, 60, 90 and 120 days at 10–25°C and 25/10°C
W	3 months, 25°C	8	After 30, 60 and 90 days during warm stratification (W), 15, 30, 60, 90 and 120 days after sowing for germination at 10–25°C and 25/10°C
C	3 months, 5°C	8	After 30, 60 and 90 days during cold stratification (C), 15, 30, 60, 90 and 120 days after sowing for germination at 10–25 and 25/10°C
W + C	3 months, 25°C (W) → 3 months, 5°C (C)	12	After 30, 60 and 90 days during warm (W), 15, 30, 60 and 90 days during cold (C), 15, 30, 60, 90 and 120 days after sowing for germination at 10–25°C and 25/10°C
GA_3	GA_3 (250 mg·l^{-1}) in germination medium	5	After 15, 30, 60, 90 and 120 days at 10–25°C and 25/10°C

Statistical analysis

Generalized linear models (GLMs) were used to evaluate the effect of pre-treatments (i.e. 0, W, C, W + C and GA_3) on embryo growth rate, E:S ratio and rate of endosperm rupture event. The GLM for final seed germination percentages was unbalanced due to many 0 values and was not carried out. The effect of incubation temperature within each pre-treatment was also assessed by GLM for embryo growth rate, E:S ratio, rate of endosperm rupture event and final germination percentages. The effect of each condition on the percentages of epicotyl–plumule emergence was analysed by GLM, based on the number of seeds with epicotyl–plumule emergence out of a total of 15 germinated seeds for each condition. GLM, based on the total of seeds with epicotyl–plumule emerged, was also used to evaluate the effects of each condition on the time between radicle emergence and epicotyl–plumule emergence. Significant differences highlighted by GLM on embryo growth rate, E:S ratio, rate of endosperm rupture event and epicotyl–plumule, were then analysed by a *post hoc* pairwise comparisons *t*-test (with Bonferroni adjustment). A log link function and quasipoisson error structure was used for analysing embryo growth rate, E:S ratio, and rate of endosperm rupture event and epicotyl–plumule emergence. A logit link function and quasibinomial error structure was used for analysing seed germination percentages while a logit link function and binomial error structure was used for analysing epicotyl–plumule 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 used R v. 2.14.0 (R Development Core Team 2011).

RESULTS

Embryo growth and root emergence

GLM highlighted a highly statistically significant effect ($P < 0.001$) of W and GA_3 treatments on embryo growth rate (Table 2). At 10 and 15°C, embryos of seeds from the W treatment grew with a mean rate (± SD) of 0.03 ± 0.02 and 0.04 ± 0.004 mm d^{-1}, respectively, significantly faster ($P < 0.001$) than at 20 and 25°C and 25/10°C (ca. 0.01 mm d^{-1}; Fig. 1A). Seeds on GA_3 had embryos extending at ca. 0.03 mm d^{-1} at warm temperatures (≥15°C), significantly faster ($P < 0.01$) than at 10°C (0.02 ± 0.01 mm d^{-1}; Fig. 1A). Seeds of the control (0) and after C and W+C treatments embryos grew very slowly (≤0.01 mm d^{-1}) at all germination conditions, with no statistical differences among temperatures ($P > 0.05$; Fig. 1A). The mean initial E:S ratio for *P. corsica* seeds was 0.27 ± 0.04, with a mean embryo length of 1.4 ± 0.2 mm and mean seed length of 5.2 ± 0.5 mm. The mean critical E:S ratio for germination was 0.58 ± 0.09, with a mean embryo length of 3.9 ± 0.7 mm and mean seed length of 6.8 ± 0.6 mm. All treatments, except C, had a moderate statistically significant effect ($P < 0.05$) on E:S ratio (Table 2). At the last measurement (after 120 days from sowing or from moving after pre-treatments; see Table 1), seeds reached their critical E:S ratio for germination at 15°C for the W pre-treatment. The mean E:S ratios were ca. 0.5 at 20°C, ca. 0.4 at 25°C and < 0.4 at 10 and 25/10°C, with these differences being statistically significant (Fig. 1B; $P < 0.001$). For control and after C and W + C pre-treatments, E:S ratios were low (< 0.5) at all the tested temperatures. Highly statistically significant differences were detected among temperatures for the 0 and W + C treatment ($P < 0.001$), while no statistical differences were detected after C ($P > 0.05$; Fig. 1B). GA_3 treated seeds reached their critical E:S ratio at warm temperatures (≥15°C) with high values (from ca. 0.6 to ca. 0.8), while at 10°C the mean E:S ratio was ca. 0.5, with these values being significantly different ($P < 0.001$; Fig. 1B).

