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RESEARCH PAPER

Interchangeable effects of gibberellic acid and temperature on embryo growth, seed germination and epicotyl emergence in *Ribes multiflorum* ssp. *sandalioticum* (Grossulariaceae)

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ABSTRACT

Morphophysiological dormancy was investigated in seeds of *Ribes multiflorum* Kit ex Roem et Schult. ssp. *sandalioticum* Arrigoni, a rare mountain species endemic to Sardinia (Italy). There were no differences in imbibition rates between intact and scarified seeds, suggesting a lack of physical dormancy, while methylene blue solution (0.5%) highlighted a preferential pathway for solution entrance through the raphe. Embryos were small at seed dispersal, with an initial embryo:seed ratio (E:S) of *ca.* 0.2 (embryo length, *ca.* 0.5 mm), whereas the critical E:S ratio for germination was three times longer (*ca.* 0.6). Gibberellic acid (GA_3, 250 mg·l^{-1}) and warm stratification (25 °C for 3 months) followed by low temperature (<15 °C) enhanced embryo growth rate (maximum of *ca.* 0.04 mm·day^{-1} at 10 °C) and subsequent seed germination (radicle emergence; *ca.* 80% at 10 °C). Low germination occurred at warmer temperatures, and cold stratification (5 °C for 3 months) induced secondary dormancy. After radicle emergence, epicotyl emergence was delayed for *ca.* 2 months for seeds from three different populations. Mean time of epicotyl emergence was affected by GA_3. Seeds of this species showed non-deep, simple (root)–non-deep simple (epicotyl) morphophysiological dormancy, highlighting a high synchronisation with Mediterranean seasonality in all the investigated populations.

INTRODUCTION

Central northern Sardinia (Italy) has been identified as one of 52 putative refugia in the Mediterranean region (Médail & Diadema 2009). Existence of these refugia implies the local long-term (one or more glacial-interglacial cycles) persistence of a species or population within a well-defined geographical area (*e.g.* mountain range, gorge). Central northern Sardinia also represents a southern European refugium (*sensu* Tzedakis *et al.* 2002) for some temperate tree species, such as *Rhamnus alpina* L., *Taxus baccata* L. and *Sorbus torminalis* (L.) Crantz, as detected in the Supramontes (a karstic region in central northern Sardinia) by Fenu *et al.* (2010). This area is also characterised by endemic species of genera typical of temperate climates, such as *Ribes sardoum* Martelli and *Ribes multiflorum* Kit ex Roem et Schult. ssp. *sandalioticum* Arrigoni, which are exclusive to the island and are the only two members of the genus in Sardinia.

Ribes L. (Grossulariaceae) is a genus of *ca.* 200 species distributed in temperate regions of the Northern Hemisphere and the Andes, with many species being cultivated for their edible fruits or ornamental purposes (Mabberley 2008). *R. sardoum*, the Sardinian currant, is a very rare and threatened species with only one small population in the Supramontes (Monte Corrasi, 1200 m a.s.l.). *R. multiflorum* ssp. *sandalioticum* is found in small populations in the Supramontes, Gennargentu Massif, Catena del Marghine and Limbara Mountain, growing at altitudes above 1000 m a.s.l., under the canopy of temperate woodlands, among mountainous streams and on wet slopes (Arrigoni 1968, 1981; Valsecchi 1977).

The seeds of Grossulariaceae are anatropous and characterised by a single layer, gelatinous sarcotesta, arillate to exarillate and albuminous. The sarcotesta is constituted of thin-walled palisade-like cells of the outer testa epidermis whose lumina are filled with mucilage. The inner epidermis of the outer integument (endotesta) consists of a layer of small cuboid crystal cells, with thickened and lignified radial and inner walls and one to three crystals per cell (Corner 1976). Of the two-layered inner integument (tegmen), the outer epidermis is crushed during seed development, whereas the inner epidermis (endotegmen) persists as a layer of enlarged thin-walled cells with firm, brown tanniniferous content. The vascular system of the seed is constituted only of a raphe bundle. The endosperm is oily, with slightly thickened walls, and the embryo is small relative to the endosperm (Corner 1976). Embryos of mature seeds of *Ribes* spp. are classified as linear underdeveloped (Martin 1946; Baskin & Baskin 2007), with a mean embryo:seed (E:S) ratio of about 0.04 (Forbis *et al.* 2002). The seeds of most *Ribes* spp. have

embryo dormancy, which can be broken by a long, cold stratification period (Young & Young 1992). This period can range from 60 to 300 days, depending on species, and consists of prechilling, warm stratification followed by prechilling, and/or a wide range of diurnal temperature to facilitate germination (Ellis *et al.* 1985; Baskin & Baskin 1998). A lower temperature can improve germination and reduce the prechilling requirement (Fivaz 1931; Pfister 1974). High germination percentages (about 100%) have been achieved for several *Ribes* spp. without any pre-treatment (Liu *et al.* 2008). However, seed coat dormancy, controlled by growth inhibitors and/or an impermeable seed coat, is also suspected to occur (Pfister 1974; Goodwin & Hummer 1993; Rosner *et al.* 2003a,b). In particular, sulphuric acid and hydrogen peroxide treatments improve germination in some species (Fivaz 1931; Adam & Wilson 1967; Pfister 1974; Rosner *et al.* 2003a,b). Nonetheless, Baskin *et al.* (2000) did not consider the Grossulariaceae among families of angiosperm with seeds showing physical dormancy (PD).

Morphophysiological dormancy (MPD) has been inferred to occur in seeds of Grossulariaceae and particularly those of *Ribes* (Baskin & Baskin 1998), but no studies have been done to substantiate this claim. In addition, if seeds of *Ribes* have MPD, then research will be required to determine which of the nine levels (non-deep simple, intermediate simple, deep simple, deep simple epicotyl, non-deep simple epicotyl, deep simple double, non-deep complex, intermediate complex and deep complex; Baskin & Baskin 1998, 2004; Baskin *et al.* 2008) is present.

To help determine the class of dormancy, information is needed on seed coat permeability to water, responses to warm and/or cold stratification and to plant hormones, in particular gibberellic acid (GA). To resolve the nature of dormancy, especially MPD, 'move-along experiments' (or double germination phenology technique) have been used to determine the temperature, or temperature sequence, required to break dormancy and promote germination. This approach mimics the environment into which dormant seeds are dispersed and subsequently exposed, and is applicable to both temperate and tropical environments (*e.g.* Baskin & Baskin 2003; Hoyle *et al.* 2008).

