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Intermediate complex morphophysiological dormancy in the endemic Iberian *Aconitum napellus* subsp. *castellanum* (*Ranunculaceae*)

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Abstract

Seeds of *Aconitum napellus* subsp. *castellanum* were physiologically dormant at maturity in early autumn, with underdeveloped embryos. Thus they have morphophysiological dormancy (MPD). Embryos in fresh seeds were on average 1.01 mm long, and they had to grow to 3.60 mm before radicle emergence. Cold stratification at 5°C for 5 months with light enhanced the mean embryo length to 2.73 mm (SE = 0.13) and seed germination to 20%. However, with higher temperatures (15/4, 20/7, 25/10, 28/14 and 32/18°C) embryo growth was small, with no seeds germinating. Optimal germination was achieved after 4 months of cold stratification at 5°C followed by incubation at 20/7°C for 1 month with light, when germination ranged between 70 and 79%, depending on seed age, locality and year of collection. Cold stratification could be substituted by the application of GA_3 solution, since mean embryo length in seeds incubated at 25/10°C for 1 month with light was 3.52 mm and the germination was 80%. Since cold stratification was the only requirement for the loss of MPD, the longest embryo growth occurred during this treatment, and GA_3 promoted MPD loss, we concluded that *A. napellus* seeds have intermediate complex MPD. Germination was higher in 4-month stored than in freshly matured seeds. A pronounced variability in germinative patterns at inter-annual and inter-population level was recorded.

Keywords: *Aconitum napellus* subsp. *castellanum*, cold stratification, dormancy break, embryo growth, gibberellic acid, morphophysiological dormancy

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Introduction

Aconitum napellus L. subsp. *castellanum* Molero and C. Blanché (*Ranunculaceae*) is a rhizomatous perennial herb, 1–1.5 m tall, which is endemic to the Central Iberian Peninsula where it inhabits marshes and stream banks, as well as shady environments of deciduous woodlands, mountain coniferous woodlands and riverbank forests, at altitudes of 1000–1400 m (Molero and Blanché, 1986; Martín *et al.*, 2003). This plant has been classified as a vulnerable taxon in the Red List of Vascular Flora of Spain (Aizpuru, 2000; Moreno, 2008) and in the Red Book of Threatened Vascular Flora of Spain (Bañares *et al.*, 2003), due to the small size of populations and the severe fragmentation of its habitat.

Information on the environmental conditions required for seed dormancy break and germination of threatened species is essential to design *ex situ* propagation protocols in order to reinforce wild plant populations, to understand the ecological life cycle of plants, and thus to predict those periods favourable for seedling emergence and establishment in nature (Lentz and Johnson, 1998; Giménez-Benavides *et al.*, 2005). Also, a good understanding of the germination ability and requirements of endemic species is important for optimal biodiversity conservation and future management of populations (Galmés *et al.*, 2006).

Studies on germination ecology of Iberian endemics up to the present (e.g. Pérez-García *et al.*, 1995; Escudero *et al.*, 1997; Albert *et al.*, 2002; Herranz *et al.*, 2002; Galmés *et al.*, 2006; Lorite *et al.*, 2007) have focused on analysis of the influence of temperature and light conditions on germinative capability. However, other important aspects of the germination process have yet to be researched, such as germination phenology, germination responses of seeds buried in the soil, effects of temperatures and GA_3 on embryo growth, and the influence of light during cold

stratification on seed germination. Since the *Aconitum* genus belongs to the botanical family of *Ranunculaceae*, we have paid special attention to the analysis of environmental conditions which promote embryo growth and subsequent germination.

Cronquist (1988) considered the *Ranunculaceae* to be a primitive family whose members have undeveloped, although fully differentiated, embryos which need to grow for germination (Baskin and Baskin, 1998). At the time of seed dispersal, embryos may be non-dormant or physiologically dormant. If embryo growth and radicle emergence are completed at suitable temperature, light/dark, and moisture conditions in about 30 d, without a dormancy-breaking treatment, seeds have morphological dormancy (MD). However, if seeds with undeveloped embryos require a dormancy-breaking pretreatment, such as exposure to moist cold (0–10°C) or to moist warm (≥15°C) stratification, before they germinate, they have morphophysiological dormancy (MPD), and embryo growth takes place during or after the breaking of dormancy (Nikolaeva, 1977; Baskin and Baskin, 1990, 1998).

Recently, Vandelook *et al.* (2009) found that seeds of *Aconitum lycoctonum*, a Eurasian perennial herb growing in deciduous forests, have deep complex MPD, and they have suggested that Asian species *Aconitum heterophyllum* and *A. sinomontanum* might have intermediate complex MPD. Clearly, all species belonging to the same genus do not necessarily have the same level of MPD, as recorded in very closely related *Sambucus* species (Hidayati *et al.*, 2000), and in species of *Osmorhiza* and of *Erythronium* (Baskin *et al.*, 1995; Walck *et al.*, 2002). Thus, one of the main goals of the present study was to elucidate whether seeds of *Aconitum napellus* subsp. *castellanum* have MD or MPD, and, if the latter, which one of the nine known levels (Baskin *et al.*, 2008) of MPD they have.

The influence of temperature on embryo growth and germination has not been analysed in any endemic Iberian *Ranunculaceae*. Moreover, additional work on conditions required for embryo growth of species with underdeveloped embryos and comprehensive knowledge on seed germination characteristics are needed before evolutionary relationships among various types of MPD can be established (Walck *et al.*, 1999). In *Ranunculaceae*, morphological dormancy and six of the nine levels of MPD are reported, so members of this family provide an opportunity to study relationships of the levels of MPD. However, detailed germination studies have been done only on *c.* 30 species in 12 genera of the approximately 2200 species in 55 genera of the family (Baskin and Baskin, 1998; Walck *et al.*, 1999).

As a contribution to understanding MPD in the *Ranunculaceae*, the germination ecophysiology of the herbaceous perennial *A. napellus* subsp. *castellanum*, and how timing of germination is controlled in nature, we have analysed the requirements for dormancy loss, embryo growth and germination in this taxon. In addition, we analysed the influence of several factors revealed as important for germination in previous studies: light conditions and duration of cold stratification (Baskin and Baskin, 1994; Baskin *et al.*, 2000; Hidayati *et al.*, 2000), seed age (Baskin and Baskin, 1998; Schütz *et al.*, 2002; Copete *et al.*, 2005), year of seed production and seed population origin (Beckstead *et al.*, 1996; Andersson and Milberg, 1998; Pérez-García *et al.*, 2003). The specific aims of this study were to: (1) determine the temperature requirements for dormancy break and embryo growth; (2) analyse the effects of gibberellic acid on dormancy break and embryo growth; (3) analyse the phenology of germination and embryo growth; (4) characterize germination responses of seeds buried in the soil and periodically recovered; (5) analyse the influence of cold stratification duration, light conditions during stratification (light/darkness) and seed age on germination; and (6) determine inter-annual and inter-population variability in final germination percentages.

