Forest Systems
29 (1), e017, 13 pages (2020)
eISSN: 2171-9845
https://doi.org/10.5424/fs/2020292-15932
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Different levels of morphophysiological seed dormancy in *Ribes alpinum* and *R. uva-crispa* (Grossulariaceae) facilitate adaptation to differentiated habitats

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Abstract

Aim of study: To study the germination ecology of two species of the genus Ribes to reveal their levels of morphophysiological dormancy (MPD) and to facilitate the production of plants from seeds, a key tool for population reinforcement.

Area of study: Experiments were carried out both outdoors and in the laboratory in Albacete (Spain) with seeds from the Meridional Iberian System mountain range.

Material and methods: Seeds from one population of *Ribes alpinum* and from other of *Ribes uva-crispa* were collected during several years. Embryo length, radicle and seedling emergence, and effects on germination of stratification and GA_3 were analysed to determine the level of MPD.

Main results: In *R. alpinum*, embryo length in fresh seeds was 0.49 mm, needing to grow to 1.30 mm to germinate. Warm stratification (25/10ºC) promoted embryo length enlargement to 0.97 mm. Afterwards, seeds germinated within a wide temperature range. Embryo growth and seedling emergence occur late summer-early autumn. In *R. uva-crispa*, embryo length in fresh seeds was 0.52 mm, being 2.10 mm the minimal size to germinate. Embryos exposed to a moderately warm stratification (20/7ºC + 15/4ºC) followed by cold (5ºC) grew to 2.30 mm. Then, seeds germinated ≥ 80% when incubated at temperatures ≥ 15/4ºC. Embryos grew in autumn/early winter, and seedlings emerged late winter-early spring.

Research highlights: These results showed that R. alpinum seeds have a nondeep simple MPD while R. uva-crispa seeds have a nondeep complex MPD. Moreover, the different germinative models found for each species help explain their installation in distinct habitats.

Keywords: *Ribes*; seed dormancy break; radicle emergence; seedling emergence; nondeep simple and nondeep complex MPD.

Abbreviation used: Morphophysiological dormancy (MPD), morphological dormancy (MD), Gibberellic acid (GA_3), months (m).

Authors' contributions: Conceived and designed the experiments: RH and JMH. Performed the experiments: RH, JMH and MC. Analyzed the data: RH, EC and MC. Wrote the paper: RH, JMH and PF. Critically reviewed the manuscript for important intellectual content: EC and MC.

Citation: Herranz, R., Copete, M.A., Herranz, J.M., Copete, E., Ferrandis, P. (2020). Different levels of morphophysiological seed dormancy in *Ribes alpinum* and *R. uva-crispa* (Grossulariaceae) facilitate adaptation to differentiated habitats. Forest Systems, Volume 29, Issue 2, e017. https://doi.org/10.5424/fs/2020292-15932.

Supplementary material: Table S1 accompanies the paper on FS's website.

Received: 24 Oct 2019 **Accepted:** 28 Sep 2020

Funding agencies/institutions	Project / Grant
Regional Plan of Castilla-La Mancha for Research and Technological Development	Germination ecology of 12 singular or threatened plant species with morphophysiological dormancy, PEII10-0170-1830

Competing interests: The authors declare that no competing interests exist.

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Introduction

Seed dormancy plays an important ecological role by allowing the fine-tuning of germination timing to favour seedling establishment (Forbis *et al.*, 2002). Requirements for seed dormancy loss and germination are specific for each species and depend on phylogeny, life cycle, geographic distribution and habitat (Finch-Savage & Leubner-Metzger, 2006). Within phylogenetically close species, variation in dormancy depth or germination

requirements has been related to habitat differences (Donohue, 2005; Porceddu *et al.*, 2017). This study analyses the correspondence between germinative ecology and the habitat in two *Ribes* genus species.

Ribes L. (Grossulariaceae) is a genus of ca. 200 species distributed in the temperate regions of the Northern Hemisphere and the Andes, with many taxa being cultivated for their edible fruits or for ornamental purposes (Mabberley, 2008; Mattana *et al.*, 2012). Upon dispersal, *Ribes* seeds are characterised by a linear underdeveloped embryo (Ruiz de la Torre, 2006). According to the seed dormancy classification system, the dormancy of these seeds is morphological (MD) or morphophysiological (MPD). If embryos grow and germinate in ≤ 30 days, they are reported to have MD, but have MPD if they require dormancy-breaking treatment to overcome their physiological component of seed dormancy (Baskin & Baskin, 2004). Mattana *et al.* (2012) have reported the presence of a nondeep simple (root)-nondeep simple (epicotyl) MPD for seeds of *Ribes multiflorum* Kit. subsp. *sandalioticum* Arrigoni. Mattana *et al.* (2014) have also documented MD for most seeds of *Ribes alpinum* L. and *R. speciosum* Pursh, and MPD for the seeds of *Ribes roezlii* Repel, *R. hudsonianum* Richardson and *R. nevadense* Kellogg, but have not specified MPD levels. Mattana *et al.* (2012, 2014) have also indicated that further studies are needed before excluding a physiological component of dormancy for the seeds of *Ribes alpinum* and *R. speciosum*, and that more extensive research is necessary to investigate MPD in the *Ribes* genus by comparing dormancy-breaking treatments and germination requirements in several this genus' species. We herein analyse the germinative patterns of one *R. alpinum* population and a *R. uva-crispa* population located in the Meridional Iberian System (central-eastern Spain) to contribute the knowledge of the germination ecology of the *Ribes* genus. No information is available about *R. uva-crispa* germination, while that available for *R. alpinum* results from studying a single population in England (UK) by Matanna *et al.* (2014). Thus, a comparative analysis from a biogeographically contrasting habitat may be a determining factor to advance in knowledge of the germination ecology of *Ribes*.

Ribes alpinum (subgenus *Berisia*) is a deciduous, non-thorny dioecious shrub that is distributed all over Europe and North Africa. In the Iberian Peninsula, it spreads over the northern region and has disjunct populations in the Baetic Mountains, where it occupies shady spots in deciduous forests and thorny scrublands. *Ribes uva-crispa* (subgenus *Grossularia*) is a deciduous, thorny hermaphrodite shrub that is distributed all over Europe, central-western Asia and North Africa. In the Iberian Peninsula, it spreads over the northern region, inhabiting *Juniperus* creeping scrublands, mountain pine forests and calcareous rocks. It tolerates sunny exposure and open gaps better than *R. alpinum* (Blanca, 1997; Ruiz de la Torre, 2006).

