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Seed germination requirements of relictic and broadly-distributed populations of *Chaerophyllum aureum* (Apiaceae): connecting ecophysiology and genetic identity

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Abstract: The main goal of this work was to relate the germinative ecology and genetic variability of *Chaerolhyllum aureum* L., an Apiaceae plant with dormant, underdeveloped embryos at the time of seed dispersal. We compared the seed germination physiology traits between a relictic geographically isolated Mediterranean population (Iberian System; central Spain) and a population located in the main, colder Atlantic Alpine core (Pyrenees; northern Spain). We analyzed both populations' genetic identity in parallel on the basis of ISSR–PCR analyses. Stratification, gibberellin treatment, and seed storage tests showed that *C. aureum* seeds have deep complex morphophysiological dormancy. The Iberian System population seeds required shorter cold stratification than the Pyrenees population seeds (16 vs. 20 weeks) to germinate. The genetic analyses suggested closer similarity between Iberian System individuals and those from the Pyrenees requiring shorter cold periods to germinate. Our findings denote a consistent parallelism between germination physiology and genetic identity. The records from these germination and genetic analyses suggested selective pressure due to the climate differences between the two geographical regions. Thus, the individuals in the Iberian System population would have been selected from the original pool to a shorter cold-stratification requiring ecotype.

Key words: Climate-mediated selection, deep complex morphophysiological dormancy, embryo growth, interpopulation variability

1. Introduction

In many plant families, e.g., Apiaceae, seeds have underdeveloped embryos, as well as an additional physiological mechanism that prevents embryo growth and germination. These seeds have morphophysiological dormancy (MPD; Baskin et al., 2004). Nine MPD levels have been described based on temperature requirements for dormancy break and embryo growth, and on the ability of the gibberellic acid to overcome dormancy (Baskin et al., 2008; Phartyal et al., 2009). In the family Apiaceae, three levels have been reported: nondeep simple MPD, nondeep complex MPD, and deep complex MPD (Baskin and Baskin, 2014). Within the genus *Chaerophyllum*, nondeep simple MPD is known in two North American species, *C. tainturieri* Hook (Baskin and Baskin, 1990) and *C. procumbens* (L.) Crantz (Baskin and Baskin, 2004), as is a deep complex MPD in two Eurasian representatives, *C. bulbosum* L. (Augé et al., 1989) and *C. temulum* L. (Vandelook et al., 2007). These authors have pointed out that additional research on the dormancy of other *Chaerophyllum* species could further elucidate evolutionary pathways of dormancy in the genus. This is indeed one of the goals of the present work, performed by studying the germinative ecology of *C. aureum* L.

Many studies have found dormancy variations among seed collections from different locations (Andersson and Milberg, 1998; Schütz and Rave, 2003). Germination responses vary with latitude, elevation, soil moisture, kind and density of plant cover, etc. (Skordilis and Thanos, 1995; Baskin and Baskin, 2014). Thus, populations of the same species may differ in terms of the duration of cold stratification required to overcome seed dormancy (Meyer and Monsen, 1991; Meyer and Kitchen, 1994). However, studies on interpopulation variability of germinative requirements are scarce in species with MPD, e.g., *Aconitum napellus* subsp. *castellanum* Molero and C. Blanché (Herranz et al., 2010), *Centaurium somedanum* M. Laínz (Fernández-Pascual et al., 2013), and *Narcissus eugenieae*

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Fern. Casas (Copete et al., 2014). This study represents an attempt to shed light on this topic by comparing the germination responses of two geographically far-distant *C. aureum* populations.

There is evidence that variation in germination responses among populations may be genetically-based and might represent an adaptation to local habitat conditions (Mondoni et al., 2009). However, germinative differences may also be a shorter-termed response: the maternal environment effect or preconditioning (Fenner, 1991; Baskin and Baskin, 2014). To determine whether the reason for variation is due to genetics or preconditioning, it is recommended that seeds used in germination studies be collected from plants that have grown in a common garden (Bender et al., 2003). In this work however, due to the difficulty of preventing cross-pollination among individuals of different populations in our experimental garden, we explored whether there was any relationship between the genetic background of populations and their physiological affinity in relation to the environmental requirements for breaking seed dormancy.

