Acta Physiologiae Plantarum (2023) 45:137
https://doi.org/10.1007/s11738-023-03617-5

ORIGINAL ARTICLE

Influence of environmental conditions on embryo growth, dormancy breaking, and germination in seeds of *Helleborus foetidus* (Ranunculaceae)

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Received: 14 March 2022 / Revised: 20 June 2023 / Accepted: 23 October 2023
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Abstract

One of the most critical phases in the life cycle of plants encompasses early stages, since success in seed dormancy overcoming and germination as well as in seedling establishment represents a key process filtering the participation of species in plant communities. In this study, the requirements for dormancy breaking and germination of seeds of *Helleborus foetidus* were analyzed. The seeds were dormant with underdeveloped embryos (0.54 mm) at the time of dispersal and radicle emergence did not occur until embryos reached full size (3.6 mm). The light–temperature requirements for embryo growth and radicle emergence were studied correlating experiments under near-natural conditions with others under laboratory-controlled conditions. The embryos completed their growth and the radicle emerged when seeds were warm → cool stratified in darkness under a thermal sequence meeting late summer to early autumn temperature conditions. Conversely, cold stratified seeds did not germinate at winter temperatures (5 °C). The highest seed germination response (i.e., 75%) occurred in 6-month-old seeds incubated at 15/4 °C in darkness after being warm + cool stratified for 3 months (i.e., 32/18 °C for 1 month + 28/14 °C for 1 month + 25/10 °C for 1 month). In outdoor conditions, the embryo grew during late summer–autumn and the radicle emerged in late autumn, but the shoot did not emerge immediately because it was physiologically dormant too, so it required cold winter temperatures for dormancy breaking. Seedling establishment commenced at late winter–early spring, which is a more suitable season for seedling survival. In conclusion, the seeds of *Helleborus foetidus* exhibit deep simple epicotyl morphophysiological dormancy (MPD), which is ecologically well adapted to temperate forest regions. This is the second report of this level of MPD in Ranunculaceae.

Keywords Dormancy · Embryo growth · Germination · Seedling phenology · Stratification

Introduction

In temperate-zone forests, some species have seeds with complicated requirements for dormancy break and germination, which influence the floristic composition of the forests. Species in Ranunculaceae have seeds with rudimentary underdeveloped embryos. Therefore, they exhibit morphological dormancy (MD) or MPD depending on whether they germinate in less than 30 days under optimal temperature conditions or require a longer dormancy-breaking period (Baskin and Baskin 2004). Seven of the nine known levels of MPD have been found in this family: non-deep simple (i.e., *Thalictrum mirabile*, Walck et al. 1999), deep simple (i.e., *Aquilegia barbaricina*, Mattana et al. 2012), deep simple epicotyl (*Actaea spicata*, Eriksoon 1994), deep simple double (*Clematis albicoma*, Platt 1951), non-deep complex (*Hepatica nobilis* var. *japonica*, Nomizu et al. 2004), intermediate complex (*Delphinium fissum* subsp. *sordidum*, Herranz et al. 2010b), and deep complex (*Aconitum lycoctonum*, Vandelook et al. 2009).

Moreover, variation in the germination capacity between different populations of the same species is well known in

Communicated by A. Gniazdowska-Piekarska.

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Published online: 11 November 2023

species whose seeds have developed embryos at the time of dispersal (Andersson and Milberg 1998; Pérez-García et al. 2003; Baskin and Baskin 2014). However, inter-population variability has rarely been studied in species with MPD, where the only examples are *Aconitum napellus* subsp. *castellanum* (Herranz et al. 2010a), *Narcissus eugeniae* (Copete et al. 2014), *Ilex maximowicziana* (Chien et al. 2011), and *Centaurium somedanum* (Fernández-Pascual et al. 2013). This inter-population variability may be due to: (1) thermal changes during the seed maturation process leading to phenotypic plasticity to in degree of dormancy; or (2) local climatic oscillations in the long term resulting in genetic (heritable) dormancy differences, with clinal variation according to the altitude (Fernández-Pascual et al. 2013) or latitude (Wagmann et al. 2012). Similarly, intra-population variability is attributed to genetic differences between individuals in species with developed embryos at the time of dispersal (Pérez-García 1997; Mira et al. 2017), but no previous studies have investigated this in species with MPD.

At present, germination plasticity, including inter- and intra-population variability, is considered to have a buffering effect against global warming (Reed et al. 2011; Fernández-Pascual and Jiménez-Alfaro 2014) because the extent of variability increases biological efficiency (fitness) of a plant to counteract the effects of temperature increases linked to climatic change (Mira et al. 2017).

This study analyzes the case of *Helleborus foetidus* L. (Ranunculaceae), a myrmecochorous rhizomatous perennial herb with a wide distribution in Central and Western Europe, and North Africa (Werner and Ebel 1994). The seeds of *H. foetidus* have underdeveloped dormant embryos at the time of dispersal, and thus they exhibit morphophysiological dormancy (MPD) (Sánchez-Yélamo and Ayerbe 1984). Germination percentages > 50% were achieved by Sánchez-Yélamo and Ayerbe (1984) at 11 °C over periods from 12 to 24 weeks, using mixtures of different proportions of hormonal substances (gibberellic acid (GA_3), indoleacetic acid, kinetin, and fusicoccin). However, no previous studies have investigated how seed dormancy is broken, using a sequence of natural environmental conditions (temperature and illumination). Nikolaeva et al. (1985) suggested that seeds of a Russian population of *H. foetidus* had non-deep simple MPD, but they did not specify the optimal temperature conditions for dormancy break and germination.

Helleborus foetidus is a forest species and grows in habitats of priority conservation concern, such as pine forest of *Pinus nigra* subsp. *salzmannii* and deciduous Euro-Siberian communities (forests of *Tilia platyphyllos* and forests of *Populus tremula*), which are protected by European Union conservation regulations (Directive 92/43/EEC). The maintenance of these habitats in a favorable conservation state may require population reinforcement programs for the species included in them (Martín-Herrero et al. 2003). In addition, this species has ornamental potential because it flowers early during the winter, and its showy flowers have a green campanulate perianth and persistent palmate compound leaves (Sánchez-Yélamo and Ayerbe 1984; Meiners and Winkelmann 2012). Finally, this herb has medicinal interest as a heart tonic (Stübing and Peris 1998; Chevallier 1996). Nursery plant production of this taxon for all of these purposes requires in-depth knowledge of its germination ecology.

Therefore, the aims of the present study were: (a) to determine the optimal temperature and light conditions for dormancy breaking, embryo growth, and germination; (b) to determine whether the seeds from the study populations exhibited any of the levels of MPD described in Ranunculaceae or others that have not yet been described in this family; and (c) to analyze the intra- and inter-population variabilities in the germination capacity.

Materials and methods

Plant material and seed source

Helleborus foetidus (hereafter hellebore) occurs at altitudes from 800 m a.s.l. to 2000 m a.s.l. at forest edges and in clearings. Adults reproduce sexually after several seasons of vegetative growth, and many die after a season of intense flowering. The flowers have 1–5 carpels (most commonly 2–3), which yield fruits with a similar number of follicles, each of which develops 10–15 elaiosome-bearing seeds, and thus each fruit contains around 25–45 seeds. The diaspores are released during late June to mid-July when many potential ant dispersers are attracted (Garrido et al. 2007).