Germination followed the same trend as that detected for internal embryo growth, with high germination at 15°C for W and GA_3 treatments (63 ± 10% and 63 ± 3%, respectively), and low germination (7 ± 3%) in Control seeds (0; Fig. 1C). Seeds also germinated at 10°C in W and GA_3 treatments (10 ± 9 and 15 ± 0%, respectively; Fig. 1C), but no germination

Table 2: GLMs results of embryo growth rate and E:S ratio of the following factors: 0, control; C, pre-chilling; W, warming; W + C; GA_3, 250 mg l^{-1}

Embryo growth rate (mm d^{-1})	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.00185	0.16425	0.011	0.99100
C	−0.36637	0.23869	−1.535	0.12480
W	0.70429	0.19543	3.604	0.00031 ***
W + C	0.08485	0.21762	0.390	0.69660
GA_3	1.00847	0.18634	5.412	6.24e−08 ***
E:S ratio	**Estimate**	**Std. Error**	***t* value**	**Pr(>|t|)**
(Intercept)	−1.02452	0.04103	−24.972	<2e−16***
C	0.01602	0.06068	0.264	0.79190
W	0.18858	0.05777	3.264	0.00122**
W+C	0.13565	0.05829	2.327	0.02060*
GA_3	0.46214	0.05238	8.823	<2e−16***