Chaerophyllum aureum is a perennial herb distributed over central and southern Europe, western Asia, and North Africa. It is abundant in the northern region of the Iberian Peninsula, with relictic populations in the central and southern peninsular areas (Jury and Southam, 2003). We selected one of those relictic geographically-isolated populations located in the meridional Iberian System (central eastern Spain; Gómez-Serrano and García-Berlanga, 2004), and a Pyrenees population (northern Spain) in the main Iberian geographical-distribution core, which is well-connected to the world species range, for comparison reasons. The population in the Iberian System is 200 km away from the closest populations and inhabits a mixed broadleaf deciduous forest, which is a refuge for several temperate Euro-Siberian species. These plants became isolated in exceptional mountainous locations with favorable local conditions after the dry Mediterranean climate consolidated in the central southern peninsula (Hernández-Bermejo et al., 2011).

The habitat of the meridional *C. aureum* population in this study is also considered a priority by European Union conservation regulations (Directive 92/43/EEC) and in the Castilla–La Mancha region (Martín-Herrero et al., 2003). Conservation habitat strategies may eventually require population reinforcements (Martín-Herrero et al., 2003), in which case, germination ecology knowledge of the target taxa is essential to produce ex situ plants for restitution programs.

The main goal of this work was to test the hypothesis that the fragmentation of the original Iberian distribution range of *C. aureum* into relictic isolated populations exposed to different ecological conditions, along with the disruption of genetic flow, has resulted in the differentiation of both physiological dormancy-breaking mechanisms and genetic identity. Accordingly, we explored two hypotheses: (a) seeds from the Pyrenees, with longer and colder winters, may require longer cold stratification periods to break dormancy than those of the Iberian System; (b) Iberian System individuals may display closer genetic affinity to those from the Pyrenees requiring shorter cold stratification times to germinate. For this purpose, we defined the following specific aims:

- Analyze the effects of cold stratification on breaking dormancy and on embryo growth;
- Determine the influence of the duration of cold and warm stratification, light conditions during seed incubation, seed age, and gibberellic acid on germination;
- Characterize the phenology of embryo growth and seedling emergence;
- Evaluate the germination responses of the seeds buried in soil and periodically exhumed;
- Determine genetic differences among populations according to ISSR–PCR analyses;
- Know whether the individuals from seeds that require longer exposure to cold stratification in order to germinate have a differentiated genetic identity.

2. Materials and methods

2.1. Seed collection

We collected 12,000 *C. aureum* seeds in summer 2010 from both of the 500-km distant Iberian populations: an isolated relictic one located in the southern Iberian System, and another one in the Pyrenees, in the species' European geographical-range area. Details of both localities are described below.

The Southern Iberian System population (IS): Meridional Iberian System, Masegosa (Cuenca Province), the Río Chico Valley. Substratum: quartzitic soil; Elevation: 1460 m a.s.l.; UTM: 30TWH8787. *Chaerophyllum aureum* plants grow on the edges of Euro-Siberian forests of *Fraxinus excelsior* L., *Populus tremula* L., *Sorbus aucuparia* L., and *Corylus avellana* L., and form perennial herb communities with *Trollius europaeus* L., *Paris quadrifolia* L., and *Aconitum vulparia* subsp. *neapolitanum* (Ten.) Muñoz Garm. Date of seed collection: 20 August 2010.

The Pyrenees population (P). Central Pyrenees, Jaca (Huesca Province), Peña Oroel. Substratum: calcareous soil; Elevation: 1240 m a.s.l.; UTM: 30TYN0011. *Chaerophyllum aureum* grows in gaps of mixed forests of *Pinus sylvestris* L., *P. nigra* subsp. *salzmannii* (Dunal) Franco, *Abies alba* Mill., and *Fagus sylvatica* L., along with other perennial herbs, such as *Gentiana lutea* L., *Helleborus foetidus* L., and *Primula veris* L. Date of seed collection: 26 August 2010.

After collection, seeds were spread on laboratory trays at 22 °C to complete seed drying until tests were started on 1 September 2010.

2.2. Germination ecology

2.2.1. General test conditions

Experiments were conducted under controlled temperature and light conditions [±0.1 °C, cold white fluorescent light, 25 μmol m^{-2} s^{-1} (1350 lux)] in germination chambers (F-4 model, Ibercex, Madrid, Spain) equipped with a digital control system. Chambers were programmed for a daily 12-h photoperiod. Seeds were placed inside the chambers in Petri dishes on double-layer filter paper, which was saturated continuously with distilled water, and were sealed with parafilm to avoid water loss. Darkness conditions, when required in the experiments, were achieved by wrapping the Petri dishes that contained seeds with two layers of aluminum foil.