Materials and methods

Plant material

A. napellus subsp. *castellanum* shows a profuse vegetative reproduction capability from a napiform rhizome, which resprouts vigorously during March and April. The flowering phase starts in July and extends to September. Fruits are poly-folliculous, containing 30–40 seeds per fruit. Seeds are dispersed by fruit opening around late October. In November, the above-ground parts die and underground roots remain dormant from December to March, often being covered by snow for a long part of this period.

We tested seeds collected from two sites, both with a population of over 2000 adult plants, as follows: (1) Laguna del Marquesado (Cuenca, central Spain), 1350 m above sea level (a.s.l.), 30SXK1349, where *A. napellus* grows in gaps of *Populus* and *Salix* riparian forests, alternating with temporally flooded grasslands (LM hereafter); (2) Río Pelagallinas, Condemios de Arriba (Guadalajara, central Spain), 1380 m a.s.l., 30TUL9160, where *A. napellus* grows on bog edges and marsh grasslands in gaps of *Pinus sylvestris* forests (RP hereafter).

Seeds were collected in LM on 21 October 2001 and 24 October 2002 and in RP on 25 October 2002. In all cases, seeds came from about 30–40 apparently vigorous and healthy plants growing in well-lit situations. Seeds were dried at room temperature (22–23°C) in the laboratory up to the beginning of

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tests, on 2 November 2001 and 2002, respectively, at which time seeds were considered to be 0 months old.

Germination analysis

Seeds were submitted to moist cold (5°C) stratification for 3 and 4 months, both with a 12-h daily photoperiod (= light) and in continuous darkness (= darkness), at two ages: 0 months (freshly matured seeds) and 4 months (seeds were stored in paper bags under laboratory conditions: 22–23°C and 50–60% air humidity). After the stratification treatment (described below), seeds were tested for germination in light and in darkness at a constant temperature of 5°C and at 12/12 h daily temperature regimes of 15/4, 20/7, 25/10, 28/14 and 32/18°C.

In treatments including both light and fluctuating temperatures, the light phase was programmed to coincide with the higher temperature, and the dark phase with the lower one. A 100-seed lot was assigned to each treatment, distributed into four 25-seed replicates. Each replicate was incubated in a 9-cm-diameter Petri dish, sealed with Parafilm, on a double layer of filter paper, within germination chambers. Tests lasted 30 d, according to the recommendations on test length of Baskin and Baskin (1998). Darkness during stratification and incubation was achieved by wrapping Petri dishes in a double layer of aluminium foil. In treatments with light, seeds were checked for germination every 2–3 d. Seeds incubated in darkness were checked only at the end of the test. Ungerminated seeds were checked for viability on the basis of the embryo appearance, paying special attention to the colour and firmness. Percentages of germination were computed based on number of viable seeds. Germination chambers (Ibercex F-4 Model, Madrid, Spain) were equipped with a digital temperature and light control system [± 0.1°C, cool white fluorescent light, 25 μmol m^{-2} s^{-1} (1350 lux)].

The fluctuating temperatures used in the germination tests simulated mean maximum and mean minimum monthly temperatures, respectively, characterizing the annual climate cycle in the seed-source region (lowlands of the Serranía de Cuenca) of most seeds used in the study: 15/4°C, November and March; 20/7°C, October and April; 25/10°C, September and May; 28/14°C, August and June; and 32/18°C, July. The 5°C treatment simulates the mean temperature recorded during winter months: December, January and February.

Influence of temperature on embryo growth

Twenty seeds were placed on two sheets of filter paper moistened with distilled water in a 9-cm Petri dish. After seeds had imbibed at room temperatures for 24 h, embryos were excised with a razor blade and their length measured using a dissecting microscope equipped with a micrometer.

On 2 November 2001, 150 seeds from LM were placed on two sheets of filter paper moistened with distilled water in each of six 9-cm Petri dishes and placed in light at 5, 15/4, 20/7, 25/10, 28/14 and 32/18°C. After 30, 60, 90, 120 and 150 d, 20 apparently healthy seeds were extracted from the Petri dishes at each temperature and their embryos were excised and measured. Mean length and standard error were calculated for each sample of 20 embryos.

Also, on 2 November 2001, 400 seeds were placed on two sheets of filter paper moistened with distilled water in a 16-cm Petri dish, which was wrapped with plastic film and placed in light at 5°C. After 3 months (treatment A) and 4 months (treatment B) at 5°C, 20 ungerminated seeds with a healthy appearance were placed in each of five 9-cm Petri dishes and placed in light at 15/4, 20/7, 25/10, 28/14 and 32/18°C for 30 d. After that period, mean length and standard error were calculated for each sample of 20 embryos.

In addition, after 90 d at 5°C, 100 seeds were placed on two sheets of filter paper moistened with distilled water in a 9-cm Petri dish and incubated in light at 20/7°C for 30 d. Embryos of seeds that had germinated at that time (52%) were recorded as fully elongated and for each of 40 seeds, the radicle was cut off and the length of the portion of the embryo remaining inside the seed coat was measured. Mean length of the portion of the embryo inside the seed coat (i.e. critical embryo length, *sensu* Baskin *et al.*, 2001; Kondo *et al.*, 2004) was 3.6 mm (SE = 0.06 mm, $n = 40$, range = 2.9–4.5 mm, where 2.9 mm is the minimal embryo length registered in germinated seeds, 'threshold embryo size' hereafter).

Effects of GA_3 on dormancy break and embryo growth

On 2 November 2001, 20 seeds were placed on two sheets of filter paper moistened with a solution of 1000 mg l^{-1} of gibberellic acid (GA_3) in distilled water in each of 24 9-cm Petri dishes. Four dishes were placed in light at 5, 15/4, 20/7, 25/10, 28/14 and 32/18°C. After 7, 15, 22 and 30 d of incubation, a dish was removed from each chamber, and the percentage of germination and the length of embryos in ungerminated seeds were recorded. Mean length and standard error were calculated for each sample of 20 embryos, assuming an embryo length of 3.6 mm in germinated seeds (critical embryo length).

For the control, 20 seeds were placed in each of six 9-cm Petri dishes on two sheets of filter paper moistened with distilled water and incubated in light at 5, 15/4, 20/7, 25/10, 28/14 and 32/18°C for 30 d.

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Phenology of embryo growth

On 2 November 2001, six groups of 50 *A. napellus* seeds from LM were mixed with fine sterilized sand, and each group was placed in a fine-mesh polyester cloth bag. Bags were buried 5-cm deep in a pot and placed in a non-heated greenhouse where they were watered to field capacity once a week, except when the substratum was frozen and watering was interrupted. Such a water regime simulated soil moisture conditions in the natural habitat of the taxon. Bags were recovered at monthly intervals as of 1 December 2001. Bag content was sieved at 1 mm in order to separate seeds from sand. Twenty seeds with a healthy appearance were excised immediately and measured, calculating mean length and standard error of embryos. The critical length of embryos (i.e. 3.6 mm) was assumed for seeds germinated within the bag while buried, with a percentage of embryos similar to that in germinated seeds.