The balance between abscisic acid (ABA) and GA and sensitivity to these hormones regulates the onset, maintenance and termination of dormancy. ABA synthesis and signalling (GA catabolism) dominate the dormant state, whereas GA synthesis and signalling (ABA catabolism) dominate the transition to germination (Finch-Savage & Leubner-Metzger 2006). For this reason, Baskin & Baskin (2004) considered the ability of exogenous GA to overcome dormancy a decisive element in distinguishing among levels of PD in their dormancy classification systems.

Dormancy breaking and germination requirements are specific for each species and depend on phylogeny, distribution and habitat (Baskin & Baskin 1998, 2004; Finch-Savage & Leubner-Metzger 2006). Even closely related species, either growing in a variety of habitats (*e.g.* Vandelook *et al.* 2008) or co-occurring in a given habitat, may differ in germination response to environmental signals (*e.g.* Daws *et al.* 2002; Karlsson *et al.* 2008). Intra-specific variation in the depth of dormancy or germination requirements among populations/ecotypes has been related to differences in post-dispersal environment (Donohue 2005), mainly due to altitude or habitat (*e.g.* Milberg & Andersson 1998; Giménez-Benavides *et al.* 2005; Mondoni *et al.* 2008).

The main aim of this study was to investigate the seed germination ecology of *R. multiflorum* ssp. *sandalioticum* in order to: (i) identify the class of dormancy, *sensu* Baskin & Baskin (2004), and if MPD is present, at what level, and (ii) confirm whether seedlots from different populations vary in their response to treatments.

MATERIALS AND METHODS

Seedlot details

Ripe fruits of *R. multiflorum* ssp. *sandalioticum* (hereafter, *R. m. sandalioticum*) were collected directly from plants in three populations, representative of its distribution, at the time of natural dispersal in 2008 and 2009 (Table 1). Seeds were immediately separated from the pulp by rubbing fruits through sieves under running water. The cleaned seeds were then spread out and left to dry at room temperature. Subsequently, seeds were placed in a dry room at 15% RH and 15 °C. Seedlot OR08 was then stored at +5 °C, until experiments with all seedlots commenced in 2009. Average seed mass was calculated for each seed lot by weighing 10 replicates of 20 seeds each (Table 1).

Climatic data for the three population site locations were acquired using the 'extract values to points' tool in GIS software (ESRI ArcMap version 9.2) from available data at a spatial resolution of 1 km^2, downloaded from the WorldClim website (http://www.worldclim.org), as documented by Hijmans *et al.* (2005).

Imbibition

The rate of water uptake was monitored for scarified and non-scarified seed in three replicates of 20 seeds each from seedlot OR08 (see Table 1). Seeds were longitudinally scarified

Table 1. Seed lot details.

population	code	collection date	substrate (Carmignani *et al.* 2001)	altitude (m a.s.l.)	seed fresh weight (mg)
M.te Novo San Giovanni, Orgosolo (NU)	OR08	23/08/2008	Mesozoic limestone	1225	7.57 ± 0.90
	OR09	20/08/2009			6.08 ± 0.43
N.ghe Ortachis, Bolotana (NU)	BO09	08/08/2009	Cenozoic basalt	1025	7.52 ± 0.76
M.te Limbara, Tempio (OT)	TE09	17/08/2009	Palaeozoic granite	1300	5.01 ± 0.17

through the testa and the tegmen, using a scalpel, and change in mass was monitored during incubation on the surface of 1% agar water in 60-mm diameter plastic Petri dishes at 20 °C in the light (12 h light/12 h dark). Seeds were weighed at 1-, 2-, 4-, 6- and 20-h intervals and then every 24 h for the next 6 days (168 h in total). Percentage water uptake was calculated following Hidayati *et al.* (2001) in relation to seed mass at t_o: $\%W_s = [(W_i - W_d)/W_d] \times 100$, where W_s = increase in mass of seed, W_i = mass of seed after a given interval of imbibition, and W_d = seed mass at t_o.

An increase in seed mass could be caused through uptake of water by the sarcotestal gelatinous pulp, and not through imbibition of water by the embryo. Therefore, seeds were imbibed in a watery methylene blue solution (0.5%) to clarify whether sufficient water penetrates the endosperm to reach the embryo (Tillman-Sutela & Kauppi 1998). After 2, 15 and 30 days of imbibition in methylene blue, longitudinal and cross-sections were then made on seeds, both manually with a scalpel and sectioned at −20 °C using a cryotome (Leica CM3050S) and checked for staining under a Stemi SV 11 microscope (Carl Zeiss, Welwyn Garden City, Herts, UK), equipped with a digital microscopy camera (AxioCam HRc; Carl Zeiss MicroImaging, Germany). Measurements were carried out using the image analysis software Axiovision 3.1.2.1 (Carl Zeiss Vision).

Germination tests

In an attempt to break seed dormancy, a 'move-along experiment' (Baskin & Baskin 2003) was carried out (Table 2), starting in May 2009, on seedlot OR08 (see Table 1). Three replicates of 20 seeds each were sown on the surface of 1% agar water in 60-mm diameter plastic Petri dishes and incubated in the light (12 h light/12 h dark) for a minimum of 4 months and a maximum of 6 months at a range of germination temperatures (10, 15, 20, 25, 30 and 25/10 °C), and further replicates were given different pre-treatments (Table 2). In the alternating temperature regime, the 12-h light period coincided with the elevated temperature period. The low number of seeds per replicate (20) in each experiment was due to limited seed availability because the species is rare and has small populations. 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 in the light (12 h light/12 h dark, at the above germination temperatures.

Three extra replicates were incubated at 10 °C after chipping with a scalpel, and to confirm the effect of light on germination, another three replicates were also sown at 10 °C in darkness (Petri dishes wrapped in aluminium foil).

Germination was defined as visible radicle emergence. Germinated seeds were scored three times at week, except for dark-incubated seeds that were only scored once, at the end of the test, to avoid any exposure to light. At the end of the germination tests, when no additional germination had occurred for 2 weeks, a cut-test was carried out to determine viability of remaining seeds and number of empty seeds. The final germination percentage was calculated on the basis of the total number of filled seeds and T_{50} values expressed as time to reach 50% maximum germination in one replicate.