Fruits were collected in two localities in the Meridional Iberian System (Central-Eastern Spain) and Alcaraz Mountains (Baetic Mountain ranges, South-Eastern Spain), as follows.

- Meridional Iberian System: Masegosa (Cuenca province), 1480 m a.s.l., 30TWH8787, limestone substrate, in a *Pinus sylvestris* forest with *Juniperus sabina*, *J. communis*, *Rosa sicula*, *Prunus spinosa*, and *Berberis vulgaris* subsp. *seroi*.
- Alcaraz Mountains: Salobre (Albacete province), 1240 m a.s.l., 30SWH4264, limestone-dolomitic substrate, from the edges of a mixed forest with *Pinus pinaster*, *Quercus ilex* subsp. *ballota*, *Q. faginea*, *Daphne laureola*, and *Cytisus scoparius* subsp. *reverchonii*.

On 11 July 2015, 10 July 2016, 8 July 2017, and 17 July 2018, we collected 100, 250, 400, and 300 fruits, respectively, from 25–60 plants in Masegosa locality. In addition,

on 17 July 2018, the fruits from each of five plants were collected and kept separate.

On 27 June 2018, we collected 300 fruits from 25 to 30 plants in Salobre locality. In addition, the fruits from each of five plants in this population were collected and kept separate.

In all cases, the fruits were collected when the green follicles became yellow, and some had started to open to allow seed dispersal. The fruits were stored under laboratory conditions (22–24 °C, relative humidity = 50–60%) and they opened gradually. After cleaning the seeds, they were spread out on trays until 1 August following their collection (seed age = 0 months old). The seeds were then stored in paper bags in the laboratory until the experiments commenced; the starting date of each experiment is given below.

Preliminary analyses in the laboratory with hellebore seeds from the year 2015 confirmed that they contained an underdeveloped embryo at the time of dispersal (embryo length = 0.54 ± 0.02 mm, seed length = 4.71 ± 0.06 mm, mean ± standard error (SE), $n = 25$). In addition, the seeds were physiologically dormant (PD) because they did not germinate within 30 days under a wide range of temperature–light conditions, which simulated those in the natural habitat of the species throughout the year (i.e., 5 °C, 15/4 °C, 20/7 °C, 25/10 °C, 28/14 °C, and 32/18 °C in light and in darkness). Therefore, the hellebore seeds exhibited some level of MPD. These preliminary experiments also showed that the seeds germinated after three months of warm stratification (28/14 °C) followed by incubation at a cool temperature (15/4 °C). In addition, the viability of the seeds during the long stratification period (> 2 months) was maintained better when preserved on wet sand rather than wet filter paper.

Outdoor experiments

We aimed to determine the timing of the main phases in the seed germination and seedling establishment of the hellebore life cycle relative to near-natural temperature conditions in a non-heated metal frame shade-house, which is located 75–175 km from the collection sites at an altitude of 690 m a.s.l. The air temperature was recorded continuously in the shade-house by a data logger.

The seeds were sown in pots and trays, using as growing medium a mixture of sterilized peat and sand (2:1 v/v). The watering system was programmed to simulate the natural regime of soil humidity, including summer drought, so watering frequency was designed based on one irrigation event a week to soil capacity, except during summer, when the inter-watering period was increased to two weeks. Besides, watering was interrupted during the winter frosting period.

Phenology of embryo growth, dormancy breaking, and radicle emergence

On 1 August 2016, ten groups of 100 hellebore seeds (from Masegosa) were each mixed with sterilized sand within a permeable polyester cloth bag and buried to a depth of 5 cm in a pot. Then, the pot containing all the bags was placed in the shade-house. From 1 September 2016, one bag was exhumed monthly. Seeds were separated from the sand and seeds with emergent radicles counted. Then, we randomly selected 25 healthy-looking seeds, whose embryos were excised and length-measured using an ocular micrometer.

In seeds with MPD, embryo must grow and reach the critical embryo length for radicle emergence. To avoid oversize this parameter, the critical embryo length is measured at the time when the seed coat splits but before radicle emerges (Vandelook and Van Assche 2008). The 25 seeds used to determine the critical embryo length were subjected to warm (28/14 °C) followed by cool (15/4 °C) stratification treatments. In the hellebore, the critical embryo length was 3.6 ± 0.09 mm (mean ± SE, $n = 25$, range 2.9–4.3 mm). Following Copete et al. (2011), we realistically assumed that morphological seed dormancy was overcome when the length of embryos reached the minimum value in this range (i.e., 2.9 mm for Hellebore seeds).

To determine the dormancy stage of non-germinated exhumed seeds, we incubated the rest of the seedlot during 30 days at the most favorable germinating conditions (15/4 °C in darkness). After incubating the seeds, we calculated the following seed status percentages: (1) seeds with radicles that emerged within the bag; (2) viable non-dormant seeds, i.e., those that germinated in the incubation phase at 15/4 °C; (3) viable dormant seeds, i.e., those that did not germinate at 15/4 °C but with healthy embryos; and (4) non-viable seeds, i.e., those with a rotten appearance or a dead embryo after excision.

Phenology of seedling emergence

On 1 August 2016, three seed starting trays (20 cm × 30 cm × 8 cm) were filled with the growing medium. In each tray, 100 seeds from Masegosa were sown to a depth of 5 mm and equidistant from each other. The trays were placed in the shade-house. From August 2016 to June 2018, the seed trays were examined once each week and any seedlings were counted and removed.

Laboratory experiments

General conditions for germination experiments

Experiments were conducted in germination chambers (Ibercex model F-4, Madrid, Spain) equipped with a

digital temperature and light control system (± 0.1 °C, cool white fluorescent light, 25 µmol m^{-2} s^{-1} (1350 lx)). Radicle emergence was determined in seeds under a 12 h daily photoperiod (hereafter light) and under continuous darkness (hereafter darkness), which was achieved by wrapping Petri dishes in a double layer of aluminum foil, at constant temperature of 5 °C and at 12/12 h daily fluctuating temperature regimes of 15/4 °C, 20/7 °C, 25/10 °C, 28/14 °C, and 32/18 °C. In the 12/12 h alternating temperature treatments. To simulate day–night sequence, the light phase coincided with the higher temperature and the darkness one with the lower temperature. Seeds were incubated in 9-cm-diameter Petri dishes on two layers of filter paper moistened with distilled water. Dishes were sealed with Parafilm to prevent desiccation.

The alternating temperature regimes simulated the annual climate cycle in continental areas in the interior of the Iberian Peninsula. In particular, 15/4 °C corresponded to November and March, 20/7 °C to October and April, 25/10 °C to September and May, 28/14 °C to June and August, and 32/18 °C to July. The 5 °C treatment simulated the mean temperature recorded during the winter months (i.e., December–February; Elias and Ruiz 1981).