In the Meridional Iberian System, *R. alpinum* lives exclusively in shady spots, while *R. uva-crispa* can establish in both shady and sunny exposures, a condition that is the major differential characteristic of their habitats. It is known that seed germination in a natural environment is often restricted to locations that meet specific environmental conditions, which are often referred to as regeneration niches (Grubb, 1977; Vandelook *et al.*, 2008). The local segregation of the habitat distribution of both *Ribes* species may imply differences in the dormancy breaking and germination requirements, which would thus drive to the differentiation of regeneration niches. In line with this, Donohue (2005) indicated that germination responses to environmental factors can be exquisitely precise mechanisms of habitat choice in plants, and that certain environmental conditions must be present to enable germination after dormancy is broken.

Moreover, it is known that temperature variation during seed maturation can produce phenotypic plasticity in dormancy or result in sensitivity to dormancy breaking factors (Fenner, 1991; Fernández-Pascual & Jiménez-Alfaro, 2014). This plasticity will play a major role in plant responses to climate change (Nicotra *et al.*, 2010; Fernández-Pascual & Jiménez-Alfaro, 2014). Thus, another goal of this work is to evaluate the inter-annual variability in germination ability.

The studied species are of much forestry interest because they are relevant components of habitats of priority conservation, such as pine forests of *Pinus nigra* subsp. *salzmannii* and deciduous Euro-Siberian communities (forests of *Tilia platyphyllos* and forests of *Populus tremula*). In addition, both *R. alpinum* and *R. uva-crispa* have very small populations in the inland Iberian Peninsula (*i.e.*, Castilla-La Mancha region), which means they have been included in catalogues of threatened species (DOCM, 1998). The maintenance of these habitats and populations in a favourable conservation state may require population reinforcement programmes (Martín-Herrero *et al.*, 2003). The production of plants associated with those conservation actions needs precise knowledge about the germinative ecology of target species, which is not available for these *Ribes* taxa in the synthetic study of Pemán *et al.* (2012) on the germination and propagation of forestry Iberian trees and shrubs.

In this context, the main goal of this study is to verify the following hypotheses: (1) *R. alpinum* and *R. uva-crispa* have differentiated germinative ecologies; (2) in *R. alpinum*, the seed fraction that does not have MD has a different MPD level to that in R. uva-crispa. Consequently, to reveal the germination ecology of *R. alpinum* and *R. uva-crispa*, the specific aims of this study are to analyse:

a. Phenology of embryo growth, dormancy break and radicle emergence
b. Phenology of seedling emergence
c. Effects of temperature and gibberellic acid on embryo growth
d. Effects of stratification conditions, seed storage time and year of collection on germination
e. Dormancy induction by cold stratification temperatures.

Material and Methods

Plant material and seed source

a) *Ribes alpinum*

This species flowers from March to May and its fruits are red berries that ripen between July and August, with 4-12 seeds each. Fruits were collected in Orea (Guadalajara province, Meridional Iberian System, central-eastern Spain) in a deciduous thorny scrubland shady spot, 1550 m.a.s.l., 30TXK0690, in a population of c.a. 50 *R. alpinum* individuals living with *Rosa pimpinellifolia*, *Rosa sicula*, *Prunus spinosa*, *Lonicera xylosteum* and *Rhamnus alpina*. On 9 July 2014, 15 July 2015, and 13 July 2018, we collected 350, 600, and 1200 berries from all the seed-bearing plants, respectively. Due to its sensitivity to late frosts and/or droughts, this population did not produce fruits in 2016 and 2017. Berries were macerated in the laboratory under a cold water stream which gave a seeds/pulp mixture, and then dried for 48 h to subsequently separate seeds by a sieve set. Dried seeds were spread on trays until 1 August. At this point (seed age = 0 month), seeds were stored in paper envelopes at room temperature in the laboratory (22-24ºC; RH. = 40-50%).

For planning the subsequent germination experiments under laboratory conditions, preliminary studies with the seeds collected in 2014 were done. These studies showed null germination in the 0-month-old seeds incubated for 30 days at the mean maximum and mean minimum monthly temperatures occurring in the natural habitat all year long: 15/4ºC corresponded to months of November and March; 20/7ºC to October and April; 25/10ºC to September and May; 28/14ºC to June and August; 32/18ºC to July. The 5ºC treatment simulated the mean temperature recorded in winter months: December, January and February (Elías & Ruiz, 1981). However, seeds germinated after 1 month of warm stratification (25/10ºC), followed by incubation at autumn (20/7ªC) temperatures. At the seed dispersal time, the mean embryo length (E) was 0.49 mm (se = 0.003, n = 25). To determine this trait, embryos were excised from imbibed seeds with a razor blade and their lengths were measured under a dissecting microscope equipped with a micrometer. Endosperm length (S) was 2.92 mm (se = 0.12, n = 25). Hence, the E:S ratio was 0.17, which suggested that the embryo was underdeveloped. The critical embryo length for radicle emergence is embryo length at the time that the seed coat splits, but immediately before the radicle emerges (Vandelook & Van Assche, 2008). In *R. alpinum*, it was 1.68±0.03 mm (m ± se, n = 25) range 1.30-2.10 mm. The minimum value required to germinate (1.30 mm) is a reliable indicator that dormancy is being overcome (Copete *et al.*, 2011).

b) Ribes uva-crispa

The timing of flowering is close to that of *R. alpinum*, and its fruits are green-yellow berries containing 6-15 seeds each. Fruits were collected near the *R. alpinum* location: Orea (Guadalajara province), at 1,540 m.a.s.l., 30TXK0891, in a mixed scrubland with *Juniperus sabina*, *Rhamuns saxatilis*, *Genista scorpius* and *Berberis vulgaris* subsp. *seroi*. This community was located at the bottom of calcareous rocks with southern exposure containing a *R. uva-crispa* with about 40 individuals.

On 9 July 2014, 15 July 2015, 24 July 2016, and 19 July 2017, we collected 500, 700, 1200 and 900 berries from all the seed-bearing plants, respectively. On 2 July 2017, 300 berries were also collected at the Botanical Garden of Castilla-La Mancha, located in Albacete (central Spain). Seed extraction and storage operations were similar to those described for *R. alpinum*. The preliminary studies conducted with the fresh seedlots of 2014 also showed null germination with the temperature interval tested on *R. alpinum*. However, seeds were germinated when stratified in light at the following monthly sequence of temperatures: 20/7ºC + 15/4ºC + 5ºC + 5ºC + 5ºC, and then incubated for 30 days at 15/4 or 20/7ºC. At the seed dispersal time, the mean embryo length was 0.52±0.04 mm (m ± se, n = 25). The endosperm length (S) was 3.00±0.19 mm (m ± se, n = 25). Hence the E:S ratio was also 0.17 and underdeveloped embryos were revealed. The critical embryo length for radicle emergence was 2.42±0.09 mm (m ± se, n = 25) range 2.10-2.80 mm. So, the minimal value to germinate was 2.10 mm.