Embryo measurements

Embryo growth during pre-treatments and germination conditions described above was assessed at different times (Table 2) by measuring five seeds for each sample interval. Seeds were cut in half under a dissecting microscope and images of embryos acquired using a flatbed scanner (Epson GT-15000) with a resolution of 1200 dpi. Embryo and seed lengths were measured using the image analysis software ImageJ 1.41 (National Institutes of Health, USA) and the E:S ratio calculated. Seed length was measured ignoring the seed coat. The initial E:S ratio was calculated by measuring 20 randomly selected seeds before the start of the experiments. The E:S ratio of seeds with a split seed coat but no radicle protrusion (critical E:S ratio) was determined for 20 randomly selected seeds and used for seeds that had germinated before measurements commenced (Vandelook *et al.* 2007). The mean rate of embryo growth was expressed in mm per day and calculated on the basis of number of days necessary to achieve the maximum E:S ratio.

Population variability

To evaluate differences in seed germination and epicotyl emergence (as a delay in epicotyl emergence had been detected in a

Table 2. Experimental design.

pre-treatment		embryo growth measurements	
code	description	number of measurements	timing
0	–	4	After 15, 30, 90 and 120 days.
C	3 months, 5 °C	6	After 45 and 90 days during cold stratification (C), and 15, 30, 90 and 120 days after sowing for germination.
W	3 months, 25 °C	6	After 45 and 90 days during warm stratification (W), and 15, 30, 90 and 120 days after sowing for germination.
W+C	3 months, 25 °C (W) → 3 months, 5 °C (C)	9	After 45 and 90 days during warm (W), 15, 45 and 90 days during cold (C), and 15, 30, 90 and 120 days after sowing for germination.
C+W+C	3 months, 5 °C (C) → 3 months, 25 °C (W) → 3 months, 5 °C (C)	12	After 45 and 90 days during cold (C), 15, 45 and 90 days during warm (W), 15, 45 and 90 days during cold (C) and 15, 30, 90 and 120 days after sowing for germination.
GA_3	GA_3 (250 mg·l^{-1}) in germination medium	4	After 15, 30, 90 and 120 days.

previous experiment) among populations, a warm pre-treatment, *i.e.* 3 months at 25 °C on the surface of 1% agar water (W, see Table 2), was applied in September 2009 to four replicates of 35, 25 and 20 seeds each from seedlots OR09, TE09 and BO09, respectively (Table 1), before transferring them to 5 and 10 °C. The different number of seeds tested for each seedlot reflected seed availability. Germinated seeds were then: (i) kept at 5 and 10 °C for an additional 2-week period in order to allow root growth, before transplanting to a sterilised mixture (2:1) of sand/soil at 10, 15 and 20 °C; (ii) kept (for seeds already incubated at 5 °C) or moved (for seeds incubated at 10 °C) to 5 °C for 2 months, before transplanting to (2:1) sand/soil at 10, 15 and 20 °C; and (iii) kept at 5 and 10 °C for an additional 2 months on the surface of 1% agar water with GA_3 (250 $mg \cdot l^{-1}$), before transplanting to (2:1) sand/soil at 10, 15 and 20 °C. The seeds and seedlings were scored daily and twice per week for radicle and epicotyl emergence, respectively. The mean time to emergence (MTE) was estimated according to the formula: $MTE = \Sigma\ (ni \times di)/N$, where ni is the number of emerged seedlings at day i, d the incubation period in days, and N the total number of emerged seedlings in the treatment. When no additional germination or epicotyl emergence occurred for 2 weeks, after a minimum of 4 months, both germination and epicotyl emergence experiments were stopped.

Statistical analysis

One-way analysis of variance (ANOVA) was carried out on water uptake and rate of imbibition. Final germination (radicle emergence) and epicotyl emergence percentages and MTE values were analysed with one-way and two-way ANOVA; subsequently, a *post hoc* Fisher least significant difference test (LSD) was conducted. The non-parametric Kruskal–Wallis and Wilcoxon tests were used to test for differences in E:S ratios, seed and embryo lengths, rate of embryo growth and T_{50} values. All statistical analyses used R v. 2.11.1 (R Development Core Team 2010).

RESULTS

Imbibition

Both intact and scarified seeds reached maximum increase in mass 20 h after soaking, with a percentage water uptake of 117.5 ± 6.4% (mean ± SD) and 111.8 ± 1.2%, at a rate of 0.416 ± 0.032 $mg \cdot h^{-1}$ and 0.394 ± 0.003 $mg \cdot h^{-1}$, respectively; these differences were not statistically significant at $P > 0.05$.

Methylene blue did not reach the endosperm or embryo, even though embryos had undergone significant imbibition (data not shown). After 2 days, the dye had partially stained the gelatinous sarcotesta (Fig. 1A), and after 15 days the endotesta was also completely stained blue (Fig. 1B). After 30 days, the endosperm and embryo were still unstained (Fig. 1C–F), suggesting that penetration of methylene blue through the seed coat was prevented by the outer epidermis of the tegmen (Fig. 1B–E). The dye stained the epistase in the micropylar region (Fig. 1D), the vascular bundle in the raphe (Fig. 1E) and its point of termination in the chalaza. The differential staining of tissues also highlighted the presence of a chalazal plug (hypostase) consisting of a tissue of brown (tanniniferous) cells that remained unstained (Fig. 1F).

Embryo growth and root emergence

Germination of *R. m. sandalioticum* seeds in light (74 ± 2%) and darkness (82 ± 10%) at 10 °C was not significantly different ($P > 0.05$). Also, no differences ($P > 0.05$) were detected between germination of scarified and intact seeds (78 ± 3% and 74 ± 2%, respectively), with germination starting at about 20 days in both treatments.

The mean initial E:S ratio for *R. m. sandalioticum* seeds was 0.19 ± 0.02, with a mean embryo length of 0.52 ± 0.08 mm and mean seed length of 2.70 ± 0.23 mm. The critical E:S ratio for germination was 0.62 ± 0.12, with a mean embryo length of 1.80 ± 0.39 mm and mean seed length of 2.91 ± 0.34 mm. While mean seed length increased slightly (even if significantly: $P < 0.05$), mean embryo length increased more than three-fold ($P < 0.0001$), consequently determining a highly significant increase in the mean E:S ratio ($P < 0.0001$).

At the end of the first cycle of pre-treatments, after 90 days (I in Fig. 2), the mean E:S ratio was <0.3 for all seeds regardless of treatment temperature, and values were not statistically different from each other ($P > 0.05$). While no germination occurred during warm stratification, 13 ± 11% and 6 ± 5% of seeds germinated at 5 °C in the two treatments started at this temperature (C and C+W+C, respectively; Fig. 2).