***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$.

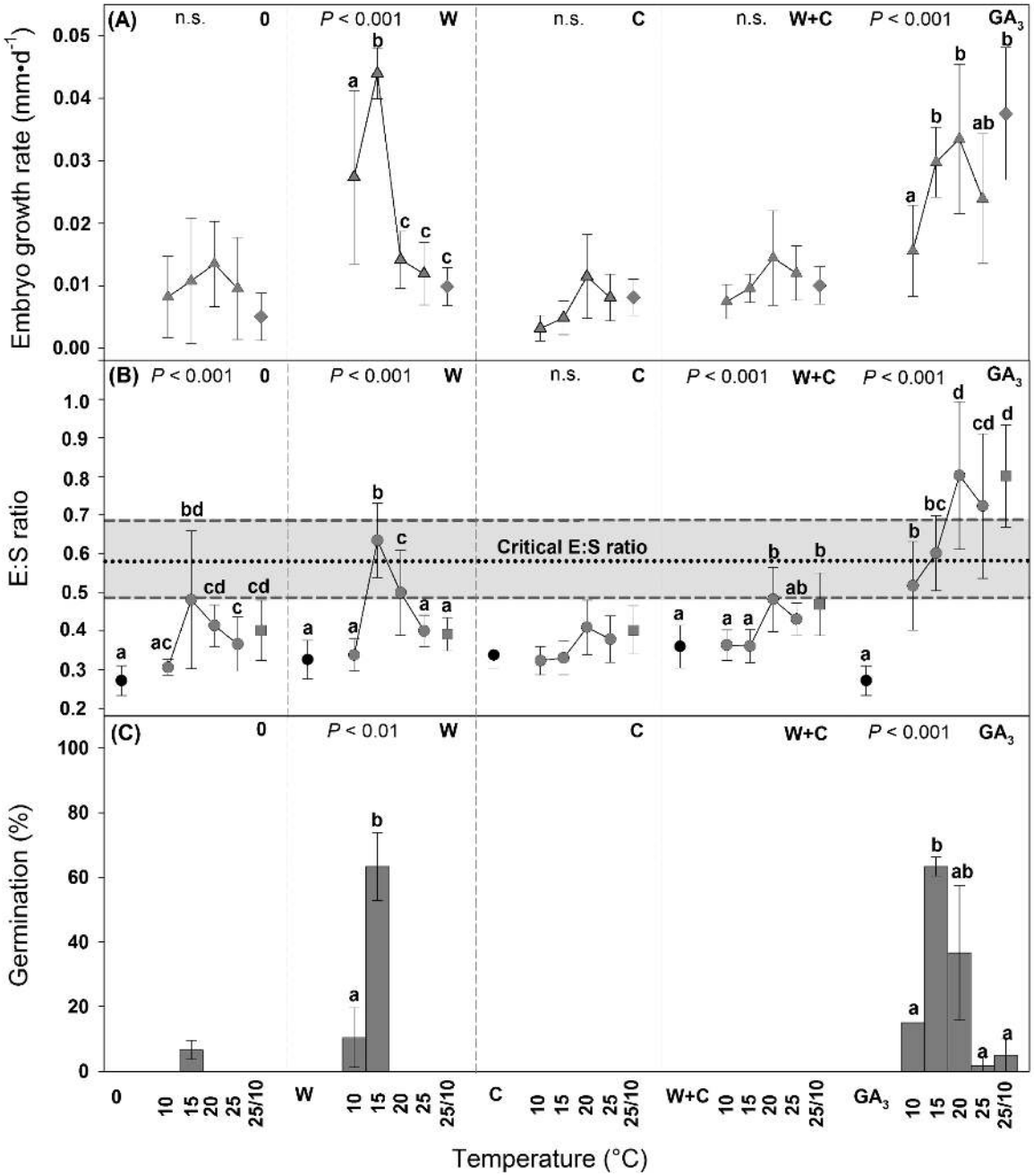

Figure 1: rate of embryo growth (**A**), final values of E:S ratio (**B**) and cumulative germination percentages (**C**) achieved at the end of germination tests (120 days), after each pre-treatment (0, control; W, 25°C for 3 months; C, 5°C for 3 months; W+C, 25°C for 3 months and then 5°C for another 3 months; GA_3, 250 mg l^{-1} of GA_3 in the germination substrate). E:S ratio measured at the start of germination tests and at the end of pre-treatments for W, C and W + C are reported here as a reference, with black circles (B). The results in the alternating temperature regime (25/10°C) are highlighted with a different symbol (diamonds and squares for embryo growth rate and E:S ratio, respectively) compared to constant temperature values. Data are the mean of 10 seeds (± SD) for embryo growth rate and E:S ratio, and of three replicates (± SD) for germination data. The gray band (B) corresponds to the range of values (i.e. 1± SD) of the critical E:S ratio, while the dotted line corresponds to the mean critical E:S ratio, calculated on 20 seeds. GLM was carried out within each pre-treatment to test differences in values of either embryo growth rate, E:S ratio and germination data. Values with the same letter are not different at $P > 0.05$ by *post hoc* pairwise comparisons *t*-test (with Bonferroni adjustment).

was detected in the Control (Fig. 1C). Seed germinated to less than 40% at 20°C in GA_3 treatment, but no germination was detected after W treatment and in the Control (Fig. 1C). Low germination (<10%) was detected at 25 and 25/10°C in GA_3 treatment (Fig. 1C), while no germination occurred at warm (>20°C) temperatures after W treatment and at temperatures

>15°C in the Control (Fig. 1C). No germination was detected at all temperatures tested after C and W + C treatments (Fig. 1C).

Testa and endosperm rupture events during germination

Paeonia corsica seeds exhibit different steps of germination, with a delay detected between testa rupture (i.e. when the endosperm was exposed by a split seed coat) and endosperm rupture (i.e. with radicle emergence). At 15°C, after W and during GA_3 treatments, the mean time course from testa to endosperm rupture were 19.00 ± 14.37 and 24.70 ± 15.23 days, respectively, with no statistical differences ($P > 0.05$). At the end of the warm stratification, no seed had a cracked seed coat.