Dormancy break in buried seeds

On 2 November 2001, six groups of 600 *A. napellus* seeds each from LM were mixed with fine sterilized sand, and each group was placed in a fine-mesh polyester cloth bag. Bags were buried and exhumed at monthly intervals. Apparently healthy ungerminated seeds within the bag during the burial experiment were divided into four lots (100 seeds if sufficient), which were incubated in light at 15/4, 20/7, 25/10 and 28/14°C for 30 d. Exhumed seeds were classified in four categories: (1) seeds germinated within the bag; (2) viable non-dormant seeds, which germinated at 20/7°C (the most favourable temperature regime for germination); (3) viable dormant seeds, which did not germinate at 20/7°C and had healthy embryos; (4) non-viable seeds, which had a rotten appearance, as well as those with a dead embryo when excised.

Germination in seedlots exhumed in April and May 2002 was ≥75%, so for these dates only 20/7°C was tested for germination.

Phenology of seedling emergence

On 2 November 2001, three 200-seed replicates from LM were sown to a depth of 5 mm in seed trays with drainage holes in a substrate consisting of a mixture of sterilized peat and sand (proportion 2:1). Seed trays were placed in the non-heated greenhouse and watered at the periodicity described above (see Phenology of embryo growth). From 2 November 2001 to 1 May 2006, seed trays were examined once a week, and if seedlings with emergent cotyledons were present, they were counted and removed.

Effect of time, light conditions during cold stratification and seed age on germination

These studies were done using LM seeds, and controls were kept in light and in darkness at 15/4, 20/7, 25/10, 28/14 and 32/18°C. For each temperature × light condition, we used four replicates of 25 seeds which were placed in 9-cm Petri dishes on two sheets of filter paper moistened with distilled water. Dishes were examined at monthly intervals and if seedlings were present, they were counted and removed. Control seeds were placed in the incubators on 2 November 2001, where they remained for 5 months. Examination of seeds incubated in darkness was carried out using a green safe light (Vandelook *et al.*, 2007).

In addition, on 2 November 2001 (age = 0 months) and 1 March 2002 (age = 4 months) 1300 *A. napellus* seeds were placed in each of four 16-cm Petri dishes on two sheets of filter paper moistened with distilled water and sealed with Parafilm. Two dishes were cold-stratified at 5°C for 3 months, one of them in light and the other in darkness. The other two dishes were also cold stratified for 4 months in light and darkness, respectively. In seeds cold stratified in darkness, filter paper was kept moist by adding distilled water when necessary, under a green safe lamp (Vandelook *et al.*, 2007). After 3 and 4 months of cold stratification (either with light or in darkness), seeds were incubated in both light and darkness at 5, 15/4, 20/7, 25/10, 28/14 and 32/18°C for 30 days.

Inter-annual and inter-population variability in germination ability

For this assay, we collected seeds from LM and RP in October 2002. We carried out a control test similar to that described in the previous section with RP seeds. To evaluate the inter-annual variability in the germination ability of *A. napellus* seeds, we compared germination at different temperature regimes of seeds collected in LM in 2001 and 2002, after cold (5°C) stratification for 4 months, both in light and in darkness during stratification and during the germination tests, with an age of 0 and 4 months at the beginning of assay. To evaluate inter-population variability in the germination ability of *A. napellus*, seeds collected in RP were cold (5°C) stratified for 4 months, using the same light conditions (light, darkness) mentioned above for assays of LM seeds in 2002, and with equal seed age (0, 4 months), in order to compare germination responses. Thus, 1300 seeds were placed in each of four 16-cm Petri dishes for cold stratification both for LM02 and RP02.

Periods of cold stratification for seeds collected in October 2002 were started on 2 November 2002 and 1 March 2003, using the same design as that described in the previous section.

Statistical analysis

The effect of duration of incubation at different temperatures and the effect of incubation temperatures on the length of embryos were analysed by a two-way analysis of variance (ANOVA).

Seed germinability was evaluated by the final cumulative germination percentage, which was compared among treatments by multifactor ANOVAs. In the comparison of the germination percentage, the effects of five factors were analysed: temperature (six levels), light (two levels), seed age (two levels), length of cold stratification (two levels), and light condition during cold stratification (two levels). In the study of the inter-annual and inter-population variability of seed germinability, two other factors were analysed: the year (two levels) and the locality (two levels) of seed collection.

When significant main effects existed, differences were detected by a multiple comparison Tukey test. Prior to analyses, normality (Cochran test) and homoscedasticity (David test) of data were checked. Values of the final cumulative germination percentage were squared-root arcsine transformed.

Results

Influence of temperature on embryo growth

Mean length of embryos from freshly matured *A. napellus* seeds was 1.01 mm (SE = 0.03; $n = 20$), while the endosperm measured 4.15 mm (SE = 0.07; $n = 20$). Mean length of embryos in seeds incubated in light at 15/4, 20/7, 25/10, 28/10 and 32/18°C for 5 months ranged between 1.48 and 2.38 mm (Table 1), never reaching the critical embryo length (i.e. mean size of embryo in seeds germinated; *c.* 3.60 mm; SE = 0.06). In addition, no embryo of seeds incubated at those conditions achieved the threshold size (i.e. minimal embryo length from which germination is possible; *c.* 2.9 mm). In consequence, no control seed germinated.

Seeds incubated at 5°C for 4 months showed a mean embryo length of 2.58 mm (SE = 0.12; $n = 20$), and 35% exceeded the threshold size. Indeed, 5% of seeds germinated. After 5 months of incubation at 5°C, the mean embryo length increased to 2.73 mm (SE = 0.13; $n = 20$), 45% of embryos surpassed the threshold size and 20% of seeds germinated (Table 1). Embryo size increased substantially and germination ranged between 20 and 70% when seeds were cold stratified in light at 5°C for 3 or 4 months prior to incubation in light at 15/4, 20/7 or 25/10°C for 30 d. Therefore, after 4-month cold stratification and incubation at 20/7°C for 30 d, the embryo length exceeded the threshold size in all seeds, and seed germination was 70% (Table 1).