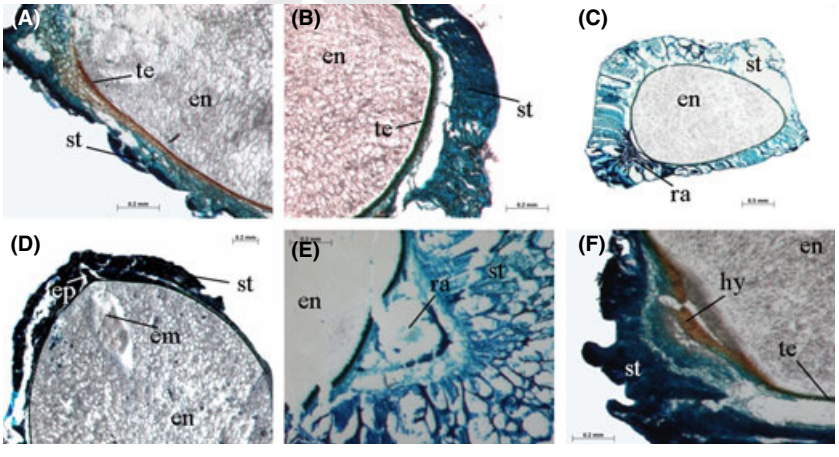

Fig. 1. Longitudinal (LS) and cross-sections (CS) of *R. m. sandalioticum* seeds (12-μm thick) taken after soaking in methylene blue dye for different periods. LS after 2 (A) and 15 days (B); middle CS of seed (C), hilum region and epistase in LS after 30 days (D); detail of raphe in CS (E) and chalazal area in LS (F) after 30 days. Reference bars are 0.2 mm, except for (C): 0.5 mm. st: sarcotesta, te: tegmen, en: endosperm, em: embryo, ep: epistase, ra: raphe, hy: hypostase.

Colour online, B&W in print

During the second cycle of pre-treatments (II in Fig. 2), seeds receiving W+C (90 day warm + 90 day cold stratification) reached a critical E:S ratio after 135 days (45 days after the end of warm stratification; Fig. 2), whereas seeds receiving C+W+C (90 day cold + 90 day warm + 90 day cold stratification), the mean E:S ratio was still <0.3 (0.23 ± 0.03) after 180 days at the end of warm stratification (Fig. 2). In seeds receiving W+C germination was 71 ± 14% after 180 days, while no more seeds incubated at 5 °C for 3 months and then at 25 °C for 3 months (C+W+C) had germinated at this time (Fig. 2).

The last cycle (III in Fig. 2) consisted of a further cold stratification (C+W+C) and, at this point, after 270 days, embryos had a mean E:S ratio of 0.54 ± 0.18, which was not statistically different to the critical E:S ratio (0.62), and 35 ± 1% of seeds germinated (Fig. 2). In summary, while no seeds germinated during warm stratification (W), 13%, 71% and 35% of seeds germinated at the end of C, W+C and C+W+C, respectively (Fig. 2), with these percentages being statistically different ($P < 0.0001$; Fig. 3C). After pre-treatments, seeds were moved to germination conditions except for seeds that had received W+C treatment, due to the high germination at the end of stratifications and scarcity of non-germinated seeds available for the experiments (see Fig. 2).

At 10 °C, embryos of untreated seeds (0) and those in the W treatment grew with at a mean rate of 0.042 ± 0.002 and 0.043 ± 0.00 mm·day^{-1}, respectively (Fig. 3A). Growth was *ca*. 0.010 mm·day^{-1} at 15 °C and always ≤0.05 mm·day^{-1} at warmer temperatures for both treatments (Fig. 3A). These differences were statistically significant within each pre-treatment ($P < 0.0001$). After C and C+W+C treatments, embryos grew very slowly (≤0.03 mm·day^{-1}) under all germination conditions, without statistical differences among temperatures ($P > 0.05$; Fig. 3A). Seeds sown on GA_3 solution showed the same trend to untreated (0) and warm stratified (W) seeds, with faster growth at cold temperatures, a mean rate of 0.043 ± 0.00, 0.011 ± 0.00 and 0.010 ± 0.001 mm·day^{-1} at 10, 15 and 25/10 (17.5) °C, respectively and lower mean rates at warmer temperatures, although above 0.006 mm·day^{-1} (Fig. 3A).

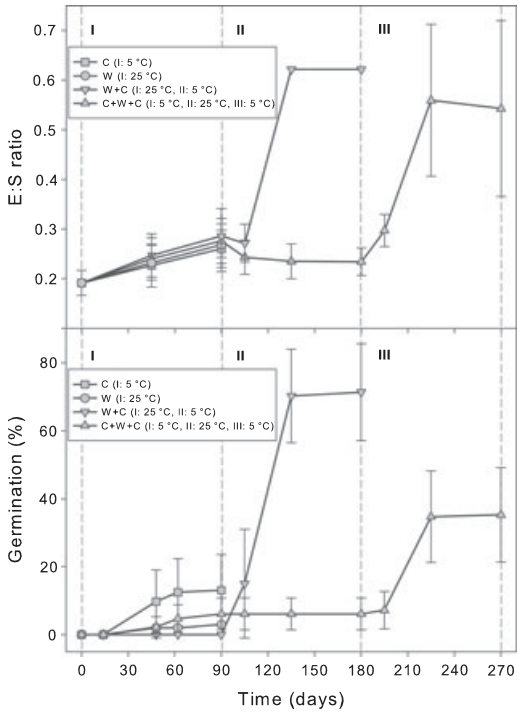

Fig. 2. Embryo:seed (E:S) ratio and germination during pre-treatments. I, II and III correspond to the three applied cycles of temperature, with I = 5 °C for C and 25 °C for 'pre-treatments for 3 months, II = 5 °C for W+C and 25 °C for C+W+C pre-treatments for another 3 months, and III = 5 °C for C+W+C pre-treatment for the final 3 months. Data are mean of five seeds (±SD) for E:S ratio values and 18 replicates (±SD) for germination data.

At the last measurement (after 120 days from sowing or from moving after pre-treatments; see Table 2), seeds reached a critical E:S ratio for germination at 10 and 15 °C for the 0 and W pre-treatment, while at warmer temperatures the mean E:S ratios were 0.4–0.5 at 25/10 (17.5) and 20 °C and <0.3 at 25 and 30 °C (Fig. 2); these differences were statistically significant ($P < 0.001$). After C and C+W+C pre-treatments, E:S ratios were low (<0.5) at all tested temperatures, with no difference between conditions ($P > 0.05$; Fig. 3B). In addition, mean E:S ratios detected at each germination temperature for C+W+C were lower than E:S ratios detected at the end of stratifications (Figs 2 and 3B). Seeds treated with GA_3 reached a critical E:S ratio at 10 and 15 °C and high values (*ca*. 0.6) at 25/10 (17.5) and 20 °C; these values were statistically significant ($P < 0.001$; Fig. 3B).