Epicotyl–plumule germination

The epicotyl–plumule of germinated seeds (i.e. radicle emerged), incubated without any treatment, emerged only at 10°C, with a value of 93% (Fig. 2A). After 2 months of warm stratification, epicotyl–plumule emerged only at 10°C in the 42% of the seeds (Fig. 2A). After 2 months of cold stratification, 92% and 58% of epicotyl–plumule emerged at 10 and 15°C, respectively, and only 1 seed at 20°C (Fig. 2A). During 2 months of GA_3 treatment, epicotyl–plumule emerged from all germinated seeds (Fig. 2A). These differences in epicotyl–plumule emergence were statistically significant at $P < 0.001$, but, except W, no statistical differences ($P > 0.05$) were detected at 10°C among treatments (Fig. 2A).

The different applied conditions of treatments and temperatures had a significant effect on the mean time for epicotyl–plumule germination ($P < 0.001$; Fig. 2B). After cold stratification, the highest mean rate was detected at 15°C (0.039 ± 0.022 $days^{-1}$), then at 10°C after C (0.031 ± 0.013 $days^{-1}$) and GA_3 treatment at 15°C (0.023 ± 0.003 $days^{-1}$; Fig. 2B). Lower mean values were

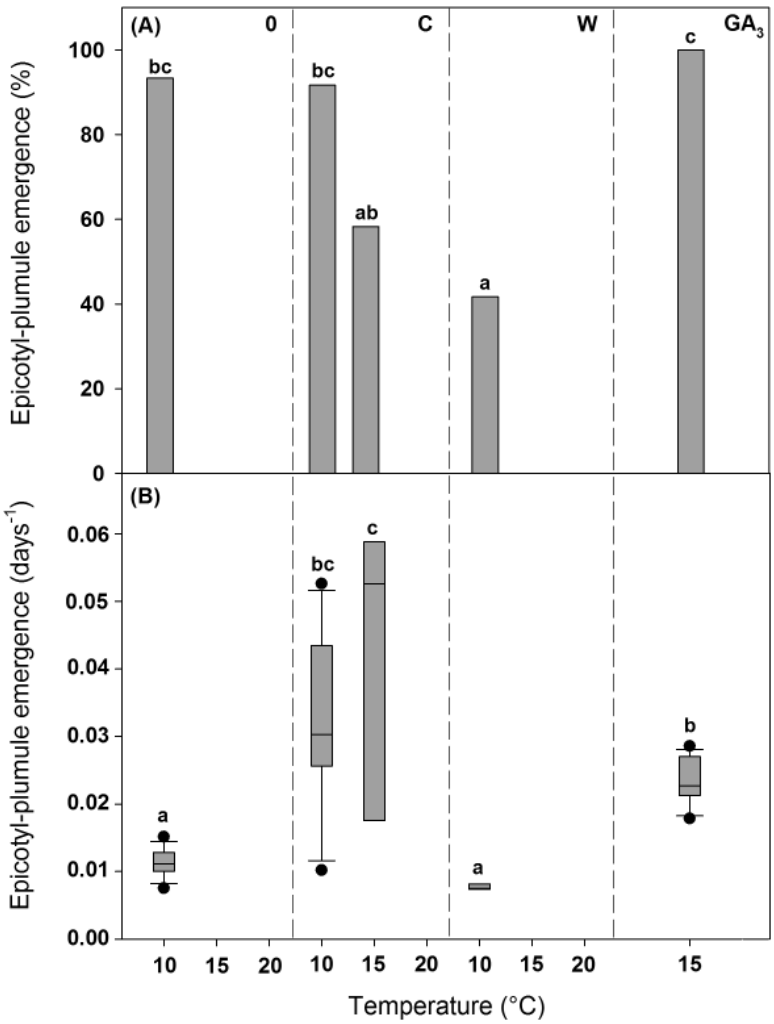

Figure 2: percentages (**A**) and rate ($days^{-1}$) (**B**) of epicotyl–plumule emergence at 10, 15 and 20°C after each pre-treatment (0, control; C, 5°C for 2 months; W, 25°C for 2 months) and at 15°C during GA_3 treatment (GA_3, 250 mg l^{-1} in the substrate). $N = 15$ germinated seeds (when available) for each condition. GLM was carried out to test differences in values. Bars or box plot with the same letter are not different at $P > 0.05$ by *post hoc* pairwise comparisons *t*-test (with Bonferroni adjustment).

detected at 10°C after 0 and W (0.011 ± 0.002 days^{-1} and 0.008 ± 0.000 days^{-1}, respectively; Fig. 2B), without statistical differences ($P > 0.05$) between these two conditions (Fig. 2B).