Table 1. Embryo growth in *Aconitum napellus* subsp. *castellanum* seeds after 1–5 months' incubation in light at various temperature regimes. Treatments A and B are 3 and 4 months of cold stratification at 5°C, respectively, followed by 30 d of incubation at the various temperature regimes

Exposure time to treatments (months)	Thermoperiods					
	5°C	15/4°C	20/7°C	25/10°C	28/14°C	32/18°C
1	1.37 ± 0.03^{Aab} (0,0)	1.50 ± 0.04^{Ab} (0,0)	1.44 ± 0.05^{Aab} (0,0)	1.39 ± 0.05^{Aab} (0,0)	1.34 ± 0.03^{Aab} (0,0)	1.32 ± 0.04^{Aa} (0,0)
2	1.86 ± 0.07^{Bb} (0,0)	1.78 ± 0.04^{Ab} (0,0)	1.54 ± 0.05^{Aa} (0,0)	1.49 ± 0.04^{Aa} (0,0)	1.42 ± 0.05^{Aa} (0,0)	1.35 ± 0.04^{Aa} (0,0)
3	2.35 ± 0.13^{Cb} (0,0)	2.21 ± 0.07^{Bb} (0,0)	1.66 ± 0.07^{Aa} (0,0)	1.50 ± 0.05^{Aa} (0,0)	1.45 ± 0.06^{Aa} (0,0)	1.39 ± 0.05^{Aa} (0,0)
4	2.58 ± 0.12^{Cc} (5,35)	2.28 ± 0.05^{Bb} (0,0)	1.68 ± 0.05^{Aa} (0,0)	1.52 ± 0.05^{Aa} (0,0)	1.51 ± 0.05^{Aa} (0,0)	1.45 ± 0.05^{Aa} (0,0)
5	2.73 ± 0.13^{Cc} (20,45)	2.38 ± 0.07B^{Cb} (0,0)	1.69 ± 0.06^{Aa} (0,0)	1.55 ± 0.06^{Aa} (0,0)	1.53 ± 0.04^{Aa} (0,0)	1.48 ± 0.05^{Aa} (0,0)
Treatment A	2.58 ± 0.12^{Ca} (5,35)	2.68 ± 0.13^{CDa} (20,45)	3.20 ± 0.11^{Bb} (50,80)	2.46 ± 0.14^{Ba} (25,25)	2.37 ± 0.12^{Ba} (5,20)	2.26 ± 0.11^{Ba} (0,15)
Treatment B	2.73 ± 0.13^{Ca} (20,45)	2.77 ± 0.13^{Da} (25,50)	3.47 ± 0.05^{Bb} (70,100)	2.84 ± 0.14^{Ca} (30,55)	2.65 ± 0.11^{Ba} (15,30)	2.61 ± 0.08^{Ca} (5,20)

The percentage of germinated seeds (first number) and the percentage of seeds with embryo ≥ 2.9 mm (embryo size at which germination is possible; second number) at each treatment are indicated between parentheses. Values of mean embryo length with different uppercase letters within columns or with different lowercase letters within rows are significantly different.

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Table 2. Effect of GA_3 on the embryo growth in *Aconitum napellus* subsp. *castellanum* seeds

Incubation length (d)	Thermoperiods				
	5°C	15/4°C	20/7°C	25/10°C	28/14°C
7	0.91 ± 0.03^{Aa} (0,0)	0.90 ± 0.04^{Aa} (0,0)	0.90 ± 0.05^{Aa} (0,0)	1.00 ± 0.06^{Aab} (0,0)	1.18 ± 0.04^{Ab} (0,0)
15	0.98 ± 0.03^{Aa} (0,0)	1.09 ± 0.08^{ABa} (0,0)	1.87 ± 0.07^{Cb} (0,0)	1.60 ± 0.08^{Bb} (0,0)	1.78 ± 0.12^{Bb} (0,0)
22	1.01 ± 0.03^{Aa} (0,0)	1.92 ± 0.16^{Cb} (10,10)	2.91 ± 0.17^{Dc} (45,55)	3.10 ± 0.12^{Cc} (35,70)	3.37 ± 0.11^{Cc} (35,80)
30	1.33 ± 0.04^{Ba} (0,0)	2.93 ± 0.17^{Db} (50,60)	3.43 ± 0.10^{Ec} (70,90)	3.52 ± 0.04^{Dc} (80,95)	3.64 ± 0.06^{Cc} (60,100)
Control	1.37 ± 0.03^{Ba} (0,0)	1.50 ± 0.04^{BCa} (0,0)	1.44 ± 0.05^{Ba} (0,0)	1.39 ± 0.05^{Ba} (0,0)	1.34 ± 0.03^{Aa} (0,0)

GA_3 solution ($1000\,mg\,l^{-1}$). Seeds exposed at different thermoperiods and with different incubation lengths (7–30 d). Control assay: $0\,mg\,l^{-1}$ GA_3 solution and 30 d of incubation. The size of the embryo at each incubation length is expressed as mean ± SE ($n = 20$) in millimetres. The percentage of germinated seeds (first number) and the percentage of seeds with embryo ≥2.9 mm (embryo size at which germination is possible; second number) at each treatment are indicated between parentheses. Values of mean embryo length with different uppercase letters within columns or with different lowercase letters within rows are significantly different. Mean embryo length in freshly matured seeds = 1.01 ± 0.03 mm ($n = 20$).

Effects of GA_3 on dormancy break and embryo growth

Gibberellic acid had significant effects on germination and embryo growth. Control seeds ($0\,mg\,l^{-1}$ GA_3) showed an embryo size ranging from 1.34 to 1.50 mm and null germination after incubation at 15/4, 20/7, 25/10 and 28/14°C for 30 d (Table 2). In contrast, seeds incubated at the same temperature regimes for the same period of time in GA_3 ($1000\,mg\,l^{-1}$) reached a 50–80% germination, had an embryo length of 2.93–3.64 mm, and the percentage of embryos with length ≥ threshold size ranged from 60 to 100%. Embryo length increased substantially between days 15 and 22 of incubation (Table 2). Most seeds incubated at 32/18°C with GA_3 were attacked by fungi and were dead after about 10 d of incubation, so valid data were not obtained for this thermoperiod.

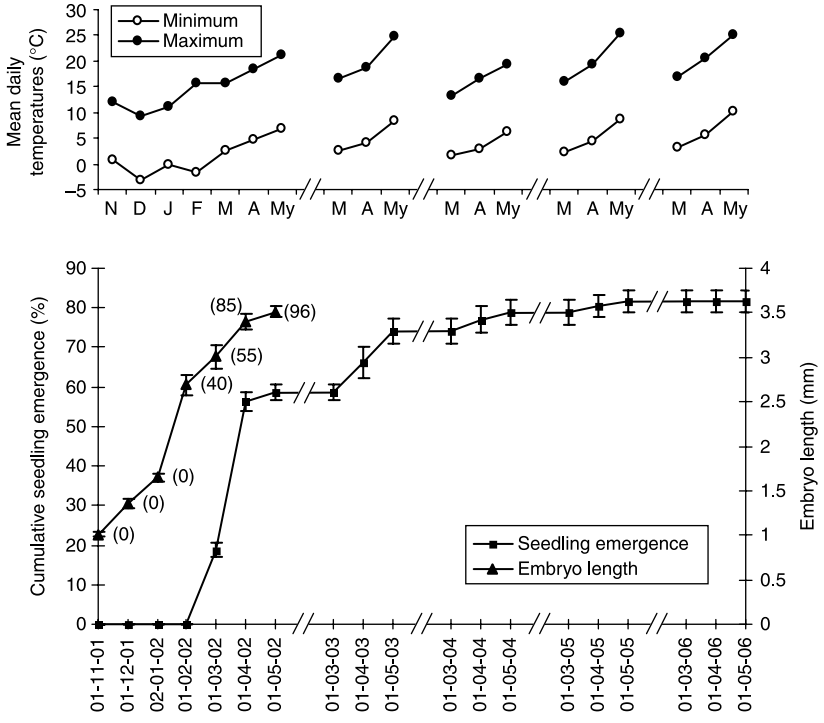

Figure 1. Changes in temperature throughout the assay in the greenhouse (mean daily maximum and minimum monthly air temperatures), germination phenology of *Aconitum napellus* subsp. *castellanum* (cumulative germination percentage, mean ± SE) and embryo length (mean ± SE if >0.1 mm; $n = 20$) on different dates. Numbers within parentheses next to embryo length indicate the percentage of seeds with embryo >2.9 mm. Date sown in the greenhouse: 1 November 2001. Location and date of seed collection: Laguna del Marquesado (Cuenca), 25 October 2001.