Experiments were carried out at a constant temperature of 5 °C, or at 15/4 or 20/7 °C for 12h/12h. In these fluctuating-temperature regimes, higher and lower temperatures were programmed to coincide with the light phase and the dark phase, respectively. Four 25-seed replicates were used per combination of temperature and light condition. Radicle emergence (>1 mm) was the criterion used for seed germination diagnosis. The percentage of germination in each replicate was calculated on the viable seed count basis. Criteria for determining seed viability were the color and turgidity conditions of the embryo.

The temperatures selected for the tests closely simulated the climate conditions in autumn, winter, and spring in the habitat of *C. aureum*, these being the seasons when germination is likely to occur naturally. Thus, a constant temperature of 5 °C simulates the mean temperature in winter months (December, January, and February). It is also the most frequent temperature used in cold stratification treatments (Baskin et al., 2004). The fluctuating 15/4 °C regime simulates the mean temperatures in November and March; 20/7 °C are typically those in October and April. For the warm stratification treatment, 28/14 °C were selected because they are the most representative temperature values in summer months (June, July, and August) in the natural habitat, and also because they have provided proper results in previous studies (Herranz et al., 2010; Copete et al., 2014).

2.3. Effect of temperature on embryo growth

In August 2009, 500 freshly matured *C. aureum* seeds were collected from the IS population to conduct tentative tests, which revealed the existence of underdeveloped embryos according to Baskin and Baskin (2014) (embryo:seed length ratio <0.20; E:S ratio hereafter), absence of germination after 30 days of incubation at 5, 15/4, or 20/7 °C, and promotion of embryo growth at 5 °C. This preliminary information was used to design the definitive tests.

On 1 September 2010, 25 seeds from each IS and P population were placed in Petri dishes on a double layer of filter paper moistened with distilled water. They were kept at constant room temperature in the laboratory (22 °C) for 24 h. After this hydration period, seeds were excised with a razor blade, and embryos were extracted and measured, along with the length of the seeds, with a dissecting microscope equipped with a micrometer, to calculate the E:S ratio at the time of seed dispersal.

On the same date, six Petri dishes with 30 seeds each per population were prepared. Three of them were submitted to cold stratification (5 °C) and the other three to warm stratification (28/14 °C) with light. After 4, 8, and 12 weeks, respectively, one dish was removed and the embryos of 25 healthy-looking seeds were measured.

In order to calculate the critical embryo length, which corresponds to seeds about to germinate with split seed coats, but immediately before radicle emergence (Vandelook et al., 2009), a 150-seed sample from each population was placed on saturated filter paper in a Petri dish at 5 °C in light. After 14 weeks, the embryos of 40 seeds with split coats were excised and measured to calculate the critical length and the critical E:S ratio (defined by the sample's mean values).

2.4. Effect of stratification temperature and dry seed storage on germination ability

On 1 September 2010 (seed age = 0), two Petri dishes (20-cm diameter) were prepared, containing 2300 seeds each per population. One dish was exposed to cold (5 °C) stratification and the other to warm (28/14 °C) stratification, both in the light. After 4, 8, and 12 weeks, respectively, 600 ungerminated seeds were extracted, divided into 100-seed lots, and then incubated at 5, 15/4, or 20/7 °C, either in the light or darkness for 4 weeks. Germination was checked at 2–3-day intervals in the light treatments, and at the end of the test when incubation was performed in the dark. A control test was carried out concurrently by incubating the nonstratified seeds with the same temperature and light combinations for 16 weeks.

To assess the effect of seed dry storage (22 °C) on germination ability, the cold stratification treatments (the warm ones did not promote germination) were repeated in May 2011, when the seeds were 8 months old.

2.5. Effect of gibberellic acid on germination ability

On 1 September 2010, 1000 seeds of each population were incubated at 20/7 °C in the light, and 100 more seeds at the same temperature in the dark, distributed into four 25-seed replicates in Petri dishes, on a double layer of filter paper saturated with a solution of 1000 ppm of gibberellic acid (GA_3) in distilled water. This concentration has

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been successfully used in previous studies (Vandelook et al., 2007; Herranz et al., 2010). At 4, 8, and 12 weeks, germination was checked using a green lamp for the counts for the dishes incubated in the dark (Vandelook et al., 2007). We replicated a parallel control test by incubating seeds under identical conditions, except for moistening, which was carried out only with distilled water.