DISCUSSION

Seed dormancy

When investigating seed germination ecology, experiments need to be started shortly after the seeds are collected, preferably within 7–10 days in order to avoid physiological changes through drying time and storage conditions (Baskin and Baskin 2014). In this study, germination tests started one week after collection.

In *P. corsica* seeds, the embryo is small and underdeveloped at dispersal and doubled in length before radicle emergence was possible. Therefore, following the dormancy classification system (Baskin and Baskin 2004, 2014), these seeds may be described as morphologically dormant (MD). *P. corsica* seeds germinated to very low levels without any treatment and more than 100 days were required to reach less than 10% of germination. After warm stratification or GA_3 treatment, seeds started to germinate (radicles emerged) in ca. 30 days, and GA_3 treatment also widened the temperature range for germination. Thus, seeds of this species also have a physiological component of dormancy (PD), and are therefore MPD. In addition, cold stratification (C) failed to break PD, and imposed secondary dormancy, also when preceded by warm stratification (W + C), delaying embryo growth and preventing seed germination. This phenomenon is not restricted to *Paeonia*, having been previously observed in *Ranunculus sceleratus* L. (Probert *et al.* 1989), *Poa laxa* Haenke subsp. *laxa* (Mondoni *et al.* 2012) and *Ribes multiflorum* Kit *ex* Roem *et* Schult. subsp. *sandalioticum* Arrigoni (Mattana *et al.* 2012).

The delay detected between onset of seed germination (root emergence) and epicotyl–plumule emergence in this species, suggests the presence of epicotyl dormancy, and may be described as a kind of simple epicotyl MPD (Baskin and Baskin 2014). In particular, seeds of *P. corsica* may be considered as deep simple epicotyl MPD, and cold stratification was required to break shoot dormancy, as previously reported for other *Paeonia* species (see Baskin and Baskin 2014 and references therein).

Roots and shoots can have different levels of PD (Baskin and Baskin 1983, 1986); therefore, to describe dormancy in seeds with simple epicotyl MPD according to the dormancy classification system (Baskin and Baskin 2004, 2014), the level of PD (deep, intermediate and non-deep; Baskin and Baskin 2004) in both root and shoot must be described (Baskin *et al.* 2009). Warm stratification and GA_3 treatment enhanced *P. corsica* embryo growth and subsequent seed germination at low temperatures, suggesting non-deep PD for roots. Furthermore, epicotyl–plumule emergence was affected positively by chilling pre-treatment and GA_3 treatment, as found also in *P. suffruticosa* seeds (Barton and Chandler 1957); in particular, it had a significant effect on the mean time for epicotyl–plumule emergence, suggesting that epicotyls of *P. corsica* have deep simple PD. Therefore, following the dormancy classification system (Baskin and Baskin 2004, 2014), seeds of *P. corsica* may be reported as non-deep simple (root)—deep simple (epicotyl) MPD.

Testa and endosperm rupture events during germination

The endosperm is known to act as a barrier for radicle protrusion and thereby the completion of germination in seeds from several angiosperm clades (Muller *et al.* 2006; Ma *et al.*, 2010). Seeds of Paeoniaceae are anatropous. The testa is constituted of many cells thick and the inner epidermis of the outer integument (endotesta) is unspecialized (Corner 1976). Data from this study highlighted that *P. corsica* seeds exhibited a two-step germination, with a lapse of time from testa to endosperm rupture. However, statistical analysis showed no difference in the mean time course from testa to endosperm rupture, both in warm stratified and GA_3 treated seeds. The inhibitory effect of ABA is counteracted by gibberellin and endosperm rupture is under the control of an ABA–gibberellin antagonism (Koornneef *et al.* 2002; Kucera *et al.* 2005; Leubner-Metzger 2003; Weitbrecht *et al.* 2011); in *P. corsica* it would appear that the effects of gibberellins promote and/or facilitate to testa and endosperm rupture at lower temperature, however, specific tests on this topic need to be conducted (see Muller *et al.* 2006).