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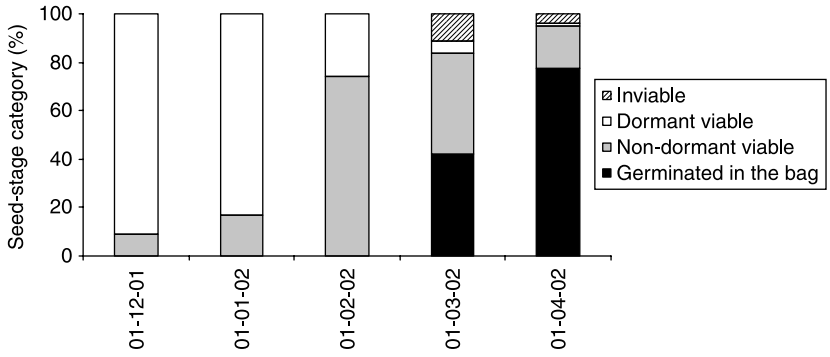

Figure 2. Stage of *Aconitum napellus* subsp. *castellanum* seeds exhumed monthly from 1 December 2001. Seed classes are: germinated within bags, non-dormant viable, dormant viable and inviable. Location and date of seed collection: Laguna del Marquesado (Cuenca), 25 October 2001. Date of seed burial: 1 November 2001.

Phenology of embryo growth

From 1 November 2001 to 2 January 2002 embryos grew little (Fig. 1), as opposed to the period from 2 January to 1 February 2002, when mean embryo length increased 63%, and 40% of embryos reached or surpassed the threshold size. Mean daily maximum and minimum temperatures during this period were 11.28 and −0.25°C, respectively. On 1 April 2002, the mean embryo length reached 3.40 mm (SE = 0.09), 85% of embryos exceeded the threshold length, and 80% of exhumed seeds had in fact germinated.

Phenology of seedling emergence

The first emerged seedlings were observed on 10 February 2002, and by the end of the month 18.83% (SE = 1.79) of the seeds had germinated. However, seedling emergence was concentrated between 1 March and 1 April 2002. At that time, cumulative germination was 56.33% (SE = 2.22). Mean daily maximum and minimum temperatures during this period were 15.57 and 2.76°C, respectively (Fig. 1). From 1 April to 1 May seedling emergence was low (cumulative emergence = 58.66%; SE = 2.13), and after 1 May 2002 no additional seedlings emerged until March 2003. On 1 May 2003, the cumulative emergence was 74.16% (SE = 3.30). During the spring of both 2004 and 2005, some residual emergence was recorded, and on 1 May 2005 cumulative germination was 81.66% (SE = 2.84). During the spring of 2006, no emergence was recorded.

Dormancy break in buried seeds

Most seeds buried on 2 November 2001 did not overcome dormancy until 1 February 2002 (Fig. 2), at which time seed germination in light was 74% (SE = 2.23) at 20/7°C and 73% (SE = 1.65) at 15/4°C (Fig. 3). Seeds exhumed in December 2001 and January 2002 only germinated noticeably at 15/4 and 20/7°C, although under 17%. Seeds exhumed in February and March 2002 showed germination percentages ≥ 73% in light at 15/4 and 20/7°C, and a notable increase of germination at 25/10 and 28/14°C (Fig. 3).

Seeds exhumed on 1 March 2002 were distributed as follows: (1) 42.21% germinated, (2) 41.60% viable non-dormant, (3) 5.20% viable dormant, and (4) 10.98% unviable. On 1 April 2002, 77.5% of seeds had germinated in the bag, and the others were mostly viable non-dormant or non-viable (Fig. 2).

Effect of time, light conditions during cold stratification and seed age on germination

Germination in control tests was null at all thermoperiods in both light and darkness for LM and RP seeds. Only seeds cold stratified germinated to ≥20% after incubation at 15/4, 20/7 and 25/10°C (Fig. 4).

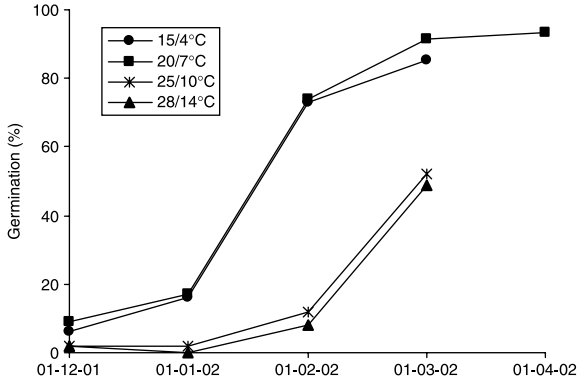

Figure 3. Changes in germination percentage in light at 15/4, 20/7, 25/10 and 28/14°C of viable *Aconitum napellus* subsp. *castellanum* seeds exhumed at monthly intervals. Date of seed burial: 1 November 2001. Date of first exhumation: 1 December 2001. Maximum SE: 3.84%.