Outdoor experiments

The aim of these studies (Table S1 [suppl.]) was to determine the timing of the main events in the seed/seedling stage of the life cycles of *R. alpinum* and *R. uva-crispa* in relation to the seasonal temperature cycle. This was determined with the seeds kept under near natural temperature conditions in a non-heated metal frame shadehouse located in the experimental field of the Technological School

of Agronomy and Forestry in Albacete (Castilla-La Mancha, central Spain), 200 km from the seed collection sites at 690 m.a.s.l. The air temperature in the shadehouse was continuously recorded by a data logger to assess the monthly averages of both maximum and minimum temperatures.

The growing medium in pots and trays containing the seeds was a mixture of sterilized peat and sand (2:1 v/v). To simulate the soil humidity conditions in the natural habitat, the water control system was programmed to water to field capacity once weekly, but it was reduced to twice monthly in July and August to simulate the summer drought that is common in the Mediterranean region. In addition, water was withheld when the substratum was frozen in winter.

Phenology of embryo growth, dormancy break and radicle emergence

On 1 September 2015, 0-month-old seeds grouped in lots of 100 seeds. Each lot mixed with fine-grained sterilised sand was placed into a fine-mesh polyester cloth bag. Five bags of *R. alpinum* were buried 5-cm deep in a pot with sand and other ten bags of *R. uva-crispa* in another pot. Both pots were placed in the shadehouse. Bags were exhumed each month, starting on 1 October 2015, and content was sieved (1 mm) to separate seeds from sand. The percentages of seeds with emerged radicles and the mean embryo length were recorded. To do so, embryos were excised from 25 healthy-looking seeds and their lengths were measured by a micrometer. In the radicle-emerged seeds, embryo length was recorded as the critical embryo length f or germination.

The non-germinated exhumed seeds, and those not used to measure embryo length, were incubated for 30 days at 20/7ºC in the light. After incubation, it was possible to calculate the following seed status percentages: (1) seeds whose radicle emerged in bags; (2) viable non-dormant seeds (*i.e.*, those germinating during incubation at 20/7ºC); (3) viable dormant seeds (*i.e.*, those that did not germinate at 20/7ºC, but had healthy embryos); (4) non-viable seeds (*i.e.*, those with a rotting appearance or showing a dead embryo when excised).

Phenology of seedling emergence

On 1 September 2015, for each species three trays (20 x 30 x 8 cm) with drainage holes were filled with the growing medium. On each tray, 100 seeds were sown 3-4 mm deep, and were placed equidistant from one another to avoid them coming into contact. The three replicates of each species were placed in the shadehouse. From September 2015 to April 2018, seed trays were examined once weekly, and the emergent seedlings were counted and removed.

Laboratory experiments

Experiments (Table S1 [suppl.]) were conducted in chambers with controlled temperature and light regimes (Ibercex model F-4, Madrid, Spain), equipped with a digital temperature and a light control system [± 0.1ºC, cool white fluorescent light, 25 µmol m^{-2} s^{-1} (1325 lux)]. Seeds were tested for embryo growth and radicle emergence during a 12-hour daily photoperiod (hereafter light) and in continuous dark (hereafter darkness). This was achieved by wrapping Petri dishes in a double layer of aluminium foil, and being placed in a constant temperature (5ºC) and in several alternating temperature regimes (15/4, 20/7, 25/10, 28/14, and 32/18ºC). In the 12/12 h alternating temperature treatments, the higher temperature coincided with the light phase, and the lower temperature with darkness. Seeds were stratified and incubated in 9 cm-diameter Petri dishes on two layers of filter paper moistened with distilled water. Dishes were sealed with parafilm to minimise water loss.

The alternating temperature regimes (5, 15/4, 20/7, 25/10, 28/14, 32/18ºC) simulated the mean maximum and mean minimum monthly temperatures characterising the annual climate cycle in continental inland regions of the Iberian Peninsula, as was done in the preliminary experiments.

The germination percentage (radicles emerged ≥ 0.5 mm, clearly visible) was computed based on the number of apparently viable seeds. The non-germinated seeds were checked for viability on the basis of embryo appearance by paying special attention to both colour and turgidity. Seeds were considered viable if the embryo was white colour and resisted slight pressure applied by using tweezers. This method is commonly used in germination studies based on many experiments (Hidayati *et al.*, 2001; Walck *et al.*, 2002). Moreover, these indicators of seed viability well agree with the results obtained when using a tetrazolium test (Hidayati *et al.*, 2001).

Effects of temperature and gibberellic acid on embryo growth

The aim of this section was to determine the optimal temperature for embryo growth and the effect of GA_3.

a) *Ribes alpinum*

The experiment started on 1 August 2015 to evaluate the effect of moderately warm (20/7 or 25/10ºC) or cold

(5ºC) stratification on embryo growth. Three 100-seed lots were prepared and each was placed in a Petri dish. Dishes were distributed at 5ºC in light, 20/7ºC in light and 25/10ºC in light, respectively. After 30 and 60 days of stratification, 25 seeds were extracted from each dish, and embryos were excised and measured, to assess mean lengths. After 30 days of stratification at 5ºC or 25/10ºC, a sample of 25 seeds from each temperature was transferred to 20/7ºC for 30 days (Treatment A and Treatment B, respectively). After incubation, embryos were excised and measured.

To determine the effect of gibberellic acid (GA_3) on embryo growth, on 1 August 2015, 100 seeds were placed at 20/7ºC in light on two sheets of filter paper moistened with a solution of 1,000 mg l^{-1} of GA_3 for 30 and 60 days. Then the embryos of 25 seeds were measured by assuming the critical embryo length as the real embryo length in the germinated seeds for the calculations. The results were compared with those of the seeds incubated at 20/7ºC in light with distilled water.

b) Ribes uva-crispa

This experiment was conducted to determine the effect of warm (25/10ºC), cold (5ºC) or moderately warm + cold (20/7 ºC + 15/4ºC + 5ºC) stratification on embryo growth. On 1 August 2015, three 200-seed lots were each placed in a Petri dish on two layers of filter paper moistened with distilled water. Dishes were stratified in light for 6 months: one at 25/10ºC, the second at 5ºC, and the third was exposed to the following monthly sequence of temperatures, 20/7ºC + 15/4ºC + 5ºC + 5ºC +5ºC +15/4ºC (Treatment A), which simulates the temperatures of autumn, winter, and early spring. A 25-seed sample was extracted every 30 days from each dish, and embryos were excised and measured.