Percentage germination (radicle emerged ≥ 1 mm and clearly visible) was assessed based on the number of apparently viable seeds. The viability of non-germinated seeds was assessed based on the embryo's color and turgidity. The reliability of these indicators of seed viability was corroborated using the tetrazolium test.

Influence of temperature on embryo growth

On 1 August 2016, five nylon bags were buried to a depth of 5 cm in each of five pots. Each bag contained 50 seeds from Masegosa mixed with sand. The substrate in the pots was peat and sand (2:1, v/v), and it was permanently wet. Each of the five pots was exposed to different temperature conditions for 4 months: (1) 5 °C, (2) 15/4 °C, (3) 28/14 °C, (4) treatment A at 32/18 °C (1 month) + 28/14 °C (1 month) + 25/10 °C (1 month) + 20/7 °C (1 month), and (5) treatment B at 28/14 °C (1 month) + 25/10 °C (1 month) + 20/7 °C (1 month) + 15/4 °C (1 month). After 4 months, the pots were transferred to 15/4 °C (treatment C) for 1 month.

Each month, a bag was removed from each pot and its contents were sieved (1 mm) to separate the seeds from the sand. Embryos were excised from 25 healthy-looking seeds and their lengths were measured. The percentages of seeds with emerged radicles were recorded.

Influence of GA_3 on embryo growth

On 1 August 2016, two lots of 50 seeds from Masegosa were each placed in Petri dishes on two sheets of filter paper moistened with a solution of 1000 mg L^{-1} GA_3 in distilled water and incubated at 15/4 °C in darkness. After 30 and 60 days, embryos from 25 seeds in a Petri dish were measured, and the percentages of germinated seeds were recorded. The results were compared with those obtained for a control test treated at 15/4 °C containing seeds incubated in distilled water.

Influence of stratification conditions and seed storage duration on germination

This experiment was conducted using seeds collected from Masegosa in 2017. Tests using seeds collected in 2016 demonstrated the effects of temperature on embryo growth and that different warm stratification conditions were more effective for promoting embryo growth and germination than cold stratification (5 °C).

The warm stratification treatments were: treatment *D* = 32/18 °C (1 month) + 28/14 °C (1 month) + 25/10 °C (1 month); treatment E = 28/14 °C (1 month) + 25/10 °C (1 month) + 20/7 °C (1 month); and treatment *F* = 28/14 °C (3 months). Treatments D and E were similar to treatments A and B, respectively, in our analysis of the effects of temperature on embryo growth but the fourth month of stratification was omitted because more than 40% of the seeds germinated during this period.

To avoid losing viability during the warm stratification period, the seeds were mixed with sand, placed in nylon bags, and then buried in the pots instead of placing the seeds on wet filter paper in the Petri dishes.

On 1 August 2017, the seed age was 0 months. For each treatment, 300 seeds were mixed with sand and placed in each of five nylon bags that were buried in a pot, where the substrate was permanently wet. Each month, a pot from each treatment was moved to another chamber according to the temperature sequence. After the stratification period, the seeds were separated from the sand and incubated on filter paper in Petri dishes under light and darkness for 30 days at the six temperatures specified in Sect. "General conditions for germination experiments".

On 1 February 2018, the seed age was 6 months and the experiment was repeated under the same conditions.

Inter-population variability of radicle emergence

Previous preliminary experiments with seeds collected during 2017 in Masegosa showed that treatment D was the most effective at promoting germination and germination percentage also increased for 6-month-old seeds.

This experiment was conducted with seeds collected during 2018 from Masegosa and Salobre. On 1 February 2019, the seed age was 6 months and 1500 seeds from each population were warm stratified (treatment D), before transferring batches of 100 seeds each to incubation under the conditions specified in Sect. "General conditions for germination experiments".

Intra-population variability in radicle emergence

In both seed source populations (Masegosa and Salobre), ten mature fruits (yellow follicles) were collected separately from five different plants in 2018. On 1 February 2019, the seed age was 6 months and 250 seeds from plants in each of the populations were warm stratified according to treatment D, as described above. The seeds were then incubated at 15/4 °C in light and darkness, which were the optimal temperatures for germination.

Influence of cold stratification on cotyledons emergence

The phenology experiments initiated in the shade-house on 1 August 2016 showed that the delay between radicle and seedling emergence was 2 months (December and January).

In this experiment, we determined whether cold stratification is required for cotyledon emergence in radicle-emerged seeds (and the necessary duration of stratification), or if winter temperatures only slow down cotyledon development.

On 15 December 2017, seeds from Masegosa with emerged radicles (2–3 mm in length) were used for this experiment. Three batches of 100 seeds, each distributed into four 25-seed replicates, were stratified at 5 °C light for 0, 30, or 60 days. Following each cold stratification treatment, the seeds were transferred to 20/7 °C in the light for 90 days. Cotyledon emergence was recorded each week.

Statistical analysis

Means and SEs were calculated for the radicle and shoot emergence percentages and embryo lengths. The effects of stratification temperature, duration of stratification, and incubation temperature on the embryo length were analyzed by three-way analysis of variance (ANOVA). Seed germinability was compared among treatments by multifactor ANOVA. Previously, the normality (Cochran test) and homoscedasticity (David test) of the data were tested and germination percentages were arcsine square-root transformed. The effects of four main factors were analyzed: temperature (six levels), light condition (two levels), stratification treatments (three levels), and seed age (two levels). The inter-population and intra-population variabilities were analyzed separately: seed source population (two levels) and individual mother plant (five levels). For significant factors, differences were explored using the multiple comparison Tukey test.

Results

Outdoor experiments

Phenology of embryo growth, dormancy breaking, and radicle emergence

At the beginning of the burial experiment on 1 August 2016, mean embryo length was 0.54 ± 0.02 mm. Embryos grew slowly until 1 October, when the mean embryo length was 0.59 ± 0.02 mm. During this period, mean maximum and minimum daily temperatures were 30 °C and 13.75 °C, respectively (Fig. 1). However, the embryos grew faster between 1 October and 1 December when the maximum and minimum temperatures were 17.3 °C and 5.35 °C, respectively. Thus, on 1 December, embryo length was 1.81 ± 0.29 mm and 20% of the seeds has an emerged radicle, whereas 36% of the seeds had yet to overcome their dormancy (Figs. 1, 2). Between 1 December 2016 and 2 January 2017 when mean maximum and minimum temperatures were 9.2 °C and 1.3 °C, respectively, embryo length increased from 1.81 mm to 2.37 ± 0.29 mm, and the seed radicle emergence percentage reached 56%. Between 2 January 2017 and 1 April 2017, the embryos barely increased in size and the seed radicle emergence percentage did not exceed 60% (Figs. 1, 2). Thus, embryo growth and radicle emergence mainly occurred during the autumn months: October, November, and December.

On 1 April 2017, 20% of the recovered seeds were non-viable. After 1 May 2017, only a few intact seeds were found, which were surrounded by an amorphous mass made of sand, radicles, and decaying seeds. Non-viable seeds could not be differentiated from decayed seeds with an emerged radicle in this mass.