2.6. Field tests under seminatural conditions

The goal of these tests was to determine changes in the dormancy stage, as well as the seed–seedling transition time, when seeds were exposed to seasonal temperature cycles that simulated conditions in nature. Tests were performed in a metal frame shadehouse located on the Albacete University campus (UTM coordinates 30SWJ9713, 690 m a.s.l.), 210 km south of the IS population. Temperatures were recorded throughout the study by a meteorological station placed inside the shadehouse. The substrate in the trays and pots where seeds were sown consisted of a mixture of sterilized peat and sand (2:1). It was watered to field capacity once a week, except: (i) in July and August, when it was watered twice a month to simulate summer drought; (ii) during winter frost periods, when watering was interrupted.

2.7. Phenology of embryo growth and dormancy break

On 1 September 2010, twelve 100-seed lots per population were mixed with sand, and each was kept in an individually tagged nylon bag. Bags were buried at a depth of 5 cm in pots and were placed in the shadehouse under the conditions described above.

Bags were exhumed monthly from the month following seed burial to calculate the percentage of seeds whose radicles had emerged, and to measure embryo length in a 25-seed sample. In the germinated seeds, embryo length was assumed to be that which corresponded to critical embryo length (Vandelook et al., 2007). The rest of the ungerminated seeds were incubated at 5 °C in the light for 4 weeks. After seed incubation, we were able to calculate the following seed status percentages: (1) seeds whose radicles emerged inside the bag; (2) viable nondormant seeds (i.e., those that germinated in the 5 °C incubation phase); (3) viable dormant seeds (i.e., those with healthy embryos, but which failed to germinate at 5 °C); (4) nonviable seeds (i.e., those with a rotten appearance or showing a dead embryo when excised).

2.8. Phenology of seedling emergence

On 1 September 2010, 200 seeds of each population were sown at a depth of 5 mm in all three trays (30 × 40 × 8 cm) filled with the peat–sand substrate and cultivated inside the shadehouse as described above. Trays were inspected weekly and emerged seedlings were counted and removed. Emergence counts continued until April 2012.

2.9. Population genetic analysis

We assigned 109 individuals to the genetic analysis: 14 from the IS and 95 from the P population. Those individuals consisted of randomly selected seedlings coming from a pool of seeds germinated in individual pots. The Pyrenees plants were divided into two subpopulations according to differences in the cold-condition requirements to break seed dormancy:

– Pyrenees subpopulation 1: seeds requiring 12 weeks of cold stratification, plus 4 weeks of incubation at 5 °C (58 individuals; Table 1);

– Pyrenees subpopulation 2: seeds requiring 16 weeks of cold stratification, plus 4 weeks of incubation at 5 °C (37 individuals; Table 1).

Subpopulation differentiation was not necessary in the IS population because all of the seeds germinated after 16 weeks at 5 °C.

2.10. DNA extraction

DNA was extracted from 150–300 mg of leaf material using a modified method (Doyle, 1987). Leaf material was then ground to a fine powder in liquid nitrogen and placed in a microcentrifuge tube with 2 mL of extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, and 0.01% proteinase K), plus 40 mL of 2-mercaptoethanol. Following incubation at 65 °C for 30 min, 1.4 mL of chloroform:isoamyl alcohol (24:1) was added, mixed, and centrifuged at 8000 rpm for 30 min. The supernatant was transferred to a new tube; the process was repeated three times. DNA was precipitated with isopropanol (2/3 volume of the supernatant) and then centrifuged at 8000 rpm for 30 min. The supernatant was discarded and the pellet was washed in 70% ethanol containing 10 mM ammonium acetate for 20 min. The pellet was dissolved in 100 mL of TE buffer (10mM Tris-HCl, pH 7.4, 1 mM EDTA) and DNA was reprecipitated with ½ volume of ammonium acetate 3 M and 2.5 volumes of ethanol. After centrifuging at 8000 rpm for 30 min, the pellet was redissolved in TE buffer with 10 μg mL^{-1} RNase and incubated at 30 °C for 30 min. The extracted DNA was quantified in a spectrophotometer, diluted to 30 ng mL^{-1} in TE and then stored at –20 °C for further analyses.