To determine the effect of GA_3 on embryo growth, on 1 August 2015, two 150-seed lots were each placed in a Petri dish on filter paper moistened with a solution of 1,000 mg^{-1} of GA_3 in distilled water and stratified in light. One dish was permanently exposed to 15/4ºC. A 25-seed sample was extracted after 30, 60, and 90 days, and embryos were excised and measured. The other dish was exposed to the following monthly sequence of temperatures: 5ºC + 5ºC +5ºC +15/4ºC (Treatment B). Embryo length was measured every month as indicated above to test if the embryos of the seeds hydrated with GA_3 and submitted to Treatment B could develop without the mediation of moderately warm stratification (20/7ºC + 15/4ºC) prior to cold stratification. As a control, we measured the embryo growth of seeds not watered with GA_3 but submitted to a similar temperature treatment to Treatment B or submitted to stratification at 15/4ºC.

Effects of stratification conditions, seed storage time and year of collection on germination

a) *Ribes alpinum*

In 2015, as only a few seeds were collected (about 3500), only a control test was performed with the 0-month-old seeds incubated at the temperature sequence indicated for the preliminary tests in 2014, along with another test with the 0-month-old seeds submitted to warm stratification. On 1 August 2015, 1,250 seeds were placed in a 16 cm-diameter Petri dish and were stratified at 25/10ºC in light for 30 days. Then seeds were incubated for 30 days at the six temperature conditions in both the light and darkness.

On August 2018, the above-described test was repeated with the 0-month-old seeds. As more seeds were collected that year (about 6,500), it was possible to evaluate the influence of seed age on germination ability. On 1 February 2019 (seed age= 6 months), 1,400 seeds were placed in a 16 cm-diameter Petri dish and stratified at 25/10ºC in the light for 30 days. Then they were incubated for 30 days under the six temperature conditions.

b) *Ribes uva-crispa*

The stratification treatment that the seeds collected in 2015 underwent started on 1 May 2016 (seed age= 9 months), once the test of the effect of temperature on embryo growth had finished. Firstly, 1,250 seeds were placed in both of the 16 cm-diameter Petri dishes to be stratified in the light. One dish was submitted to cold (5ºC) stratification for 5 months and another to moderately warm plus cold stratification according to the following monthly sequence: 20/7ºC + 15/4ºC + 5ºC + 5ºC + 5ºC. This was done to simulate autumn-winter conditions. After those treatments, the seeds in both dishes were incubated under the six temperature conditions in both the light and darkness.

The seeds collected in 2016 and 2017 were also submitted to the same moderately warm plus cold stratification treatment described above. A sufficient available amount of seeds enabled the influence of seed age on germination ability and its inter-annual variability to be evaluated.

Dormancy induction by cold stratification temperatures

This test was conducted only on *R. alpinum* as its seeds surpass dormancy after an exposure period to warm temperatures. The aim of this trial was to assess if low temperatures (5ºC) occurring in winter could induce secondary physiological dormancy in seeds whose primary physiological dormancy has been overcome and their embryos have started growing, but are not completely elongated.

On 1 August 2015, 200 seeds were placed in a 9 cm-diameter Petri dish and stratified for 1 month at 25/10ºC in the light + 1 month at 5ºC in the light. After this period, 100 of these seeds (4 replicates of 25 seeds) were incubated at 20/7ºC for 30 days, and 100 seeds were incubated at that temperature for 60 days. The results (germination percentage and embryo length) were compared to those obtained in the control test: 1 month at 25/10ºC in the light + 1 month 20/7ºC in the light.

Statistical analysis

Means and standard errors were calculated for embryo length and germination percentage. In each plant species, the effects of different stratification treatments and GA_3 on embryo length were analysed by multifactor analysis of variance (ANOVAs) using Statgraphics centurion XVI. Germination was evaluated by the percentage of the number of apparently viable seeds and was compared among different factors (incubation conditions, seed storage time, year of seed collection and seed population) by a multifactor ANOVAs. When the effect of a factor was significant, differences were detected by a multiple comparison Tukey test. Prior to the analyses, data normality (Cochran test) and homoscedasticity (David test) were checked. The values expressed as percentages were arcsine square root-transformed before the analyses.

Results

Outdoor experiments

Phenology of embryo growth, dormancy break and radicle emergence

a) *Ribes alpinum*

In the test carried out on 1 September 2015, embryos grew from 0.50 to 1.25 mm in September. During this month, the maximum and minimum mean temperatures were 25.7 and 11.3ºC, respectively. In October, embryos grew from 1.25 to 1.58 mm; early in October, 82% of the seeds had already broken dormancy; and 78% of the seeds had emerged radicles by the end of this month (Fig. 1A, 1B). Early in December, embryos had reached the critical length (1.70 mm) and 99% of the seeds had emerging radicles (Fig. 1B, 2A).

b) *Ribes uva-crispa*

At the beginning of the burial experiment on 1 September 2015, the mean embryo length was 0.52±0.04 mm. Embryos grew slowly between this date and 1 November, when the mean embryo length was 1.09±0.06 mm. During this period, the mean maximum and minimum daily temperatures were 23ºC and 10ºC, respectively. However, between 1 November 2015 and 1 February 2016, when the mean maximum and minimum temperatures were 12ºC and 1ºC, respectively, embryos grew faster. Thus, on 1 February, embryo length was 2.33±0.05 mm (Fig. 1A, 1C), but most seeds were still dormant (Fig 2B). On 1 March 2016, embryo length was 2.39±0.05 mm, 17% of the seeds had emerged radicles, and 60% of the seeds had already overcome dormancy. On 1 May 2016, embryos had achieved the critical length (2.40 mm), 62% of the seeds had emerged radicles, and 21% of the seeds were non-dormant viable. These values hardly varied in the seeds exhumed on 1 June (Fig. 1A, 1C, 2B).

Phenology of seedling emergence

a) *Ribes alpinum*

Although no seedlings had yet emerged on 1 October 2015, at the end of this month emergence was 72%. Seedling emergence was 82% on 1 December 2015. This figure did not increment during the rest of the study and, thus, some seeds with emerged radicles (17%) failed to produce seedlings. There was barely any delay between

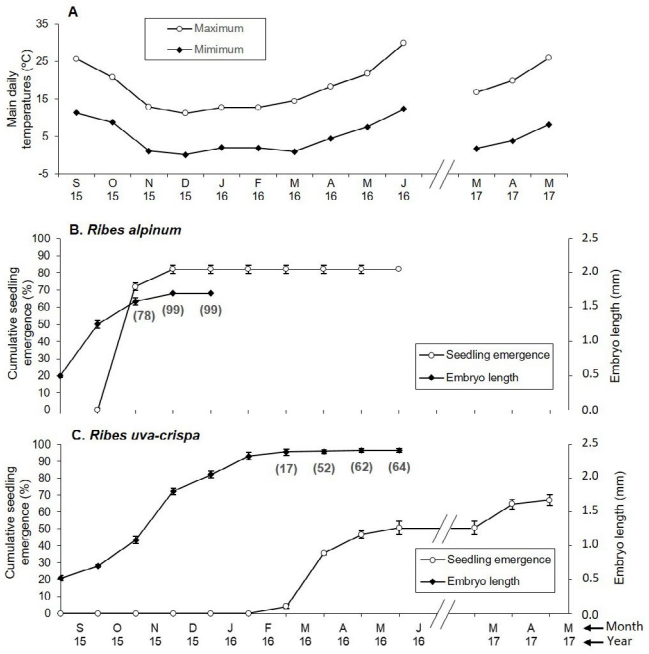

Figure 1. Mean monthly minimum and maximum air temperatures (A) and phenology of embryo growth (mean±SE; n=25) and seedling emergence (mean±SE; n=3) in the *Ribes alpinum* (B) and *R. uva-crispa* (C) seeds sown in September 2015. Numbers in parentheses next to embryo length indicate radicle emergence (%, if > 0).