Germination followed the same trend as detected for embryo growth, with high germination at 10 °C of 74 ± 2%, 87 ± 13% and 88 ± 3% for 0, W and GA_3, respectively, and 61 ± 22% for C treatment (Fig. 3C), with no difference detected among treatments ($P > 0.05$). Seeds also germinated at 15 °C in 0, W and GA_3 treatments (40 ± 5%, 33 ± 8% and 76 ± 10%, respectively, and not statistically different to germination at 10 °C ($P > 0.05$). Low germination (<6.0%) was also detected at 25/10 °C (17.5) for W and GA_3 and 20 °C for GA_3 treatments. No germination occurred after C+W+C treatment (Fig. 3C).

Although there were no statistical differences in the final germination percentages achieved at 10 °C after different pre-treatments (Fig. 3C), the seeds germinated at different rates. Untreated seeds (0) needed 42 ± 6 day to reach 50% of maximum germination (T_{50}). After W and GA_3 treatment, this time was approximately halved to 21 ± 3 and 27 ± 1 day, respectively. After C treatment, seeds germinated very slowly, with a T_{50} of 158 ± 13 day, nearly four-fold longer than the control (0). The T_{50} values were statistically different ($P < 0.05$; Fig. 4).

Population variability

After W pre-treatment, seeds of Bolotana (BO09) and Orgosolo (OR09) populations had high germination percentages at 10 °C (94 ± 2% and 96 ± 2%, respectively), while at the same temperature, germination of seeds from Tempio (TE09) was 41 ± 14% (Fig. 5). Low germination (<30%) was achieved in all seedlots after moving from 25 °C (W) to 5 °C (Fig. 5), with final values of 25 ± 5%, 18 ± 7% and 3 ± 4% for Bolotana, Orgosolo and Tempio populations, respectively. The two-way ANOVA highlighted that population and temper-

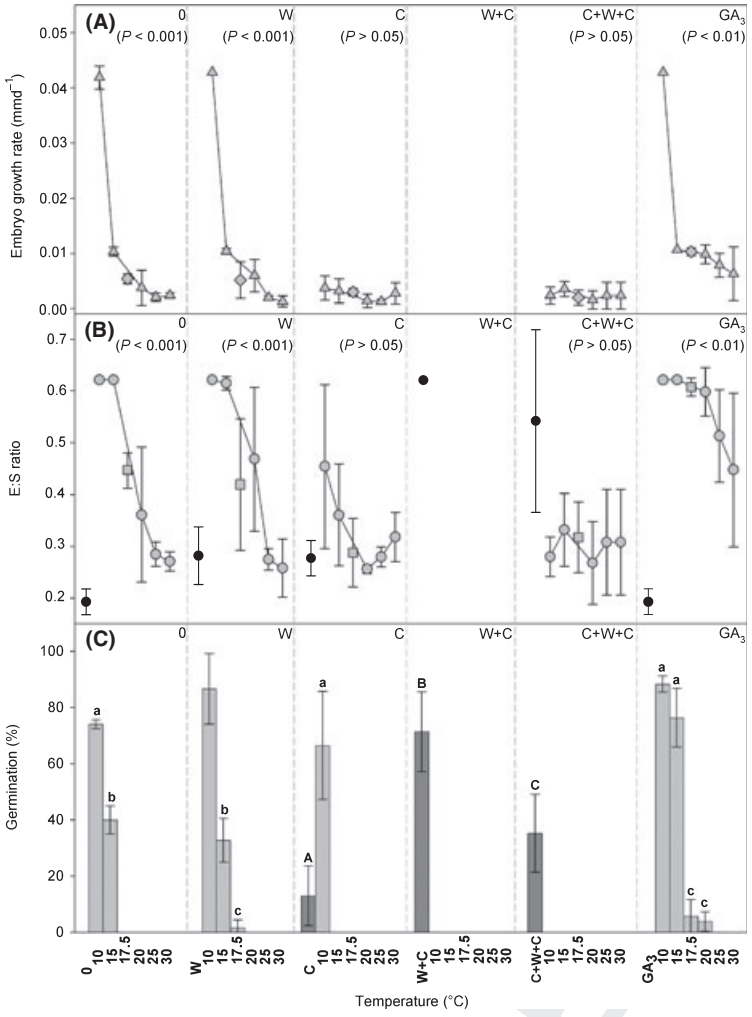

Fig. 3. Rate of embryo growth (A), final values of E:S ratio (B), and cumulative germination percentages (C) achieved at the end of germination tests, after each pre-treatment (0, control; W, 25 °C for 3 months; C, 5 °C for 3 months; W+C, 25 °C for 3 months and then 5 °C for another 3 months; C+W+C, 5 °C for 3 months, 25 °C for another 3 months and 5 °C for the last 3 months; see Table 2). E:S ratio measured at the start of germination tests (and therefore the end of pre-treatments for W, C, W+C and C+W+C), as well as seeds germinated during pre-treatments (see also Fig. 1) are reported here as a reference, with black circles (B) and dark grey bars (C). The results in the alternating temperature regime (25/10 °C) are here reported as 17.5 °C and 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 five seeds (±SD) for embryo growth rate and E:S ratio, and of three replicates (±SD) for germination data. A non-parametric Kruskal-Wallis test was carried out within each pre-treatment to test differences in values of either embryo growth rate or E:S ratio, and one-way ANOVA was used for germination data. In (C) bars with the same letter are not different at $P > 0.05$ with *post hoc* Fisher's LSD (capital letters used for seeds germinated during pre-treatments).

ature and their interaction had a significant effect on seed germination ($P < 0.001$). Intra-population variability was also detected among seedlots from Orgosolo. Seedlot OR08 had high germination percentages after W pre-treatment, both at 10 °C (*ca.* 85%, see Fig. 3C) and 5 °C (*ca.* 70%, see Fig. 2). While no differences in germination were detected at 10 °C ($P > 0.05$), germination of the two seedlots at 5 °C was statistically different ($P < 0.001$).