Phenology of seedling emergence

Seedling emergence was delayed until 1 February 2017 (4%), although 20% of the seeds had an emerged radicle on 1 December 2016. The cumulative seedling emergence percentages were 38% and 54% on 1 March and 1 April in 2017, respectively. No more seedlings emerged during the rest of the year. On 1 February 2018, the cumulative seedling emergence percentage increased to 57% and no subsequent new seedling emergences were recorded (Fig. 1).

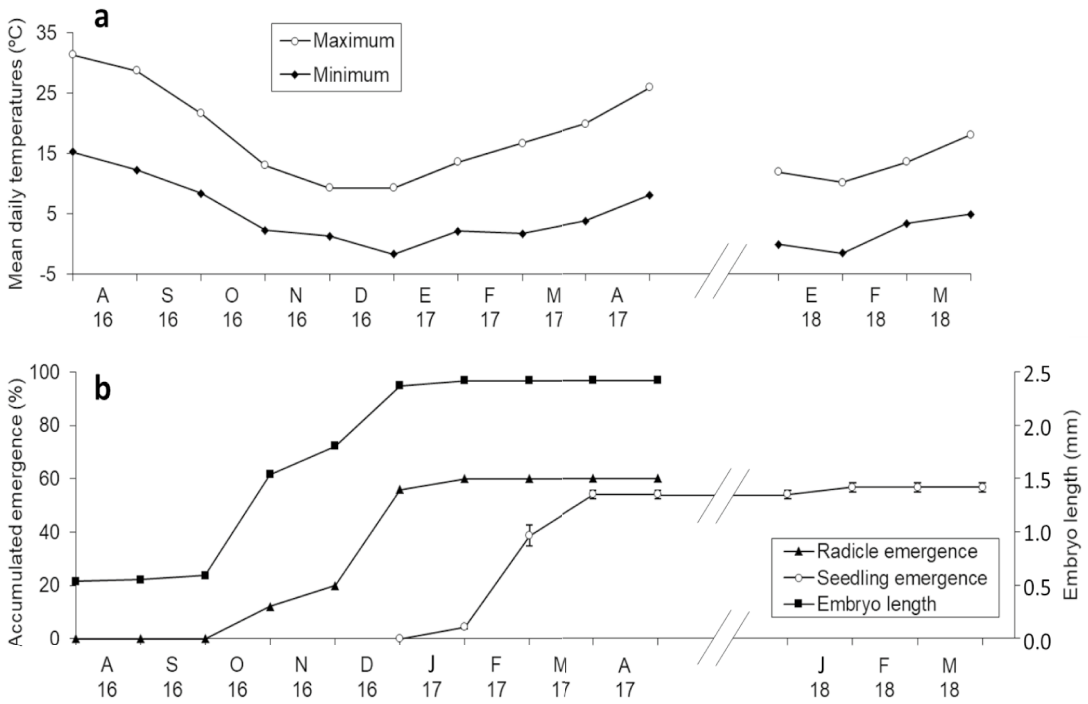

Fig. 1 **a** Mean monthly minimum and maximum temperatures. **b** Phenology of embryo growth and radicle and seedling emergence for *Helleborus foetidus* seeds sown on 1 August 2016

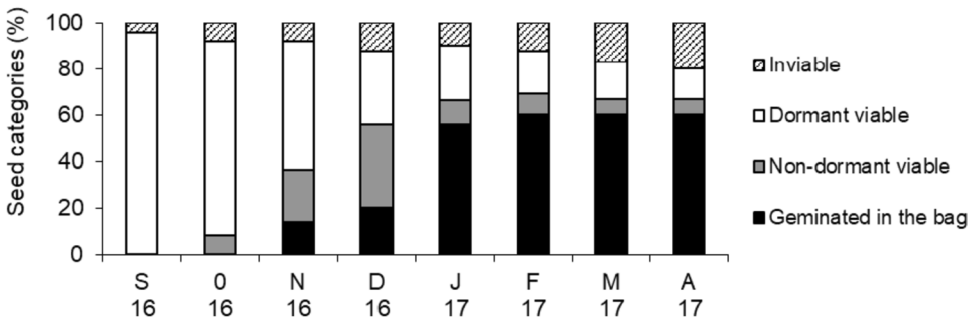

Fig. 2 Percentages in *Helleborus foetidus* seed categories: (1) non-viable, (2) dormant viable, (3) non-dormant viable, and (4) germinated in the bag. Seeds were buried on 1 August 2016 and recovered each month for 8 months. Seeds from Masegosa were collected in 2016

Laboratory experiments

Influence of temperature on embryo growth

In the seeds stratified in wet sand under darkness, the embryos barely grew under both cold (5 °C) and cool temperatures (15/4 °C) during the 4-month stratification period, or during the following fifth month at 15/4 °C (Table 1). However, the embryos grew continuously throughout the warm stratification treatments (28/14 °C, treatment A, treatment B). The growth was greater under treatments A and B compared with 28/14 °C. Both treatments simulated the typical temperature sequence in the summer–autumn months, and the seeds began to germinate from the third month. The greatest embryo growth (3.09 ± 0.26 mm) and radicle emergence percentage (68%) occurred under treatment C (1 month at 15/4 °C following treatment A). After stratification for 4 months at 28/14 °C followed by one extra month at 15/4 °C, the radicle emergence percentage reached 40% (Table 1).

Influence of GA_3 on embryo growth

GA_3 stimulated embryo growth, but it did not promote radicle emergence. After 60 days at 15/4 °C in darkness, embryo length was significantly longer (2.32 ± 0.16 mm) in seeds imbibed in GA_3 solution (1000 mg L^{-1}) than in seeds incubated in distilled water (0.58 ± 0.01 mm). However, the greatest length achieved was still much lower than the

Table 1 Effects of temperature and stratification period on embryo growth (mean ± standard error, mm) in *Helleborus foetidus* seeds

		Stratification temperatures in darkness				
		5 °C	15/4 °C	28/14 °C	TREAT. A (32/18 °C + 28/14 °C + 25/10 °C + 20/7 °C)	TREAT. B (28/14 °C + 25/10 °C + 20/7 °C + 15/4 °C)
Stratification period (months)	1	0.56 ± 0.01^{aA} (0, 0)	0.58 ± 0.01^{aA} (0, 0)	0.56 ± 0.01^{aA} (0, 0)	0.72 ± 0.02^{bA} (0, 0)	0.56 ± 0.01^{aA} (0, 0)
	2	0.58 ± 0.01^{aAB} (0, 0)	0.59 ± 0.01^{aAB} (0, 0)	0.68 ± 0.05^{aA} (0, 0)	0.91 ± 0.08^{bAB} (0, 0)	0.66 ± 0.06^{aA} (0, 0)
	3	0.60 ± 0.01^{aABC} (0, 0)	0.63 ± 0.01^{aBC} (0, 0)	1.13 ± 0.10^{abA} (0, 0)	1.60 ± 0.28^{bBC} (16, 28)	2.37 ± 0.15^{cB} (4, 32)
	4	0.62 ± 0.01^{aBC} (0, 0)	0.63 ± 0.01^{aBC} (0, 0)	2.29 ± 0.27^{bB} (8, 44)	2.12 ± 0.29^{bC} (48, 48)	2.54 ± 0.24^{bB} (44, 48)
	5 (Treat. C)	0.63 ± 0.01^{aC} (0, 0)	0.65 ± 0.01^{aC} (0, 0)	2.32 ± 0.26^{bB} (40, 48)	3.09 ± 0.26^{cD} (68, 72)	2.80 ± 0.23^{bcB} (64, 64)

Values followed by different uppercase letters within a column or different lowercase letters within a row are significantly different ($P < 0.05$). The first number in parentheses is the germination percentage and the second is the percentage of seeds with an embryo length greater than the minimal value required for radicle emergence (2.9 mm)

critical embryo length (3.6 mm) required for germination, and thus no radicle emergence occurred.