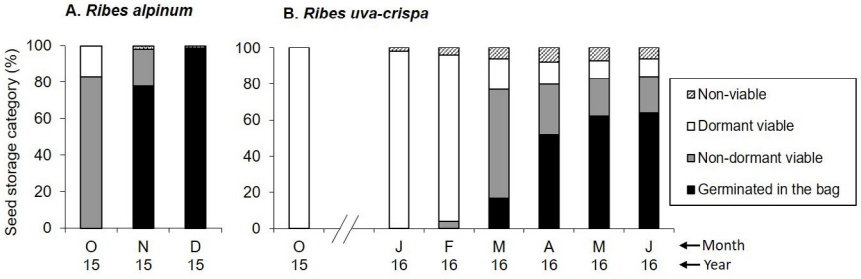

Figure 2. Changes in the percentage of dormant, non-dormant, non-viable and germinated seeds of *Ribes alpinum* (A) and *R. uva-crispa* (B) buried in September 2015 and exhumed monthly over a 9-month period.

radicle and seedling emergence, and on 1 November 2015 the radicle and seedling emergence percentages were similar (Fig. 1B).

b) *Ribes uva-crispa*

Cumulative seedling emergence was 4% on 1 March 2016 and 35% on 1 April 2016. On these dates, radicle emergence was 52%, with hardly any delay (1-2 weeks) between radicle and seedling emergence. Cumulative seedling emergence increased to 50% on 1 June 2016. Then seedling emergence stopped until March and April 2017, before reaching an accumulate value of 67% at the end of this period. No seedling emerged from 1 May 2017 (Fig. 1C).

Laboratory experiments

Effects of temperature and gibberellic acid on embryo growth

a) *Ribes alpinum*

In the cold-stratified seeds (5ºC in light) left for 2 months, embryos hardly grew and their length was 0.56±0.03 mm at the end of this period. The seeds exposed to stratification Treatment A (1 month at 5ºC in the light + 1 month 20/7ºC in the light), embryos hardly grew, and no seed reached the minimum embryo length required to germinate. In the seeds submitted to warm stratification (25/10ºC in light) for 1 month, embryo length doubled in the first month (to 0.97±0.10 mm), but growth in the second month was almost null. The most marked embryo growth was recorded in the seeds undergoing Treatment B (1 month at 25/10ºC in the light + 1 month at 20/7ºC in the light), with a mean of 1.66±0.04 mm that comes very close to the critical embryo length value (1.70 mm). Indeed 96% of the germinated seeds and the embryos grew to the minimal length required to germinate in them all (Table 1).

Gibberellic acid (GA_3) stimulated embryo growth and promoted germination. In the seeds treated with this hormone and incubated at 20/7ºC for 1 month, embryo length and germination were 1.68±0.02 mm and 88%, respectively. In contrast, the embryo elongation in the seeds not treated with GA_3 reached 0.92±0.04 mm and germination was null under the same incubation conditions (Table 1).

b) *Ribes uva-crispa*

In the cold-stratified seeds (5ºC in light) left for 6 months, embryos hardly grew and their length was 0.67±0.01 mm at the end of this period. Similarly, embryos barely responded in the seeds submitted to warm stratification (25/10ºC in light) for 6 months: they had grown to 0.75±0.02 mm at the end of this period. In contrast, in the

Table 1. Embryo growth (mean±SE, n=25) in the *Ribes alpinum* seeds undergoing different stratification treatments for 2 months (m). Treatment A: 5ºC (1 m) + 20/7ºC (1 m). Treatment B: 25/10ºC (1 m) + 20/7ºC (1 m). Values followed by different uppercase letters in a column or different lowercase letters in a row significantly differ ($P < 0.05$). The first number in parentheses is the germination percentage, and the second number is the percentage of seeds whose embryo is longer than or equals the critical embryo length required to germinate (1.3 mm)

		Stratification temperature					
		5ºC	**20/7ºC**	**25/10ºC**	**TREAT. A**	**TREAT. B**	**20/7ºC+GA_3**
Stratification length (m)	1	0.51 ± 0.01^{aA} (0,0)	0.92 ± 0.04^{bA} (0,4)	0.97 ± 0.04^{bA} (0,4)	0.51 ± 0.01^{aA} (0,0)	0.97 ± 0.04^{bA} (0,4)	1.68 ± 0.02^{cA} (88,96)
	2	0.56 ± 0.01^{aB} (0,0)	1.37 ± 0.09^{cB} (36,60)	0.99 ± 0.04^{bA} (0,12)	0.70 ± 0.02^{aB} (0,0)	1.68 ± 0.02^{dB} (96,100)	1.7 ± 0.00^{dA} (100,100)

seeds submitted to the moderately warm-cold-moderately cold stratification sequence to simulate autumn-winter-early spring conditions (Treatment A), embryo growth was sustained throughout the trial, although the most pronounced growth was recorded during cold stratification, when length increased from 1.12±0.16 to 2.30±0.24 mm. In the month following exposure to 15/4ºC, embryos hardly increased, but the germination percentage went from 4% to 60%, and 84% of the seeds achieved the minimal embryo length (2.10 mm) required to germinate (Table 2).

The GA_3 treatment elongated embryos and promoted germination without having to submit seeds to a moderately warm stratification phase preceding a cold one. Thus, in the seeds imbibed in the GA_3 dissolution and submitted to Treatment B, embryo length reached 2.02±0.12 mm and 64% of seeds germinated, in contrast to seeds not hydrated with GA_3, where embryo length and seed germination were 0.77±0.02 mm and 0%, respectively. In the seeds hydrated with GA_3 and permanently stratified at 15/4ºC in the light, embryos grew to 2.22±0.06 mm and the seeds germinated to 56% after 60 days (Table 2). The seeds imbibed in distilled water and stratified under the same conditions gave an embryo length of 1.18±0.06 mm and null germination after the same period (data not shown in Table 2).