A delay of about 2 months was detected between the onset of seed germination (radicle emergence) and epicotyl emergence in the three investigated populations. Only seeds that germinated at 10 °C were monitored for epicotyl emergence, due to the low germination percentages at 5 °C in all populations (Fig. 5). In BO09 (n = 9), 67%, 89% and 33% of seeds that germinated produced elongated epicotyls at 10, 15 and 20 °C, respectively, without any treatment (Fig. 5A). After 2 months of cold stratification (Fig. 5B), epicotyls emerged from all seeds that germinated (100%) at 10 and 15 °C and from 89% of those germinated at 20 °C. After GA_3 treatment (Fig. 5C), only 67%, 56% and 22% of epicotyls emerged at 10, 15 and 20 °C, respectively, mainly due to high microbial growth and seedling mortality (*ca.* 50%, data not shown) at all tested temperatures (Fig. 5). In OR09 (n = 15), the same pattern was detected (Fig. 5), with more epicotyls emerging at 10 °C (67%, 93% and 71% for control, cold stratification and GA_3 treatment, respectively) and 15 °C (67%, 67% and 71%) than at 20 °C (7%, 53% and 50%). Also in this case, high seedling mortality (*ca.* 26%, data not shown) was detected for GA_3 treatment at all temperatures. Considering the low germination even at 10 °C for seeds from the TE09, a very low number of germinated seeds was sown at the different conditions for epicotyl emergence (n = 4) in this population. However, also in TE09, epicotyl emergence was higher at 10 and 15 °C than at 20 °C (Fig. 5), except for the control (A), where no emergence occurred at 15 °C and high seedling mortality was detected after GA_3 treatment (*ca.* 42%, data not shown). No significant differences were detected among populations ($P > 0.05$); however, epicotyl emergence was significantly affected by both applied treatment and temperature ($P < 0.05$), although their interaction was not significant ($P > 0.05$, two-way ANOVA).

The climate diagrams show a bi-seasonal Mediterranean trend for all three population sites, with a rainy and cool season from September to June and 3 months of dry and warm summer weather (Fig. 5). By synchronising the start of warm stratification with the month of dispersal (August), it was possible to predict that seeds would reach maximum germination in November, at the beginning of the winter season for all populations (Fig. 5). Without any treatment, maximum epicotyl emergence was achieved in April–May for

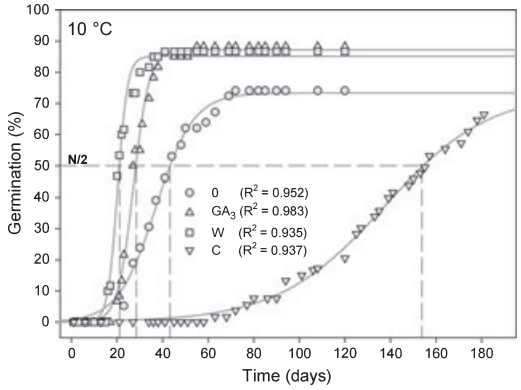

Fig. 4 Germination trends at 10 °C after each pre-treatment (0, control; W, 25 °C for 3 months and C, 5 °C for 3 months; see Table 2). Points correspond to actual data and solid lines indicate the fitted lines from sigmoidal regressions ($P < 0.0001$ for all lines). Data are mean of three replicates. For clarity, SD error bars are not shown, but were generally about 10%. Dashed lines indicate T_{50} values: times to achieve 50% (N/2) maximum germination.

Bolotana and March for Orgosolo and Tempio populations. After 2 months of cold stratification, the maximum would be delayed to May at 20 °C for all populations, while at lower temperatures (10 and 15 °C) it would be reached 1 month earlier than the control (February–March) for Bolotana, and in March–April for Orgosolo and Tempio populations. After GA_3 treatment, maximum epicotyl emergence would be achieved before April at all temperatures for all populations (Fig. 5).

The applied treatments had a significant effect on mean time to epicotyl emergence (MTE; $P < 0.001$), with lower mean values detected after both cold stratification and GA_3 treatment (74 ± 28 days and 28 ± 14 days for C and GA_3, respectively) than in the control (94 ± 30 days), even if only the latter was significantly different ($P < 0.05$; Fig. 6). Temperature and population had no effect on MTE ($P > 0.05$).

DISCUSSION

Physical dormancy and water uptake

The similar increase in seed mass for both intact and scarified seed confirms that the seed coat of *R. m. sandalioticum* is water-permeable. Thus seeds of this taxon do not have physical dormancy (PY). Although several studies have shown a positive effect of scarification on germination of *Ribes* seeds (Fivaz 1931; Adam & Wilson 1967; Pfister 1974; Rosner *et al.* 2003a,b), members of the Grossulariaceae do not have a water-impermeable palisade layer of cells in the seed coat (Baskin *et al.* 2000, 2006). Thus, promotion of germination following scarification is good evidence for the presence of physiological dormancy (PD) (Baskin & Baskin 1998).

Although methylene blue failed to reach the endosperm and embryo, it was clear that embryos had undergone significant imbibition. Methylene blue staining has been used in