Influence of stratification conditions and seed storage duration on germination

After the warm stratification treatments started on 1 August 2017 (seed age = 0 months), the radicle emergence percentage was only 40% following treatments *D* (incubation at 5 °C darkness) and *F* (incubation at 15/4 °C darkness). After the warm stratification treatments started on 1 February 2018 (seed age = 6 months), the radicle emergence percentage increased significantly for 0-month-old seeds under all temperature–illumination following treatments *D* and *F*. The trend was similar following treatment *E*, except under 20/7 °C in darkness, 25/10 °C in darkness, and 28/14 °C. A significant positive effect of darkness on radicle emergence was found in 6-month-old seeds at 15/4 °C, 20/7 °C, and 25/10 °C following treatments *D* and *F* (Fig. 3, Table 2).

The highest radicle emergence percentages (> 75%) were obtained for 6-month-old seeds after stratification treatments *D* and *F* followed by incubation at 15/4 °C in darkness. Therefore, this temperature was identified as optimal for radicle emergence, although good results were also obtained under 5 °C in darkness and 20/7 °C darkness (Fig. 3, Table 2).

During the warm stratification treatments, 10–15% of seeds lost viability. During the incubation period, loss of viability ranged from 0 to 16%, and the highest values were obtained for 6-month-old seeds at 25/10 °C following treatment E.

Inter-population variability in radicle emergence

Under the most favorable incubation conditions for radicle emergence (5 °C, 15/4 °C, 20/7 °C) following warm stratification treatment D, the germination percentages were significantly higher in seeds from Masegosa than Salobre in both light and darkness. The values were similar in both populations at 25/10 °C and 28/14 °C in light (Fig. 4, Table 3).

In seeds from Masegosa in 2018, the highest radicle emergence percentages were obtained at 15/4 °C, with 53% in light and 63% in darkness (Fig. 4, Table 3). These values were slightly lower than those obtained under the same conditions and for the same population but using seeds collected in 2017, i.e., 60% in light and 75% in darkness (Fig. 3, Table 2).

Intra-population variability in radicle emergence

High intra-population variability in radicle emergence was observed in both populations (Masegosa and Salobre). In Masegosa, the highest values were obtained in plants 1 and 4. In particular, the radicle emergence percentages in seeds from plant 4 were highest at 15/4 °C, with 53% in light and 68% in darkness. Lower percentages were obtained at the same temperature in seeds collected from plant 3, with 18% in light and 24% in darkness. In the Salobre population, the results differed for plant 1 at 15/4 °C, with 41% in light and 50% in darkness, whereas the radicle emergence percentages in most of the other plants did not reach 20% (Fig. 5).

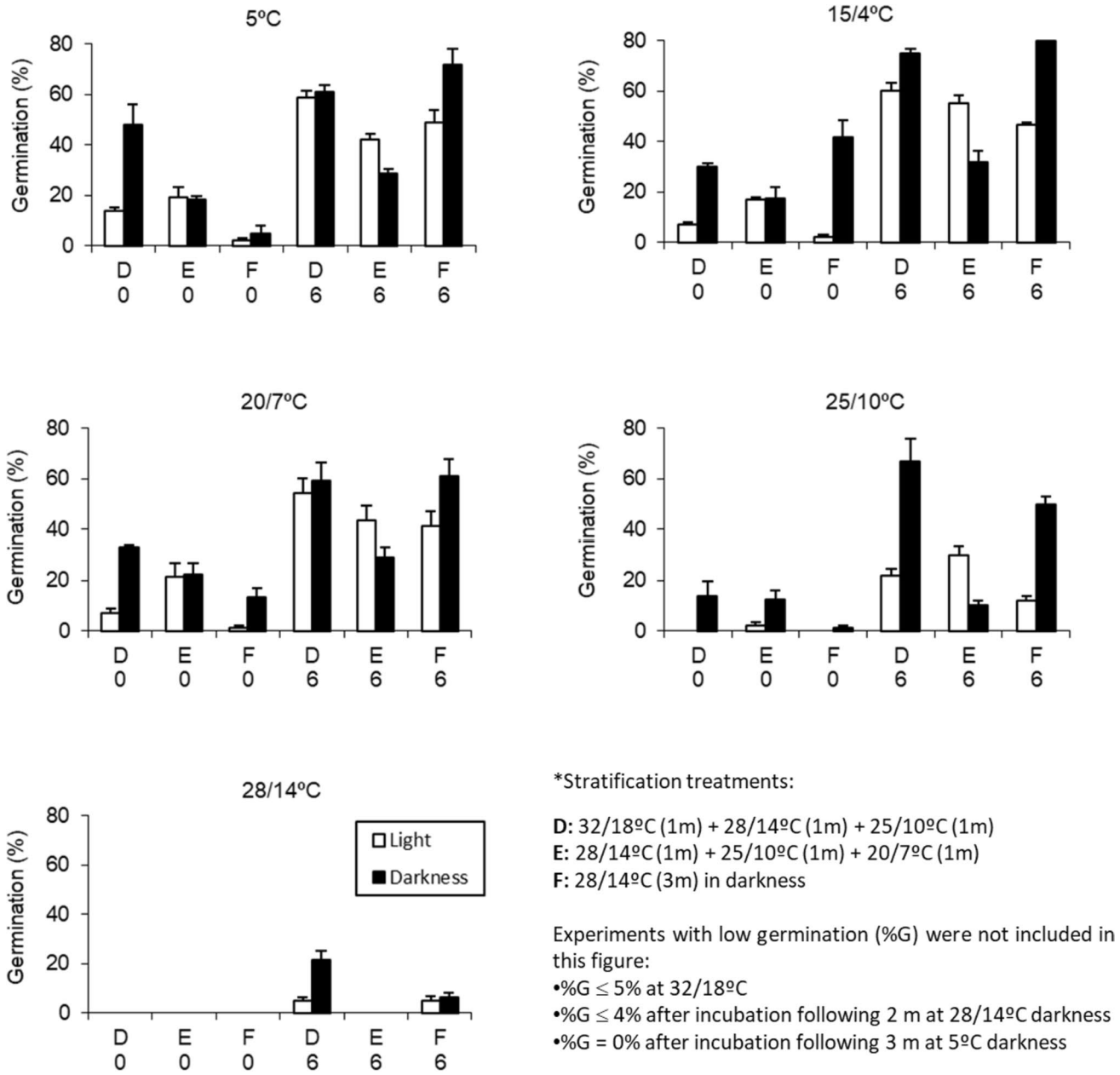

Fig. 3 Effects of seed age, stratification treatment*, incubation temperature, and illumination conditions during incubation on the germination percentage by *Helleborus foetidus* seeds

Influence of cold stratification on cotyledon emergence

Cold stratification (5 °C) promoted cotyledon emergence from seeds with emerged radicles and shortened the incubation time required for cotyledon development. Thus, cold stratification of seeds for 30 days resulted in a cotyledon emergence percentage of 45% within only 40 days after they were transferred from 5 °C to 20/7 °C (total time = 70 days). In seeds not subjected to cold stratification, the cotyledon emergence percentage at 20/7 °C after 70 days was only 7% (Fig. 6).