Effect of stratification conditions, seed storage time and year of seed collection on germination

a) *Ribes alpinum*

In the control test conducted with the seeds collected in 2015, germination was null for all temperature and lighting conditions. After the warm stratification (1 month at 25/10ºC in light), germination surpassed 90% when seeds were incubated at 20/7ºC in both the light and darkness. At 15/4ºC, germination was below 20% and was null at the other incubation temperatures (Fig. 3).

The seeds collected in 2018 showed significantly higher germination percentages than those from 2015 at any incubation temperature, except for 20/7ºC. At 25/10 and 28/14ºC, the germination percentages increased with seed age, and exceeded 90% when seeds reached the age of 6 months (Fig. 3). During the warm stratification treatment conducted with the 6-month-old seeds, 10% of them germinated.

The lighting conditions did not influence the final germination percentages (Fig. 3).

b) *Ribes uva-crispa*

The cold stratification treatment at 5ºC that lasted 5 months did not promote germination, and no seed germinated when seeds were transferred to the incubation temperatures (data not shown otherwise). In the 9-month-old seeds from 2015, the germination percentages after the moderately warm and cold temperature stratification (1 month at 20/7 + 1 month at 15/4ºC + 3 month at 5ºC) exceeded or came close to 60% at any incubation temperature, regardless the lighting conditions, except for 5ºC at which germination did not reach 30% (Fig. 4). With the seeds collected in 2016 and 2017, the germination trends after this treatment were similar, but inter-annual differences were clearly manifested. So the seeds from 2016 obtained lower values than those from 2017.

Table 2. Embryo growth (mean±SE, n=25) in the *Ribes uva-crispa* seeds undergoing different stratification treatments. Treatment A: 20/7ºC (1 m) + 15/4ºC (1 m) + 5ºC (3 m) + 15/4ºC (1 m). Treatment B: 5ºC (3 m) + 15/4ºC (1 m). Values followed by different uppercase letters in a column or different lowercase letters in a row significantly differ ($P < 0.05$). The first number in parentheses is the germination percentage, and the second one is the percentage of seeds whose embryo is longer than or equals the critical embryo length required to germinate (2.1 mm)

		Stratification temperature					
		5ºC	**25/10ºC**	**TREAT.A**	**TREAT. B**	**TREAT.B+GA_3**	**15/4ºC+GA_3**
Stratification length (m)	1	0.55 ± 0.01^{aA} (0,0)	0.64 ± 0.01^{abA} (0,0)	0.66 ± 0.02^{bcA} (0,0)	0.55 ± 0.01^{aA} (0,0)	0.76 ± 0.03^{cA} (0,0)	1.64 ± 0.06^{dA} (0,12)
	2	0.56 ± 0.02^{aAB} (0,0)	0.64 ± 0.02^{aA} (0,0)	1.12 ± 0.07^{bB} (0,0)	0.56 ± 0.02^{aA} (0,0)	1.36 ± 0.10^{cB} (0,0)	2.22 ± 0.06^{dB} (56,84)
	3	0.60 ± 0.01a^{ABC} (0,0)	0.67 ± 0.02^{aA} (0,0)	1.88 ± 0.05^{bC} (0,24)	0.60 ± 0.01^{aA} (0,0)	1.69 ± 0.14^{bCB} (8,44)	2.35 ± 0.04^{cB} (64,92)
	4	0.61 ± 0.01^{aBCD} (0,0)	0.69 ± 0.02^{aAB} (0,0)	2.19 ± 0.05^{bD} (0,68)	0.77 ± 0.02^{aB} (0,0)	2.02 ± 0.12^{bC} (64,72)	–
	5	0.64 ± 0.01^{aCD} (0,0)	0.71 ± 0.02^{aAB} (0,0)	2.30 ± 0.10^{bD} (4,80)	–	–	–
	6	0.67 ± 0.01aD (0,0)	0.75 ± 0.02^{aB} (0,0)	2.31 ± 0.05^{bD} (60,84)	–	–	–

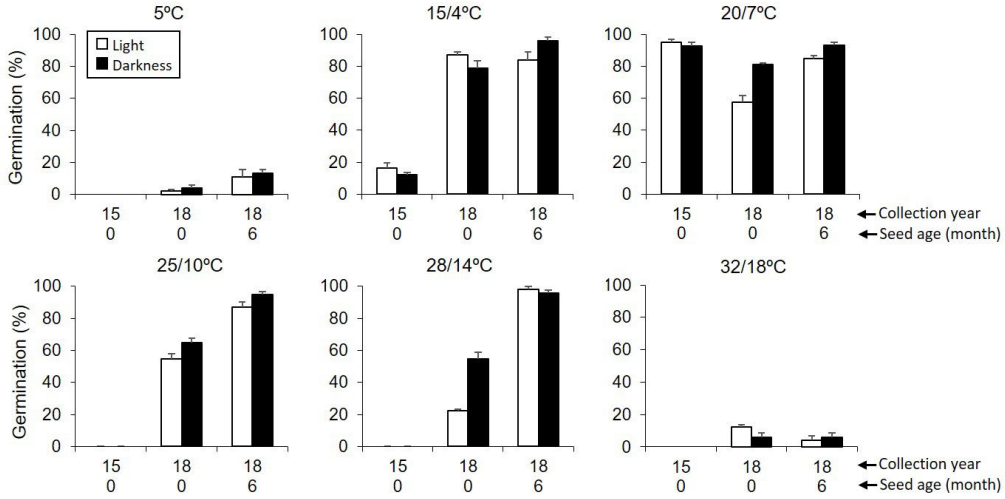

Figure 3. Effect of incubation conditions (light/darkness and temperature), seed storage time (0 and 6 months) and year of seed collection (2015, 2018) on the final germination percentage (mean±SE) in *Ribes alpinum* seeds incubated following a warm stratification at 25/10ºC in light for 30 days. Significant differences between the categories within a factor followed by different lowercase (Tukey multiple comparisons test, *p*-value < 0.05): Temperature (32/18^{a}, 5^{a}, 28/14^{b}, 25/10^{b}, 15/4^{bc}, 20/7^{c}); Collection year (2015^{a}, 2018^{b}); Seed age (0^{a}, 6^{b}).

The effect of seed age (0 vs. 9 months) on germination was tested in the seeds collected in 2016. Germination ability increased with seed age, but only at 15/4 and 20/7ºC. At these temperatures, the germination percentages were higher in the darkness than in the light, and exceeded 80% in the 9-month-old seeds. This trend was not detected at the other temperatures. In the seeds collected in 2015 and 2017, the germination percentages recorded during the incubation process were independent of the lighting conditions (Fig. 4).