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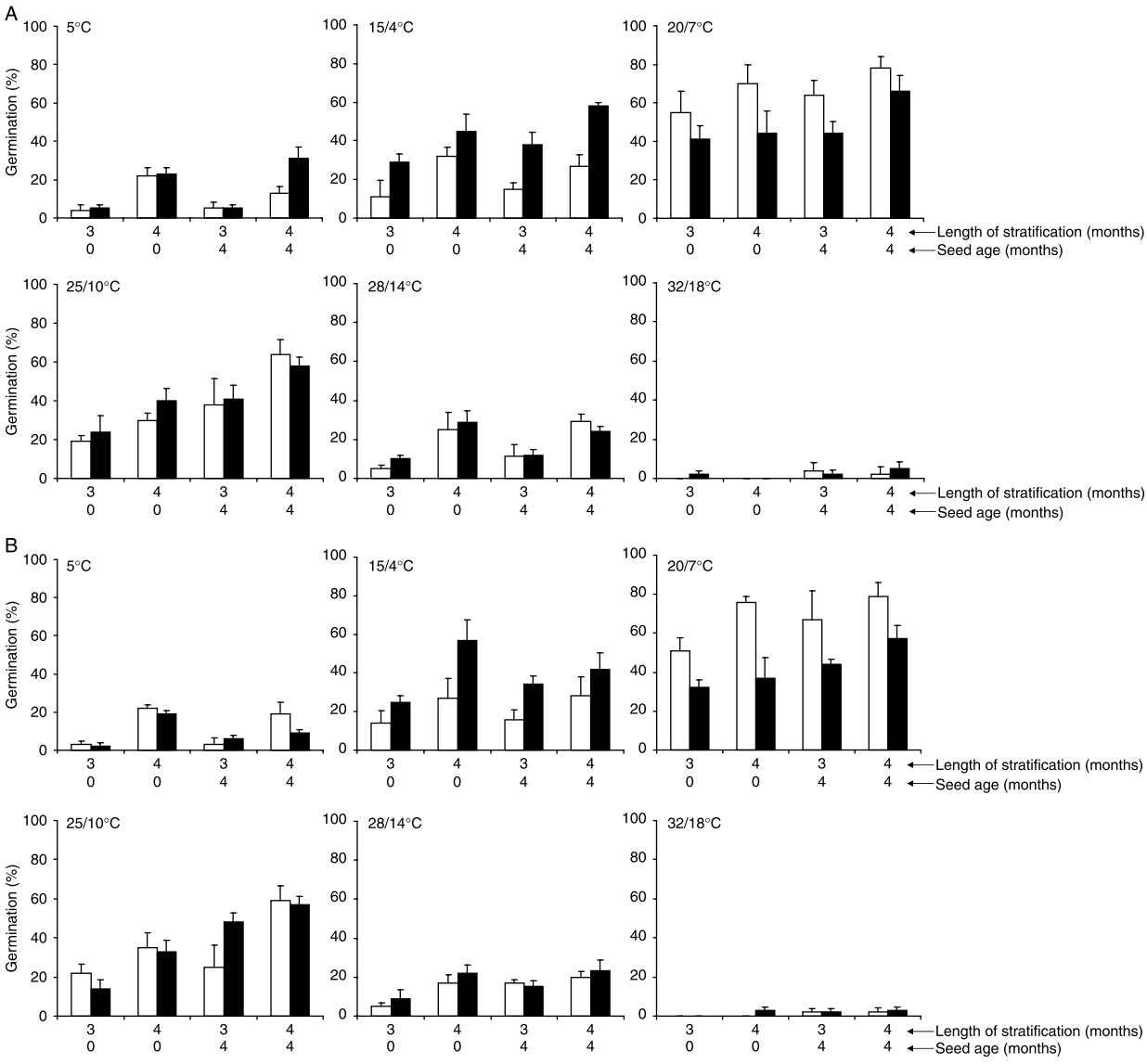

Figure 4. Influence of the length of wet cold (5°C) stratification (3 and 4 months) in both continuous darkness (A) and in light (B), as well as seed age (0 and 4 months) on seed germination (mean and SD) of *Aconitum napellus* subsp. *castellanum*. White and black bars show germination percentages in light and darkness, respectively. Locality and year of seed collection: Laguna del Marquesado (Cuenca), 2001.

Table 3 shows significant effects on final germination percentages of incubation temperature, light conditions during incubation and during cold stratification, duration of cold stratification and seed age.

The highest germination percentages were achieved by seeds incubated in light at 20/7°C, both for seeds stratified in light and in darkness. After 3 months' cold stratification in light, 0- and 4-month-old seeds germinated to 51% (SE = 3.27) and 66.83% (SE = 7.43), respectively, and after 4 months of cold stratification seeds germinated to 76% (SE = 1.41) and 79% (SE = 3.57), respectively. Seeds cold stratified in darkness exhibited a similar trend. Other thermoperiods promoting germination were 15/4 and 25/10°C. In contrast, constant low temperature (i.e. 5°C) and fluctuating thermoperiods of 28/14 and 32/18°C produced low germination values, but seldom over 20%. In the more favourable thermoperiods for germination (i.e. 15/4, 20/7 and 25/10°C), germination percentages increased with length of cold stratification and seed age. This pattern was very pronounced at 25/10°C, regardless of light conditions during the cold stratification phase (Fig. 4).

At 20/7°C, percentages of germination in light were significantly higher than those in darkness. In contrast, at 15/4°C the trend was just the opposite. No significant differences were detected in the thermoperiod 25/10°C.

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Table 3. Effects of several factors on seed germination of *Aconitum napellus* subsp. *castellanum* (locality: Laguna del Marquesado; year: 2001)

Factor	df	*F*	*P*	Categories
Incubation temperature	5	306.04	>0.001	32/18 < 5 < 28/14 < 15/4 < 25/10 < 20/7
Light conditions (incubation)	1	4.37	0.0372	Light < Darkness
Light conditions (stratification)	1	3.89	0.0494	Light < Darkness
Seed age	1	45.83	>0.001	0 < 4
Duration of stratification	1	169.43	>0.001	3 < 4

Main effects on germination of incubation temperature, light conditions during incubation and during cold stratification, seed age and duration of cold stratification in multifactor analysis of variance. The table shows degrees of freedom (df), *F*-ratio values, and categories of factors where germintation differences were significant. Residual degrees of freedom: 374.

Inter-annual and inter-population variability in germinative ability

Incubation temperature, light condition during incubation and stratification phases, seed age, population and year of collection had significant effects on germination percentages (Table 4).

Germination patterns of LM seeds collected in 2001 and 2002 showed noticeable differences: germination was significantly lower in seeds from the second year (Fig. 5, Table 4). Freshly matured seeds, cold stratified in light for 4 months, and incubated in light at 20/7°C germinated to 76% (SE = 1.41) when collected in 2001 but to 23% (SE = 0.96) when collected in 2002. Four-month-old seeds subjected to the same treatment conditions, reached a final germination of 79% (SE = 3.57) in the 2001 sample, and 60% (SE = 6) in the 2002 sample. Germination patterns were similar for thermoperiods 15/4 and 25/10°C, i.e. increasing germination percentages with seed age. Another noteworthy result with LM seeds collected in 2002 was that when they were incubated at 20/7°C, they germinated more in darkness than in light, compared to seeds collected in 2001 (Fig. 5).

As regards inter-population variability, it can be stressed that seeds collected in RP also reached high germination values when incubated at 15/4, 20/7 and 25/10°C, although the optimum was 15/4°C, in contrast to that (20/7°C) in LM seeds. However, in RP seeds the increase in germination with seed age was only manifested when they were cold stratified in light and subsequently incubated in light at 20/7 and 25/10°C (Fig. 5).