Discussion

Helleborus foetidus seeds have underdeveloped and dormant embryos at the time of dispersal, thus being affected by some level of MPD (Baskin and Baskin 2004).

We found that the embryos grew under warm stratification (28/14 °C; Treatments A and B) followed by cool temperatures (15/4 °C), at which seeds germinated. However, the embryos grew little, and no radicle emerged when the previous warm period was substituted by cold stratification (5ºC for 120 days) and then transferred to 15/4 °C (Table 1).

Table 2 Main effects of temperature, light conditions, stratification treatment, and seed age on germination by *Helleborus foetidus* seeds according to ANOVA

Factor	df	F	*P*	Category
Temperature (°C)	5	32.99	< 0.001	28/14 °C[a], 25/10 °C[b], 20/7 °C[c], 5 °C[c], 15/4 °C[c]
Light	1	10.40	0.0014	Light [a], Darkness [b]
Treatment	2	4.04	0.0189	E[a], F [ab], D [ab]
Seed age (months)	1	108.57	< 0.001	0[a], 6[b]

The table shows the degrees of freedom (df), F-ratio values, and categories with significant differences in germination ($P < 0.05$). Different letters indicate significant differences between levels for each factor (Tukey test). Residual degrees of freedom: 231

Hence, loss of MD (i.e., embryo growth) occurred during exposure to warm and then cool temperatures. This behavior supports that hellebore seeds have some simple level of MPD (Baskin and Baskin 2014).

Embryo growth commenced when the seeds were submitted to optimal temperatures, and thus MD and PD were overcome concurrently. Therefore, we could reject the possibility of non-deep simple MPD, because at this level, PD is broken first and the embryo then grows immediately (MD overcoming), as found in *Chaerophyllum tainturieri* (Baskin and Baskin 1990) and *Cyclospermum leptophyllum* (Walck et al. 2008). Nondeep simple MPD can also be rejected because GA_3 did not promote embryo growth, and intermediate and deep simple MPD too, since after embryo growth commenced, the seeds did not need cold stratification for radicle emergence (Fig. 1). Likewise, deep simple double MPD is not compatible with our results, because cotyledon emergence (shoot growth) requires a second winter in this level (Baskin and Baskin 2004).

The phenological patterns were similar in both the laboratory and outdoor experiments (Figs. 1, 2). Thus, embryo grew during the warm summer temperatures, and radicles emerged as the temperatures decreased in the autumn. However, shoots only began to emerge two months later of radicle germination. This lapse of time is consequence of the epicotyl dormancy (sensu Barton 1936). The level of this dormancy is defined by the effect of cold stratification on shoot development. For instance, seeds of *Viburnum odoratissimum* have non-deep simple epicotyl MPD since shoots emerge without a previous cold stratification requirement (Baskin et al. 2008). However, in *Helleborus foetidus*, cold stratification of germinated seeds accelerated the shoot emergence when these seeds were incubated to spring temperatures (20/7 °C) (Fig. 6). Thus, in seeds not subjected to cold stratification, the shoot emergence percentage was 0% at 20/7 °C after 60 days, but 37% when the seeds with emerged radicles were cold stratified for 30 days and then incubated at 20/7 °C for 30 days (total time = 60 days). Therefore, we conclude that the hellebore

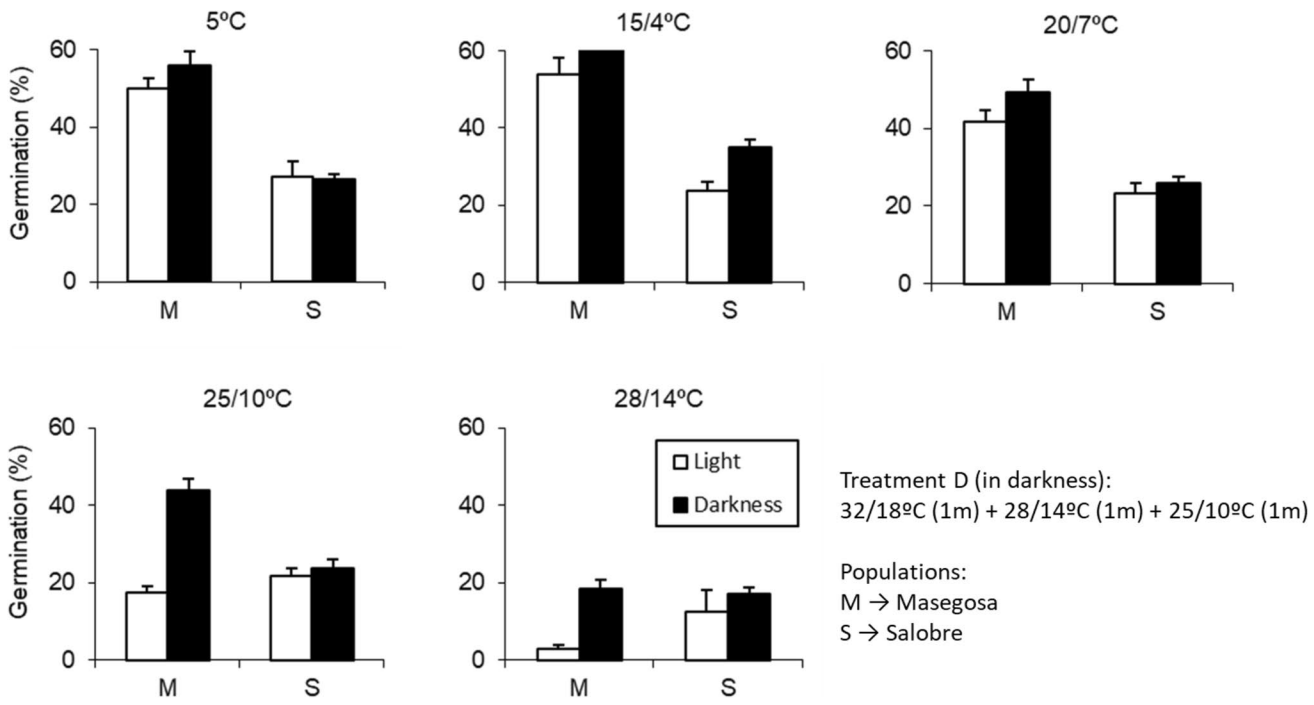

Fig. 4 Inter-population variability in germination capacity of *Helleborus foetidus* seeds after stratification treatment *D* in darkness. Seed age at the beginning of the stratification: 6 months (collection year: 2018). Germination < 2% in seeds incubated at 32/18 °C