The seeds collected in 2017 recorded the highest germination percentages in the study. The germination of the seeds from the natural population in the Meridional Iberian System exceeded 80% at any temperature, except for 5ºC at which the final germination was 60%. In the seeds from the Botanical Garden of Castilla-La Mancha (Albacete), germination went beyond 80% at all temperatures, even at 5ºC in the darkness (87%), and was 100% at 25/10ºC in the light (Fig. 4).

Induction of dormancy by cold stratification temperatures

The *R. alpinum* seeds entered secondary dormancy when cold-stratified (5ºC) for 30 days after warm stratification (25/10ºC), which interrupts primary physiological dormancy. Germination in the cold-stratified seeds was 9% when incubated at 20/7ºC in the light for 30 days, which contrasts with that recorded in the control test (96%). When this incubation treatment was prolonged to 60 days, the germination percentage (49%) remained significantly lower than that in the control (Table 3).

Discussion

The seeds of both *Ribes* species had underdeveloped embryos upon dispersal and did not germinate even though they were incubated for 30 days at a wide range of temperatures. Therefore, it can be concluded that they have MPD.

In *R. alpinum*, embryo growth occurred when seeds were exposed to moderately warm temperatures (25/10ºC), but not to typical cold stratification temperatures (5ºC), which evidences that seeds have some level of simple MPD (Baskin & Baskin, 2014). As there was hardly a delay in radicle and shoot emergence (1-2 weeks), the dormancy of epicotyl and double dormancy can be ruled out. Furthermore, levels of deep simple or intermediate simple MPD were also rejected because, once embryos had considerably developed, no cold stratification period was necessary to promote radicle emergence. Thus, as GA_3 stimulated both embryo growth and germination, it can be concluded that the *R. alpinum* seeds have nondeep simple MPD (Baskin & Baskin 2014, 2004). However in the seeds collected in 2018, a small fraction (10%) of the seed lot responded to stratification at 25/10ºC in the light for 30 days at the age of 6 months, as embryos developed and germination took place. Consequently, it may be considered that this fraction has only MD.

In *R. uva-crispa*, embryo growth occurred when seeds were exposed to a sequence of moderately warm (1 month

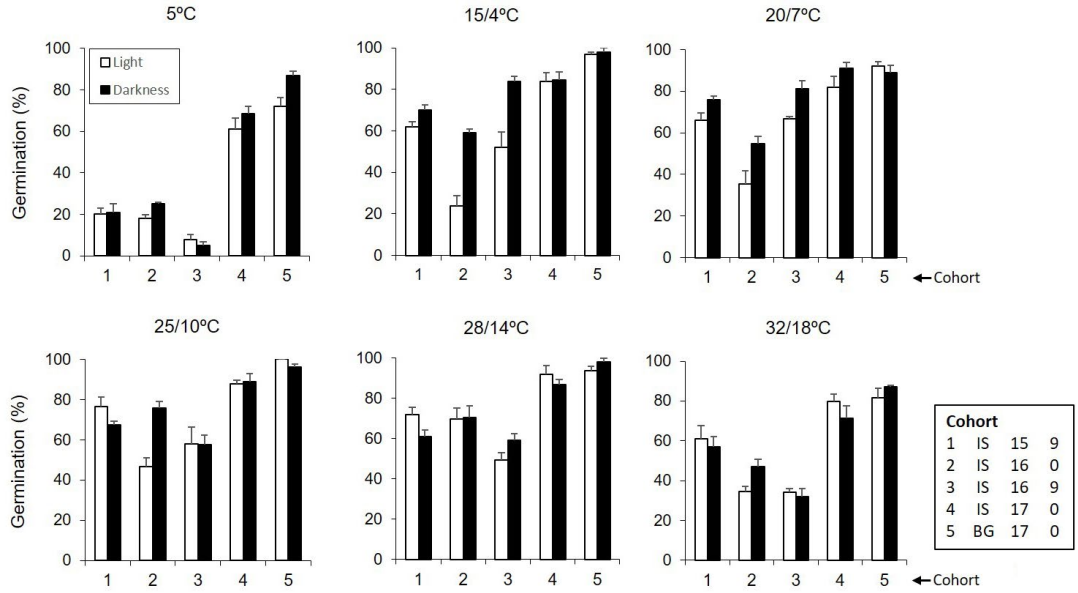

Figure 4. Effect of incubation conditions (light/dark and temperature), seed age (0 or 9 months), year of seed collection (2015, 2016 or 2017) and seed population (IS: Meridional Iberian System, BG: Botanical Garden of Castilla-La Mancha) on the final germination (mean ± SE) in *Ribes uva-crispa*. Seeds stratified for 150 d (30 d at each temperature: 20/7+15/4+5+5+5ºC) in the light and then transferred to incubation temperatures for 30 days. Seeds' origin (cohort): Cohort 1 (IS, 2015, 9 m), Cohort 2 (IS, 2016, 0 m), Cohort 3 (IS, 2016, 9 m), Cohort 4 (IS, 2017, 0 m) and Cohort 5 (BG, 2017, 0 m). Significant differences between the categories within a factor followed by different lowercase (Tukey multiple comparisons test, *p*-value < 0.05): Temperature (5^{a}, 32/18^{b}, 15/4^{bc}, 20/7^{bc}, 25/10^{c}, 28/14^{c}); Collection year (2016^{a}, 2015b, 2017^{c}); Seed age (9^{a}, 0^{b}); Seed population (IS^{a}, BG^{b}).

Table 3. Induction of dormancy by cold stratification (5ºC) in the *Ribes alpinum* seeds. Control treatment: 25/10ºC (1 m) + 20/7ºC (1 m). Treatment 1: 25/10ºC (1 m) + 5ºC (1 m) + 20/7ºC (1 m). Treatment 2: 25/10ºC (1 m) + 5ºC (1 m) + 20/7ºC (2 m). Values followed by different letters in a column significantly differ (P < 0.05)

	Germination (%±SE)	**Embryo length (mm±SE)**
Control	96 ± 2^{A}	1.68 ± 0.02^{A}
Treatment 1	9 ± 2^{B}	1.26 ± 0.05^{B}
Treatment 2	49 ± 3^{C}	1.52 ± 0.05^{C}

20/7ºC + 1 month 15/4ºC) stratification to simulate autumn months, followed by a cold (3 months 5ºC) one to simulate winter conditions. In the moderately warm phase, embryo length increased only from 0.52 to 1.12 mm, while for the cold phase growth was more pronounced (66%, from 1.12 to 2.30 mm, Table 2). This suggests that these seeds have some level of complex MPD. The slight growth of the embryos exposed to warm temperatures (25/10ºC), along with the absence of germination at any temperature after a previous cold stratification period at 5ºC for 5 months, ruled out the existence of any simple level of MPD. The fact that embryo growth was very low (from 0.52 to 0.67 mm) during the 6-month cold stratification period at 5ºC (Table 2) allowed us to rule out the existence of intermediate and deep complex MPD levels in the *R. uva-crispa* seeds. Indeed the data from both the laboratory (Table 2) and the phenological outdoor test (Fig. 1) provide compelling evidence for nondeep complex MPD for the seeds of this taxon. The effect of GA_3 on embryo growth and germination also confirmed the existence of this level. Baskin and Baskin (2014) have shown in some seeds with nondeep complex MPD that GA_3 can partly substitute for warm stratification, but not for cold stratification. In this study, 64% of the seeds germinated when incubated at 15/4ºC in a GA_3 solution after 3 months of cold stratification (Treatment B), but this did not occur when they were submitted to the same treatment in the absence of GA_3.