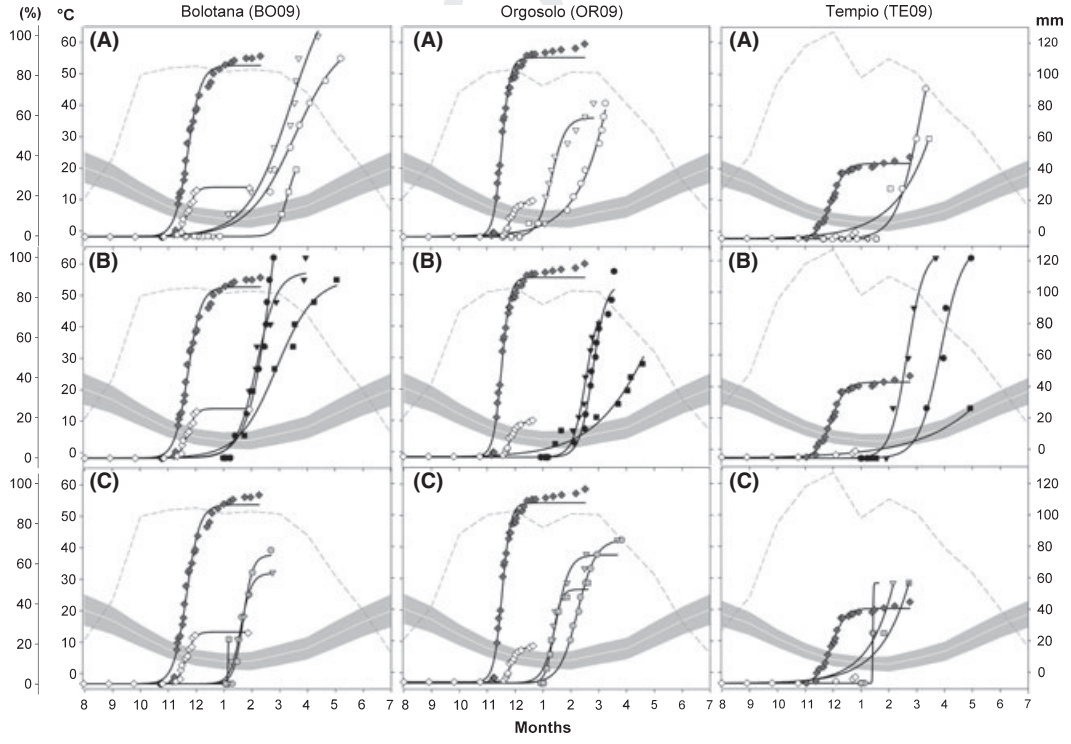

Fig. 5. Germination (root emergence) at 5 °C (white diamonds) and 10 °C (dark diamonds) after W treatment (25 °C for 90 days) and epicotyl emergence at 10 °C (circles), 15 °C (triangles) and 20 °C (squares) for each population, white symbols without any treatment (A); dark symbols after 2 months at 5 °C (B); grey symbols after 2 months with 250 $mg \cdot l^{-1}$ GA_3 solution (C): points correspond to actual data and solid lines indicate fitted lines from sigmoidal regressions. Annual trends of temperature (grey solid bands) and rainfall (grey medium dashed lines) are also reported for each population (data from WorldClim, http://www.worldclim.org); annual trends start from the month of natural dispersal (8: August).

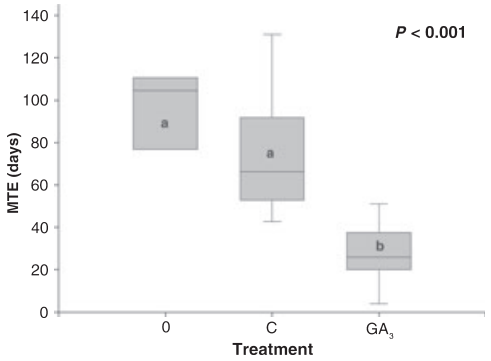

Fig. 6. Mean time of epicotyl emergence (MTE) for different treatments (0, control; C, 2 months at 5 °C; GA_3, 2 months with 250 mg·l^{-1} GA_3 solution), calculated as average values of all tested temperatures for all seedlots collected in 2009; $P < 0.001$ in one-way ANOVA; data are mean of 252 seedlings; boxes with the same letter are not different at $P > 0.05$ from *post hoc* Fisher's LSD test.

several studies to identify the pathway of water uptake into seeds (*e.g.* Tillman-Sutela & Kauppi 1998; Orozco-Segovia *et al.* 2007). However, this staining method is an indirect approach, as permeability of the seed coat may be different from that for water (MW: 18.01 g·mol^{-1}) and other, especially larger, molecules (*e.g.* methylene blue MW: 319.85 g·mol^{-1}). In a study of seed coat imposed dormancy in *Grevillea linearifolia* (Cav.) Druce (Proteaceae), Briggs & Morris (2008) found only small changes in permeability of high MW compounds (Lucifer yellow CH dilithium salt, MW: 457.25 g·mol^{-1}) between untreated and treated (heat shock and smoke) seeds, although the treatments increased germination. While potassium iodide dye (MW: 419.81 g·mol^{-1}) was the most effective marker for visualisation of water uptake into the starchy endosperm of wheat (*Triticum aestivum* L.) grains, methylene blue and Congo red (MW: 696.68 g·mol^{-1}) failed to infiltrate beyond the outer embryo tissue (Rathjen *et al.* 2009).

We identified the pathway of penetration for methylene blue solution into seed of *R. m. sandalioticum*. After cells of the gelatinous sarcotesta were imbibed, dye entered the seed through the raphe vascular bundle, reaching the chalazal region, where a tanniniferous chalazal plug (hypostase) prevented the solution from entering the endosperm. Despite the clear staining result, it is possible that water, being a much smaller molecule, reached the endosperm and embryo directly by penetrating the seed coat layers. However, it seems most likely that the bulk of water taken up by the seed followed the same pathway as the dye. Other methods, such as magnetic resonance micro-imaging (see Rathjen *et al.* 2009), will be needed to confirm the exact pathway(s) of water during imbibition of seeds of *R. m. sandalioticum*.

Morphophysiological dormancy

The embryo in seeds of *R. m. sandalioticum* is small at dispersal and must grow before radicle emergence. Therefore, following the dormancy classification system (Baskin & Baskin 1998, 2004), these seeds are morphologically dormant (MD). Generally, if embryos have only MD, growth is completed in a relatively short period, and seeds germinate in 30 days or less (Baskin & Baskin 2004). *R. m. sandalioticum* seeds germinated at low temperatures (≤15 °C) without any treatment; however, more than 60 days were required for reach maximum germination. After warm stratification and GA_3 treatment, seeds germinated (radicles emerged) in 30 days, due to increased embryo growth and seed germination rates. Pritchard *et al.* (1993) found similar germination behaviour for a geophyte from temperate deciduous woodland (*Arum maculatum* L., Araceae). These authors demonstrated that dispersed seeds of this species were receptive to a warm temperature phase prior to germination in the cold, leading to increased germination. GA_3 treatment also widened the temperature range for germination in *R. m. sandalioticum* seeds. Thus, seeds of this species also have a physiological component to dormancy (PD), and are morphophysiologically dormant (MPD). This is the first fully documented report of this class of seed dormancy in Grossulariaceae.

MPD is divided into nine levels: non-deep simple, intermediate simple, deep simple, deep simple epicotyl, non-deep simple epicotyl, deep simple double, non-deep complex, intermediate complex and deep complex (Baskin & Baskin 1998, 2004; Baskin *et al.* 2008). In addition, Jayasuriya *et al.* (2010) reported a new kind of epicotyl PD in a tropical Fabaceae species, where the delay in plumule emergence is not correlated to an underdeveloped embryo. Epicotyl MPD is found in Piperales, Liliales, Ranunculales, Saxifragales, Dipsacales and Boraginaceae (unplaced in Euasterids I), mainly in herbaceous species (Baskin & Baskin 1998; Baskin *et al.* 2008, 2009).

In the simple kinds of MPD, embryos grow at relatively high temperature (≥10 °C), while in complex kinds of MPD, embryos grow during cold stratification (Baskin *et al.* 2008). Interestingly, not only did pre-treatments starting with a cold stratification (C and C+W+C) fail to break PD in *R. m. sandalioticum* seeds but they imposed secondary dormancy, with delayed embryo growth and seed germination even at low temperatures. Thus, these seeds have a simple level of MPD. In addition, the delay detected between onset of seed germination (root emergence) and epicotyl emergence in this species can be described as a kind of simple epicotyl MPD.