Embryo growth and germination under GA_3 treatment

In non-dormant seeds of *P. corsica*, 10 and 15°C stimulated embryo growth, yielding higher E:S ratios than other temperatures. GA_3 treatment increased E:S ratios at all temperature tested, influencing also the embryo growth rate. Several authors have suggested a minimum embryo length for germination in species where embryos must elongate before radicle emergence (see Copete *et al.* 2011; Kondo *et al.* 2004; Mattana *et al.* 2012). In *P. corsica* seeds, GA_3 promoted embryo growth beyond the critical E:S ratio. Therefore, the value of the critical E:S ratio for germination in *P. corsica* was here considered as a range. Newton *et al.* (2013) showed that, in some seeds of *Narcissus pseudonarcissus* L. and *Galanthus nivalis* L., little germination was obtained even when mean germination embryo length was attained or exceeded; alternatively germination commenced before the germination embryo length was reached. It is known that gibberellins stimulate germination by inducing hydrolytic enzymes (that weaken the barrier tissues), inducing mobilization of seed storage reserves, stimulating expansion of the embryo (Finkelstein *et al.* 2008) and increasing the growth potential of the embryo (Finch-Savage and Leubner-Metzger 2006; Kucera *et al.* 2005). In *P. corsica* seeds, GA_3 promoted embryo growth at all tested temperatures (Fig. 1) but this alone was insufficient to promote endosperm weakening at high temperatures (i.e. > 20°C) and at alternating

temperature regime (25/10°C). Consequently, an additional trigger, e.g. transfer to ≤ 20°C must also be required to promote the radicle protrusion. A similar effect of GA on embryo growth but not radicle emergence at high temperature has been found in *Aegopodium podagraria* L. seeds by Vandelook *et al.* (2009). Studies at molecular level are needed to provide additional information on the role of GA_3 on the embryo growth, tissues weakening and germination on seeds of *P. corsica*.

Sequential steps of seed dormancy loss and germination

Non-dormant seeds (i.e. after warm stratification) of *P. corsica* incubated at 15°C reached their critical embryo length (step 1) in 23 ± 20 days while the seed coat splitting occurred between 8 and 14 days (step 2), with an overlap among these two steps (Fig. 3). Endosperm weakening (i.e. radicle protrusion; step 3) occurred in 30 ± 15 days and it seems subsequent to the split seed coat (Fig. 3). This suggests that the seed coat starts to split when embryos are still growing, but not yet at their 'critical length' for germination; radicle protrusion, instead, follows almost immediately the splitting of the seed coat (Fig. 3). Muller *et al.* (2006), through direct biomechanical measurement of the puncture force required to rupture the endosperm, demonstrated that also the *Lepidium sativum* micropylar endosperm has weakened before radicle emergence. The requirement for cold stratification to break epicotyl–plumule dormancy (step 4) after radicle protrusion leading to the completion of all seed germination processes, occurred by 102 ± 27 days (Fig. 3).

Ecological correlates of seed germination

Seeds of *P. corsica* are slow to mature, ripening in late summer and dispersal taking place in autumn, mainly by barochory. The seeds have a relatively high seed mass, therefore they have a lower dependence to light for germination (Flores *et al.* 2015). After dispersal, the seeds are then exposed to a mean soil temperature < 20°C without having experienced a warm stratification, and stay dormant in the ground until the next summer when the seeds are exposed at a cycle of warm temperature (i.e. >20°C). Due the proximity of mountainous waterways, the soil may remain moist throughout the year so it is very likely that seeds are imbibed also during summer. Once imbibed, embryos may continue to grow slowly inside the seeds. However, it is only when mean soil temperatures drop below 15°C in October–November, and following the periods of annual maximum precipitation, that embryo growth reaches the critical E:S ratio (mean embryo length of ca. 4.0 mm), thereby allowing seeds to germinate. Germinated seeds go through the winter with an emerged radicle, and the epicotyl–plumule emerges only after ca. 3 months (March–April), when mean soil temperatures again reach 10–15°C. Seedling establishment is completed before the end of the second wet season (May–June). Seedling growth can take place for 2 months, until the start of summer, so that the seedlings enter the dry summer