Table 3 Main effects of temperature, light conditions, and inter-population variability on germination capacity of *Helleborus foetidus* seeds according to ANOVA

Factor	*df*	*F*	*P*	Categories
Temperature (°C)	4	16.54	< 0.001	28/14 °C[a], 25/10 °C[b], 20/7 °C[bc], 5 °C[bc], 15/4 °C[c]
Light	1	6.43	0.0132	Light[a], Darkness[b]
Population	1	15.06	0.0002	Masegosa[b], Salobre[a]

The table shows the degrees freedom (*df*), *F*-ratio values, and categories with significant differences in germination ($P < 0.05$). Different letters indicate significant differences between the levels of each factor (Tukey test). Residual degrees of freedom: 73

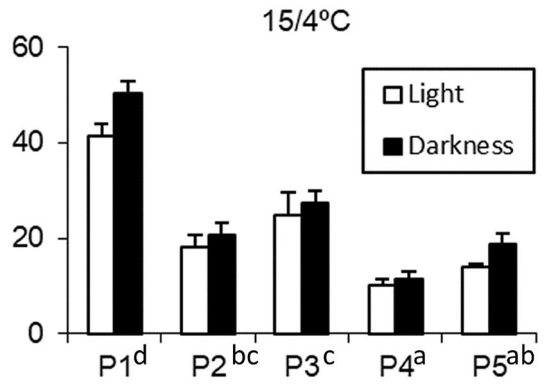

Fig. 5 Inter-population variability in germination capacity of *Helleborus foetidus* seeds after stratification treatment *D* [32/18 °C (1 month) + 28/14 °C (1 month) + 25/10 °C (1 month)] in darkness. Populations studied: Masegosa and Salobre. Seed age at the beginning of stratification: 6 months. Collection year: 2018. Number of plants: five. Different letters indicate significant differences between individuals

seeds exhibited deep simple epicotyl MPD, being the second report of this level of MPD in Ranunculaceae.

Our results differ from the preliminary level of MPD suggested by Nikolaeva et al. (1985) and corroborate the proposal of Sánchez-Yelamo and Ayerbe (1984) about the possibility of epicotyl dormancy in hellebore seeds because seeds with emerged radicles did not develop their epicotyl when incubated at 22 °C whereas they did when incubated at 11 °C.

Hellebore seeds are dispersed from mother plants in late spring–early summer as other temperate deciduous forest herbs with epicotyl dormancy (e.g., *Erythronium japonicum*, Kondo et al. 2002; *Hexastylis heterophylla*, Adams et al. 2003). This dispersal strategy satisfies the requirements for overcoming seed dormancy since seeds are warm-stratified during summer-early autumn, which is indispensable for subsequent germination as the temperatures decrease in the autumn (15/4 °C). From an ecological perspective, deep simple epicotyl MPD represents a suitable adaptation to temperate areas with a significant seasonal variation throughout the year. When the seeds are dispersed in early summer, the presence of an underdeveloped and dormant embryo prevents germination after infrequent summer rain, followed by drought which would lead to mortality of any seedlings that may have emerged. On the contrary, radicle emergence does not occur until the autumn, which is a more advantageous season for seedling survival. In addition, the existence of a shoot with PD avoids shoot emergence during the cold winter, thereby avoiding possible frost damage to young seedlings. However, the winter temperatures are warmer under the soil and the root can continue developing. Thus, when the shoot expands in spring, seedlings have a well-structured root system that can effectively explore the soil available water (Kondo et al. 2004; Copete et al. 2011).

Helleborus foetidus seeds germinated (i.e., radicles emerged) in light and darkness, although the germination percentages for 6-month-old seeds were higher in darkness than light at most incubation temperatures following the stratification period (treatments D and F). The capacity to germinate in darkness, e.g., when seeds remain buried in fissures in the soil, or covered by litter in holes, hampers the establishment of a permanent soil seed bank (Milberg et al. 2000). By contrast, for small seeds, it has been suggested that germination with a requirement for light might mean that these seeds only germinate when they are not far from the soil surface, thereby avoiding seedlings exhausting their seed storage reserves previous to emerge (Pearson et al. 2002). The weight of hellebore seeds (≈ 12 mg) is much higher than the threshold value (1.5 mg) below which temperate forest herbs require light for germination (Jankowska-Blasczuk and Daws 2007), but hellebore seeds can germinate in the light and colonize gaps, forest edges, and clearings. Another feature that supported the capacity to grow in gaps between vegetation is germination at daily fluctuating temperatures (15/4 °C, 20/7 °C, etc.) (Williams 1983). By contrast, germination in darkness promotes establishment under a closed forest canopy, and thus a wide variety of habitats can be occupied by the hellebore.

The cumulative seedling emergence percentage was 57% at the end of the study. This amount is relatively low because 20% of the seeds lost viability after sowing on 1 April. The loss of viability could have increased during the following months when seeds were exposed to warm stratification. This feature and others may hinder the formation of a seed bank, such as the capacity to germinate under darkness and the large size of hellebore seeds making them difficult to bury in the soil (Gao et al. 2021). However, 3% of the seedlings emerged in January 2018 following the second winter after sowing, thereby suggesting that the hellebore can form a small, short-lived persistent soil seed bank. This capacity may have important ecological advantages, such