Discussion

In fresh matured *A. napellus* seeds, mean length of embryos was 1.01 mm (SE = 0.03). These must grow to 3.60 mm (SE = 0.06; critical embryo length) for radicle emergence. Cold stratification at 5°C (Table 1) or gibberellic acid (Table 2) were the pretreatments required to stimulate germination of *A. napellus* seeds. Seed incubation at 15/4, 20/7, 25/10, 28/14 and 32/18°C for 5 months by itself did not induce embryo growth over the threshold size (i.e. 2.9 mm) from which seeds become able to germinate (Table 1).

Since *A. napellus* seeds do not germinate for about 30 d after being placed on a moist substrate at different temperature and light conditions, it can be concluded that they have morphophysiological (MPD) and not morphological dormancy (Baskin and Baskin, 2005). If embryos had single morphological dormancy and required low temperatures such as 5°C for growth, rapid growth should have started immediately after seeds were placed at 5°C, instead of 1 month later.

Table 4. Effects of several factors on seed germination of *Aconitum napellus* subsp. *castellanum* (inter-annual and inter-population variability)

Factor	df	*F*	*P*	Categories[1]
Incubation temperature[1]	5	166.52	>0.001	$32/18^{a}$, 5^{b}, $28/14^{b}$, $25/10^{c}$, $20/7^{cd}$, $15/4^{d}$
Light conditions (incubation)	1	1.30	0.2554	
Light conditions (stratification)	1	11.39	>0.001	Darkness < Light
Seed age	1	40.93	>0.001	0 < 4
Year	1	52.80	>0.001	2001 < 2002
Population	1	58.71	>0.001	RP < LM

Main effects on germination of incubation temperature, light conditions during incubation and during cold stratification, seed age, year of seed collection and population in multifactor analysis of variance. The table shows degrees of freedom (df), *F*-ratio values, and categories of factors where germination differences were significant. Residual degrees of freedom: 373.

[1] Categories with different letters are significantly different.

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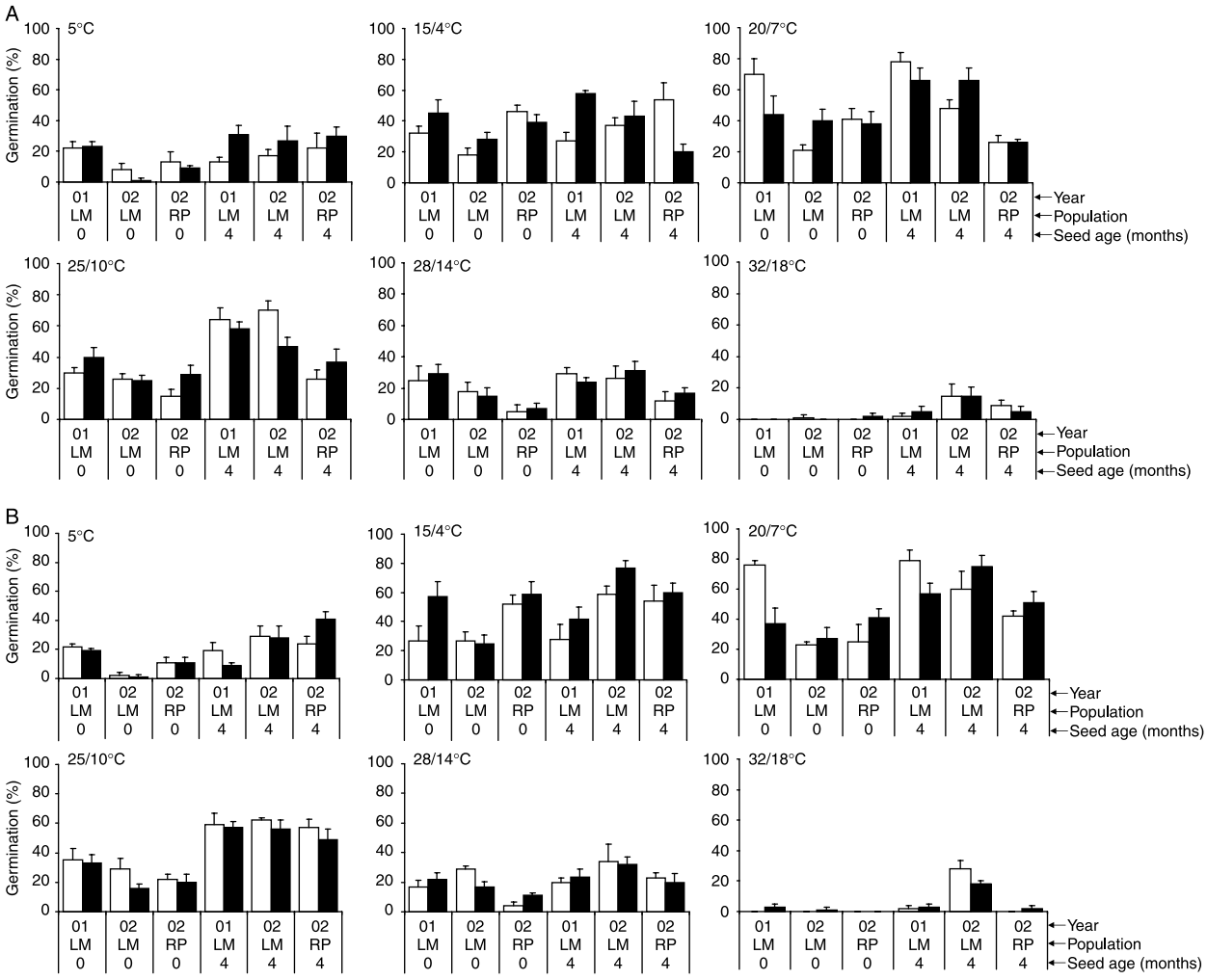

Figure 5. Inter-annual (01 = 2001; 02 = 2002) and inter-population (LM = Laguna del Marquesado; RP = Río Pelagallinas) variability in germination (mean and SD) of 0- and 4-month-old seeds of *Aconitum napellus* subsp. *castellanum* cold stratified in darkness (A) and in light (B) at 5°C for 4 months and tested at different temperatures. White and black bars show germination percentages in light and darkness, respectively.

There are two general types of MPD: simple and complex. In seeds with simple MPD, embryo growth (loss of morphological dormancy) occurs only during warm (≥15°C) stratification. In contrast, embryos of seeds with complex MPD grow only during cold stratification (Baskin and Baskin, 1998). Since embryos of *A. napellus* seeds grew to full length during cold stratification, seeds of this taxon have complex MPD.