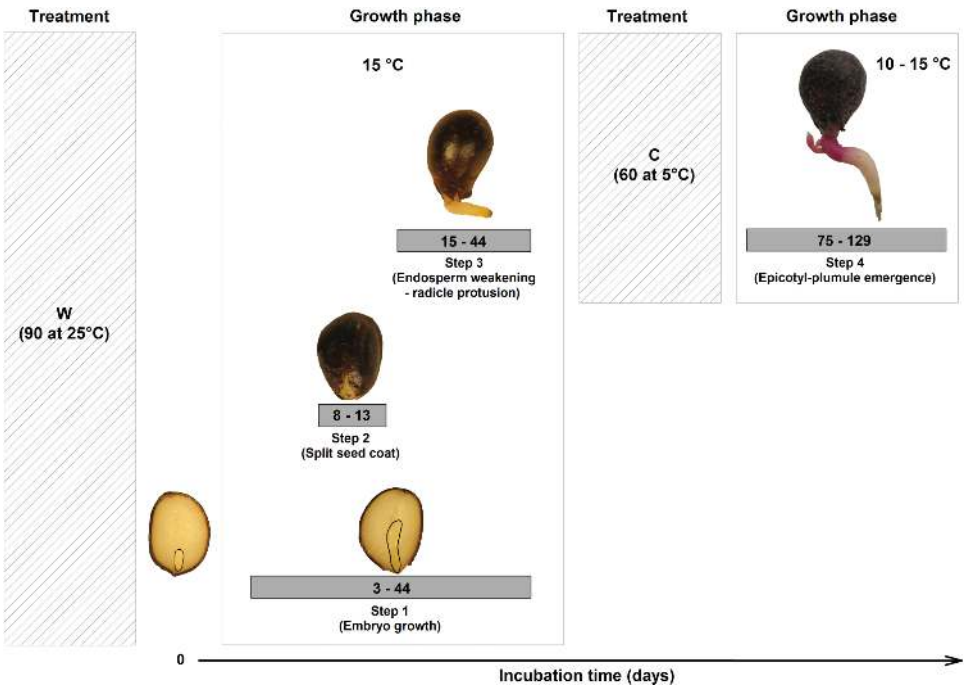

Figure 3: interval of incubation time (expressed in days) to complete embryo growth, split seed coat and radicle protrusion events in non-dormant seeds of *Paeonia corsica* during incubation at 15°C after W (25° for 90 days) and epicotyl–plumule emergence event obtained (in germinated seeds) at 10–15°C after C (5°C for 60 days). Numbers inside the grey rectangles correspond to the minimum and maximum number of days to complete each step.

period (June–August) with well-developed root and shoot systems.

Secondary dormancy of non-germinated seeds imposed by cold stratification in the first winter prevents radicle emergence in late spring and exposure of recently emerged seedlings to the dry summer conditions. This ecological response has been found in other species growing under a Mediterranean climate, with hot, dry summers and cool, wet winters (Baker *et al.* 2005; Mattana *et al.* 2012). As in the case of *R. multiflorum* subsp. *sandalioticum* (Mattana *et al.* 2012) and *Actinotus leucocephalus* Benth. (Baker *et al.* 2005), dormancy alleviation over summer and germination in autumn increases the likelihood that seeds will germinate when there is sufficient moisture available for seedling survival. However, the need for low temperatures (10–15°C) for the rapid embryo growth phase and germination of non-dormant seeds, could suggest that these phases are the most sensitive to temperature, and could impact on germination phenology and/or could reduce the level of natural emergence in the field, highlighting an increasing threat from global warming.

CONCLUSIONS

A multi-step seed germination, from dispersal to seedling establishment, was observed in *Paeonia corsica*. Further studies should be carried out also in natural conditions to confirm if the multi-phasic temperature control of embryo growth and seed germination observed here reflects the temporal emergence of plants *in situ*.

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