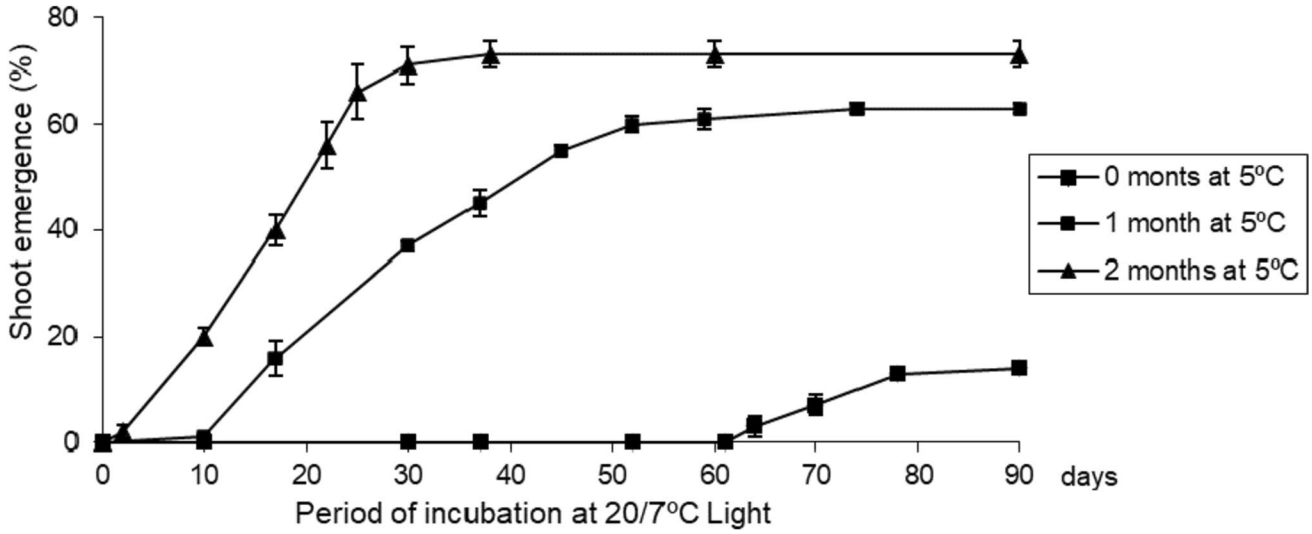

Fig. 6 Accumulated seedling emergence by *Helleborus foetidus* seeds with emerged radicles after incubation for 90 days at 20/7 °C in light following 0, 1, or 2 months of cold stratification at 5 °C

as the possibility of population reestablishment after a perturbation without the external input of propagules (Baskin and Baskin 1978). The presence of an elaiosome on seeds may facilitate their dispersal and burial in the soil by ants, despite their relatively large size (Vandelook et al. 2018). In addition, primary dormancy can prevent seeds from germinating promptly after dispersal and increase the likelihood of seeds becoming buried and incorporated in the soil seed bank (Thompson and Grime 1979). Indeed, another Ranunculaceae species, i.e., *Aconitum napellus* subsp. *castellanum*, also has large seeds that can germinate while buried in the soil with no requirement for light, and it can form soil seed banks with short-term persistence (Herranz et al. 2010a).

The improvement of germination ability with dry seed storage or after ripening, as shown in the present study, is a fairly common event in seeds with developed embryos and with non-deep physiological dormancy (Baskin and Baskin 2014). However, it is less common in species with MPD, although it is found on *Aconitum napellus* subsp. *castellanum* (Herranz et al. 2010a), *Delphinium fissum* subsp. *sordidum* (Herranz et al. 2010b), *Narcissus alcaracensis* (Herranz et al. 2013), and *Narcissus eugeniae* (Copete et al. 2014). This promoting effect of dry storage suggests that radicle emergence from hellebore seeds after dispersal is avoided by non-deep physiological dormancy and that it is independent on epicotyl dormancy, which is deep and must be overcome by cold stratification. In its natural habitat, *Helleborus foetidus* seeds would undergo dry storage after the ripening process during the summer period.

Similar to other species with MPD (Fernández-Pascual et al. 2013; Copete et al. 2014), our results confirmed high inter-population variability in the germination capacity, which may be due to maternal genetic and environmental influences during embryo formation and seed maturation (Fernández-Pascual and Jiménez-Alfaro 2014). We also found marked interannual differences in the germination of seeds collected in Masegosa locality (2017 and 2018) because of the effects of the weather patterns during the seed ripening process. The high intra-population variability may be a consequence of genetic differences between individuals, as found in other species with seeds that contain developed embryos at dispersal (Mira et al. 2017). Seed germination is subject to strong selection pressure, and thus it is highly likely to be sensitive to climatic changes (Walck et al. 2011). Thus, the inter- and intra-population variabilities detected in this study are good adaptations to the uncertain future climate change scenario, where most Mediterranean areas are supposed to become more aleatory (Mira et al. 2017). Finally, the origins of seeds used in plant propagation protocols must be considered due to the intra-specific variation in hellebore seeds.

The results obtained in this study provide accurate guidelines on how to grow hellebore plants from seeds for different purposes. The plant production protocol will vary depending on the climate to promote efficient radicle and shoot emergence (Fig. 7). In contrasting climates with cold winters, the whole process should be conducted in a non-heated shade-house with a metal frame. The seeds must be sown in early August in forestry seedling propagation containers at a depth of 1 cm using a mixture of peat and sand as the substrate, with periodic watering. Under these conditions, radicles will emerge during November and December, and seedling emergence will occur 2 months later. Finally, the new young plants must be kept in these containers for 6

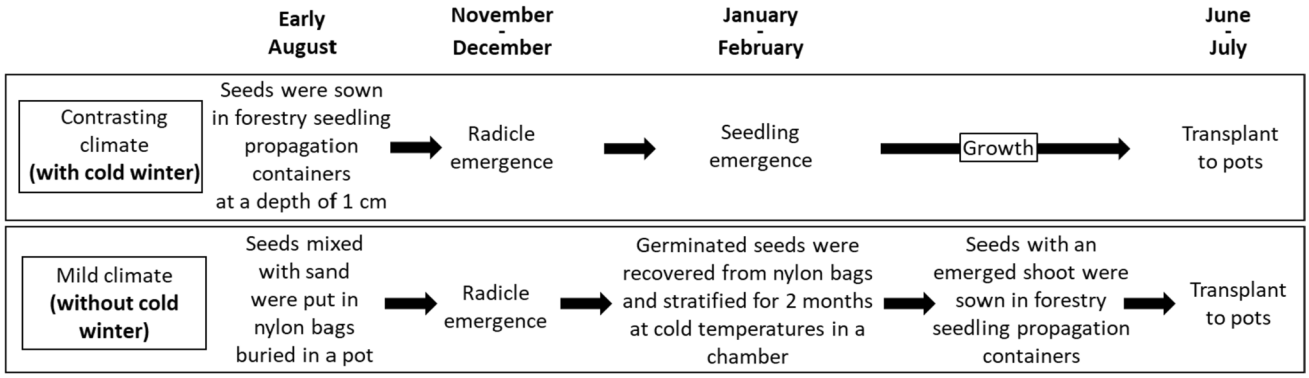

Fig. 7 Plant production guide for *Helleborus foetidus*

months, before transplanting to pots where they can complete their development. In mild climates without cold winters, in early August, the seeds should be mixed with fine sterilized sand and placed in fine-mesh nylon cloth bags, which must be buried at a depth of 5 cm in a pot and exposed to natural temperatures. In early November and December, the bags need to be removed to recover seeds with emerged radicles. These seeds must then be stratified at cold temperatures (5 °C) in a chamber for 2 months to promote shoot emergence, and the seeds can then be sown in forestry seedling propagation containers initially, before transplanting to pots.

Author contribution statement All authors have contributed throughout the different phases of this work in a coordinated way: designed and performed the experiments (JMH, RH, MAC), analyzed the data (RH, EC), wrote the manuscript (JMH, MAC, EC), and discussed the results (JMH, PF). All authors read and approved the final manuscript.

Acknowledgements We do appreciate the Reviewers' effort in checking the English throughout the entire manuscript.

Funding E.C.'s salary is co-founded by FEDER (Fondos Europeos de Desarrollo Regional) funds.

Data availability The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflicts of interest to the content of this article. The authors have no affiliations with or involvement in any organization or entity with any interest in the subject or materials discussed in this manuscript.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors consent with the publication of this article.

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