In addition, three types of complex MPD have been distinguished: (1) non-deep; (2) intermediate; and (3) deep (Baskin and Baskin, 1998). Seeds with non-deep complex MPD require warm followed by cold stratification before they germinate. As seeds of *A. napellus* do not show this requirement for embryo growth and germination, they do not have non-deep complex MPD. Seeds with deep complex MPD require cold stratification for loss of physiological dormancy and for growth of embryo, and gibberellic acid (GA_3) does not promote germination. Thus, *A. napellus* seeds do not have deep complex MPD. Seeds with intermediate complex MPD require only cold stratification for both loss of physiological dormancy and growth of embryos. However, gibberellic acid substitutes for cold stratification in promoting germination. Since GA_3 promoted germination of *A. napellus* (Table 2), it can be concluded that they have intermediate complex MPD. In addition, in seeds with intermediate complex MPD, dry laboratory storage may decrease the length of the cold stratification required to break dormancy (Baskin *et al.*, 1992). Seeds of *A. napellus* incubated at optimum thermoperiods (20/7 and 25/10°C) showed similar germination values when cold stratified for 3 months preceded by dry laboratory storage for 4 months as those cold stratified for 4 months without dry laboratory storage (Fig. 4). These results further support the presence of intermediate complex MPD in seeds of *A. napellus*.

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So, in addition to the deep complex MPD described for *A. lycoctonum* (Vandelook *et al.*, 2009), the existence of intermediate complex MPD in other *Aconitum* species is confirmed. This level of MPD may also be present in seeds of *A. heterophyllum* (Pandey *et al.*, 2000) and *A. sinomontanum* (Dosmann, 2002), although an analysis of the effects of cold stratification on dormancy break in the former and of germination promotion by GA_3 in the latter are needed.

In the laboratory, maximum germination (70–80%) occurred with 1 month's incubation at 20/7°C with light after seeds were exposed to 5°C for 4 months (2880 h) both with light and in darkness (Table 1, Fig. 4). In the non-heated greenhouse the emergence of seedlings started from 1 February 2002, just after the period of the higher growth of the embryo, with a delay of 10 d (Fig. 1). Buried seeds exhumed on that date germinated up to 73 and 74% when incubated at 15/4 and 20/7°C with light, respectively (Fig. 3). Seedling emergence was highest during March 2002. In the early part of this month, average length of embryos in buried seeds was 3.01 mm (SE = 0.13), with 55% of embryos ≥ 2.9 mm (Fig. 1). It should be stressed that, at this time, seeds had been exposed to effective temperatures for cold stratification (0–10°C) since the previous November. Stokes (1965) and Baskin and Baskin (1998) determined 5°C as the optimal cold-stratification temperature for many species. Therefore, dormancy in seeds of *A. napellus* in nature is broken during winter and seeds germinate in spring.

Although embryos of *A. napellus* seeds were 3.6 mm long in seeds with emerged radicles, they do not necessarily have to be this long at the end of a cold stratification period to germinate upon transfer to higher temperature regimes. Thus, after 3 months of cold stratification at 5°C, mean embryo length was 2.35 mm (SE = 0.13; with 0% embryos ≥ 2.9 mm) although seeds germinated to 50% when transferred to 20/7°C in light, reaching an embryo length of *c.* 3.20 mm (SE = 0.11). In seeds cold-stratified for 3 months at 5°C, embryos grew from 2.35 mm to 2.68 mm (increase: 0.33) when transferred to 15/4°C for a month, in contrast to seeds that were only incubated at the latter thermoperiod for the same period (i.e. 4 months), which grew from 2.21 mm to 2.28 mm (increase: 0.07) during the fourth month. Seeds of *A. napellus* may be similar to those of *Heracleum sphondylium* in that low (e.g. 2°C) temperatures are required for breakdown of endosperm storage proteins into the soluble amino acids and nitrogenous compounds required for embryo growth (Stokes, 1953). This hypothesis has been suggested previously for other species whose morphophysiological dormancy is broken by cold stratification, such as *Thaspium pinnatifidum* (Baskin *et al.*, 1992), *Delphinium tricorne* (Baskin and Baskin, 1994) and *Anthriscus sylvestris* (Baskin *et al.*, 2000).

When buried seeds began to break down dormancy (January 2002), they first germinated at low and medium temperatures (15/4 and 20/7°C) and later (March 2002), as seeds progressively overcame dormancy, over 40% also germinated at high temperatures (25/10 and 28/14°C; Fig. 3). This dormancy-breakdown pattern is similar to that of seeds having fully developed embryos and non-deep physiological dormancy of Type 1. Even though such a type is very common in temperate zone winter annuals, it has also been documented in seeds of various perennials in eastern temperate North America, where these seeds come out of dormancy during winter and germinate in spring (Baskin and Baskin, 1998, 2005).

A high percentage of *A. napellus* seeds did not require light to germinate (Figs 4 and 5), with many of them germinating while buried in soil (Fig. 2). Such germinative traits do not promote the formation of a persistent soil seed bank. However, seeds cold stratified in the laboratory for 4 months never exceeded 80% germination under the most favourable temperature conditions (Figs 4 and 5). Moreover, 20% of seeds sown in the non-temperature-controlled greenhouse did not germinate until the second (2003) and following springs (2004, 2005 and 2006). Hence, a small reserve of viable seeds may occur at population sites of this taxon, because it can be assumed that some seeds in the germination phenology study did not afterripen until the second and following winters after sowing. Indeed, another *Ranunculaceae* species, *Thalictrum mirabile*, whose seeds germinate while buried in the soil with no light requirement, can form a short-term persistent soil seed-bank (Thompson *et al.*, 1997). The promoting effect of environmental conditions for dormancy break may vary among years and microsites (Baskin *et al.*, 1992).

Our results reveal large variations in seed dormancy between populations and between seeds harvested in different years. Variation in dormancy level among populations is a well-known phenomenon (Naylor and Abdalla, 1982; Schütz and Milberg, 1997; Andersson and Milberg, 1998). Germination also differs among seeds collected in different years (Beckstead *et al.*, 1996; Andersson and Milberg, 1998).

Differences in dormancy levels among populations (intra-specific variation) are often considered to be genotypic and to represent adaptations to the local/regional climate or to specific habitats or edaphic factors (Pegtel, 1985; Meyer, 1992). However, the environment of the mother plant can have a great influence on the dormancy of the seeds produced, often being responsible for inter-population and inter-year differences in dormancy patterns (Fenner, 1991; Gutterman, 1992). In our study, differences in dormancy level were as pronounced between years within a population (significant interactions; Table 4) as between populations. Maternal effects may be

responsible for such differences, since variation cannot be explained explicitly as a result of genetic adaptation to a local environment: in that case variation between years should have been small. Andersson and Milberg (1998) also attribute the inter-annual germinative differences detected in *Silene noctiflora*, *Sinapis arvensis* and *Spergula arvensis* to maternal effects. However, validation of the hypothesis requires separation of genotypic and phenotypic effects by growing plants from different populations under the same conditions.

The ecological consequence of intermediate complex MPD in seeds of *A. napellus* does not differ from that of seeds of many temperate nemoral species with physiological dormancy that is broken by cold stratification during winter. In nature, the cold stratification requirement for dormancy break in seeds of *A. napellus* occurs during winter, and seeds germinate in late winter and early spring about 2 months before the canopy closure. Germination at the beginning of the warm season means that young plants have the maximum frost-free period for growth and development before the onset of winter.

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