The presence of not only nondeep simple MPD in most of the *R. alpinum* seeds, but also of nondeep complex MPD in the *R. uva-crispa* seeds confirms our hypotheses: both species have a different germinative ecology and *R. alpinum* shows a level of MPD different from that of *R. uva-crispa*.. This, in addition to the existence of nondeep simple (root)-nondeep simple (epicotyl) MPD in *R. multiflorum* subsp. *sandalioticum* (Mattana *et al.*, 2012) and of MD in *R. speciosum* (Mattana *et al.*, 2014), poses a wide variability in the germination ecology of *Ribes* species.

Future studies on other species of this genus will probably detect other MPD levels.

Mattana *et al.* (2014) pointed out that 67% of *R. alpinum* seeds may have MD, as opposed to the 10% recorded in this study. These very marked inter-population differences may be due to differences in the seed storage times and/or morphological seed traits. In Mattana *et al.* study (2014), seeds were stored for 13 years in a seed bank, while the longest dry storage period at room temperature in our study was 6 months. Surpassing nondeep physiological dormancy after dry seed storage has been well recorded (Baskin & Baskin, 2014). In species of *Ribes* genus, Mattana *et al.* (2014) detected that dormancy index of seeds to be inversely proportional to embryo length upon seed dispersal, and shorter embryos were more dormant. In the *R. alpinum* population from the UK (Mattana *et al.* 2014), embryo length was 0.8 mm upon dispersal compared to 0.5 mm in our population from the Meridional Iberian System. These differences may be due to the lower mean annual temperature (8º vs. 9.2ºC) in the Meridional Iberian System (Elías & Ruiz, 1981). According to Mattana *et al.* (2014), embryo length is related to the mean annual temperature, which may explain the higher dormancy index of the seeds herein analysed.

The *R. alpinum* seeds that had overcome their dormancy after being exposed to moderately warm (25/10ºC) temperatures were induced to secondary dormancy if they were then exposed to 5ºC for 30 days. Although this is a poorly known phenomenon in species with MPD, there is background for some species of the *Narcissus* genus with deep simple epicotyl MPD, such as *N. hispanicus* (Copete *et al.*, 2011) and *N. eugeniae* (Copete *et al.*, 2014). It is also likely to be manifested in other species with nondeep simple MPD whose dormancy is broken by exposure to high temperature. This aspect deserves future research.

Ribes alpinum seedlings emerge in autumn, like other species with nondeep simple MPD whose seeds overcome dormancy at high temperature, *i.e.*, *Lonicera etrusca* (Santiago *et al.* 2013). In contrast, *R. uva-crispa* seeds emerge late in winter and early in spring, which typically occurs in species with nondeep complex MPD (Baskin & Baskin, 2014). The autumn emergence of *R. alpinum* seedlings requires the presence of uninterrupted wet spells in the soil for 45-60 days after rainfall late in summer and early in autumn. These water conditions are more likely to occur in shady environments than in sunny areas which, in combination with other ecological requirements (relative humidity, soil moisture, shadow) during other plant development stages of this species, could help us to understand its preference for shaded spots in the Meridional Iberian System. In contrast, the delay in *R. uva-crispa* seedling emergence late in winter-early in spring would allow their seeds to find favourable wet conditions in both shadow and sunny exposures for this species to be able to colonise environments under different light conditions. Hence the different germinative models detected in *R. alpinum* and *R. uva-crispa* help explain the existence of well differentiated habitats observed for these species in the Meridional Iberian System. These results confirm that germination traits can play an important role in the processes that filter regional species pools into local communities, and that divergence in germination traits of sympatric and closely related species helps explain variation in microenvironmental conditions (Jiménez-Alfaro *et al.*, 2016).

Wide inter-annual variability has been observed in the germinative ability of both *R. alpinum* and *R. uva-crispa*. Although all the seeds of both species are dormant at dispersal, their sensitivity to dormancy breaking factors (stratification treatments) varies widely between seed collection years. In a single population, these differences in dormancy usually result from temperature variation during seed maturation or maternal effect, which produces phenotypic plasticity in dormancy or in sensitivity to dormancy breaking factors (Fenner, 1991; Gutterman, 2000). For *R. uva-crispa*, the seeds collected in 2017 were more sensitive to stratification treatments than those from 2015 and 2016. With *R. alpinum*, the seeds from 2018 germinated better than those from 2015 after the stratification treatment breaking dormancy. Although no annual data on temperatures in the seed-collection locality are available, 2017 and 2018 were probably hotter during the seed maturation period, particularly if the traditional dormancy variation rule is considered: warmer temperatures equal less dormant seeds (Wagmann *et al.*, 2012; Fernández-Pascual & Jiménez-Alfaro, 2014). The Cohort 5 of *R. uva-crispa* seeds was collected at the Botanical Garden of Castilla-La Mancha from the 4-year-old plants that originated from the seeds collected in the same locality under study. Thus, both populations should have the same genetic background, and the differences in germinative behaviour (*i.e.*, higher germinative ability at 5ºC in the Botanical Garden population) may be due to the maternal effect. Although the individuals chosen for seed collection could have varied slightly each year, the genetic makeup of each seed collection should be similar as populations were very small and the fruits of all the plants were collected each time. Thus, we can assume that most of the variation herein detected was caused mainly by the phenotypic plasticity in sensitivity to dormancy breaking factors. In a changing climate scenario, this phenotypic plasticity is of upmost importance as it may be key for quick plant responses to new conditions (Walck *et al.*, 2011; Fernández-Pascual & Jiménez-Alfaro, 2014). For this reason, future studies should focus on testing such germinative plasticity in other populations of these species, and on verifying inter-population variability in the germination ability among the populations living in

habitats with contrasting temperature (different altitude) or humidity conditions.

Acknowledgements

We thank Helen L. Warburton for her suggestions and improvements to the English text. We also thank the reviewers for their exhaustive revision of the manuscript which contributed to the enhancement of its quality.

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