Roots and shoots can have different levels of PD (Baskin & Baskin 1983, 1986); therefore, to describe dormancy in seeds with simple epicotyl MPD, the level of PD (deep, intermediate and non-deep; Baskin & Baskin 2004) in both the root and shoot must be described (Baskin *et al.* 2009). Warm stratification and GA_3 treatment enhanced embryo growth and subsequent seed germination at low temperatures; therefore, *R. m. sandalioticum* roots have non-deep PD. GA_3 treatment also positively affected epicotyl emergence, while the effect of cold stratification, even if positive, was not statistically significant. Therefore, epicotyls of *R. m. sandalioticum* have non-deep PD, and seeds have non-deep simple (root) – non-deep simple (epicotyl) MPD. Baskin *et al.* (2009) recently described this level of epicotyl MPD for another woody member of the Saxifragales (*Daphniphyllum glaucescens* Blume subsp. *oldhamii* (Hemsl.) var. *oldhamii* (Hemsl.), Daphniphyllaceae), and it was first described by Baskin *et al.* (2008) in another woody species, *Viburnum odoratissimum* Ker Gawl. (Caprifoliaceae). These authors found that seeds of the latter species did not match the 'classical'

deep simple epicotyl MPD for which cold stratification of seeds with emerged radicles is required for epicotyl emergence. In seeds of *V. odoratissimum* with an emerged radicle, there was a delay of one to several weeks for epicotyl emergence, depending on incubation temperature. However, unlike other species of *Viburnum* (such as *V. acerifolium* L., *V. dentatum* L. and *V. dilatatum* Thunb.), no cold stratification was required for epicotyl emergence (Baskin *et al.* 2008).

Ecological correlates of seed germination and seedling establishment

Berries of *R. m. sandalioticum* ripen in summer and dispersal takes place in August, mainly by endozoochory (birds and/or mammals), although many fruits simply drop to the ground (unassisted dispersal). Following dispersal, in late summer the seeds are exposed to warm temperatures (mean air temperature >20 °C), and from September onwards they also experience increased availability of water through higher rainfall. In intact seeds, water presumably first causes the matrix of the sarcotesta to swell so as to produce temporary water storage around the seed in the form of a gelatinous pulp, and then water penetrates through both the inner layers of the seed coat and the hilum, from where it travels through the raphe into the chalaza before entering the endosperm and finally reaching the embryo.

Once imbibed, embryos may start to grow inside the seeds. However, it is only when mean temperatures drop below 10 °C in November–December and following annual maximum precipitation that embryo growth reaches the critical E:S ratio (mean embryo length of *ca*. 1.80 mm), thereby allowing seeds to germinate. Germinated seeds go through the winter with an emerged radicle, and epicotyls emerge only after 2 months (April), when mean air temperatures again reach 10–15 °C. Seedling establishment is completed before the end of the wet season (June). Seedling growth can take place for 2 months, until the start of summer, so that the seedlings enter the dry summer period (June–August) with well-developed root and shoot systems.

Secondary dormancy of non-germinated seeds imposed by cold stratification prevents radicle emergence in late spring and exposure of recently emerged seedlings to the dry summer conditions that would most likely kill them.

Intraspecific variability

While seeds of the Bolotana and Orgosolo populations had a similar response to treatments and germination conditions, those from the highest population (Tempio) had low seed germination at both 5 and 10 °C. Variability in seed dormancy between and within seedlots in *Ribes* has been observed by several authors (Pfister 1974; Young & Young 1992; Rosner *et al.* 2003a,b). In particular, Rosner *et al.* (2003a,b) detected a latitudinal and altitudinal gradient in the effect of cold stratification on seeds of *Ribes cereum* Dougl from New Mexico. However, it is unlikely that the difference detected among *R. m. sandalioticum* populations was due to differences in dormancy breaking or germination requirements. Seeds were all collected in the same year (2009) and the three climatic diagrams highlight the same trends in temperature and rainfall. The low germination detected for the Tempio population may instead be due to poor quality of the seedlot, as suggested by the lower seed mass of these seeds compared to the other seedlots (see Table 1).

Variability in the level of seed dormancy was also detected between seeds collected in different years from the same population (Orgosolo). Seeds collected in 2008 were also able to germinate at 5 °C, with high percentage germination (>70%) after warm stratification; while seeds collected in 2009 failed to achieve more than 30% germination after the same pre-treatment. A widening of the temperature range for germination is effect of after-ripening, *i.e.* a period of usually several months of dry storage at room temperature of freshly harvested, mature seeds (Finch-Savage & Leubner-Metzger 2006). Mira *et al.* (2011) found that *Silene diclinis* (Lag.) M. Laínz seeds need more than 964 days of dry storage at 5 °C to lose dormancy. Therefore, it is likely that the higher germination at 5 °C in *R. m. sandalioticum* seeds after 9 months (*ca*. 270 days) of dry storage at 5 °C may be due to incipient after-ripening during storage. The same process could also explain the high germination without any pre-treatment reported in Liu *et al.* (2008) for seed bank collections of several *Ribes* species. Therefore, regardless of age, it is likely that the seeds had the same level of MPD at the time of dispersal.

CONCLUSIONS

Embryo growth and seed germination of *R. m. sandalioticum* were positively affected by both warm stratification followed by cold temperatures and by GA_3 treatment. Embryos were small at dispersal and needed to increase length three-fold to reach the critical embryo:seed ratio required for germination. GA_3 also had a positive effect on epicotyl emergence, which was delayed with respect to root emergence. Based on these results, the presence of a non-deep simple (root)–non-deep simple (epicotyl) MPD is reported for seeds of this species. A more extensive study should be carried out in order to investigate MPD in the genus *Ribes*, as also suggested by Baskin & Baskin (2007), through a comparison of dormancy-breaking treatments and germination requirements in several species of this genus. Seeds of the investigated species showed high specialisation with respect to water uptake, seed germination and seedling establishment, which were synchronised with the Mediterranean seasonality in all the investigated populations. Moreover, the sensitivity of this species to low temperatures for seed germination highlighted an increasing threat from global warming, which could reduce the level of natural emergence in the field. The reported data may help establish *ex situ* conservation programmes for this rare species using seed banking and multiplication in nurseries and to initiate seed ecology studies on the very threatened congener *R. sardoum*.

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