2.11. DNA amplification

First, 15 and 30 ng of genomic DNA were amplified in a 25-mL volume that contained 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl2, 200 μM each dATP, dCTP, dGTP, dTTP, 0.4 mM primer, and 1 unit of Taq DNA polymerase, in a thermal cycler (MJ-Mini, BioRad, Hercules, CA, USA). The cycling program began with an initial 2-minute step at 94 °C, followed by 40 cycles at 94 °C for 45 s, 52–62 °C for 45 s, and 72 °C for 2 min, with a final 10-min step at 72 °C and storage at 4 °C. A negative control was added to each run to test for contamination. Sequences of primers are shown in Table 2. Amplification products were separated

Table 1. Effects of cold stratification treatments on germination. Effects of cold (5 °C) stratification treatments on the final mean germination (%) of freshly matured seeds (seed age = 0 week) of *C. aureum* (mean ± SE, n = 4). After 4, 8, and 12 weeks, every four 25-ungerminated-seed replicates were incubated at 5, 15/4, or 20/7 °C, in light (L) or darkness (D) for 4 weeks. In the control, we incubated every four 25-seed replicates, nonstratified previously, in the same combinations of temperature and light conditions for 16 weeks for the IS-population, and for 20 weeks for the P-population. The capital letters in the columns and the lowercase letters in the rows denote the pairs of cases responsible for significant differences ($\alpha = 0.05$).

Stratification at 5 °C		IS population			P population		
		5 °C	15/4 °C	20/7 °C	5 °C	15/4 °C	20/7 °C
4 weeks	L	8 ± 1^{Bb}	0 ± 0^{Aa}	0 ± 0^{Aa}	0 ± 0^{Aa}	0 ± 0^{Aa}	0 ± 0^{Aa}
	D	9 ± 2^{Bb}	0 ± 0^{Aa}	0 ± 0^{Aa}	0 ± 0^{Aa}	0 ± 0^{Aa}	0 ± 0^{Aa}
8 weeks	L	31 ± 2^{Cc}	6 ± 2^{Bab}	22 ± 2^{Bb}	11 ± 2^{B}	3 ± 1^{Ba}	2 ± 0^{Ba}
	D	36 ± 1^{Cc}	10 ± 3^{Bab}	24 ± 5^{Bb}	21 ± 1^{Bb}	5 ± 4^{Ba}	4 ± 1^{Ba}
12 weeks	L	92 ± 2^{Dd}	69 ± 5^{Cc}	60 ± 3^{Cc}	46 ± 3^{Cb}	10 ± 1^{Ca}	10 ± 3^{Ca}
	D	99 ± 1^{Dd}	72 ± 2^{Cc}	62 ± 1^{Cc}	52 ± 2^{Cb}	12 ± 3^{Ca}	11 ± 4^{Ca}
Control							
16 weeks	L	88 ± 2^{Dc}	2 ± 0^{Ba}	6 ± 5^{Ba}	46 ± 3^{Cb}	5 ± 1^{Ba}	4 ± 5^{Ba}
	D	99 ± 1^{Dc}	6 ± 5^{Ba}	5 ± 1^{Ba}	52 ± 1^{Cb}	5 ± 2^{Ba}	9 ± 1^{Ca}
20 weeks	L	-	-	-	84 ± 4^{Db}	22 ± 1^{Ca}	29 ± 2^{Da}
	D	-	-	-	95 ± 1^{Db}	26 ± 2^{Ca}	31 ± 5^{Da}

Table 2. The ISSR primers and their sequences used in the study.

Primer name	Sequence (5'–3')	Tm (°C)
ISCS8	TCTTCTTCTTCTTCTTCTTCTTCTTCTA	
ISCS11	CTCTCTCTCTCTCTCTT	52
ISCS17	DBDBCACCACCACCACCAC	62
ISCS20	DHBCGACGACGACGACGA	62
ISCS21	BDBACAACAACAACAACA	52
ISCS30	ACACACACACACACACYT	52
ISCS31	ACACACACACACACACYA	52
ISCS32	ACACACACACACACACYG	52
ISCS33	TGTGTGTGTGTGTGTGRT	52
ISCS34	TGTGTGTGTGTGTGTGRC	52
ISCS37	AGCAGCAGCAGCAGCAGC	62
ISCS41	CTCCTCCTCCTCCTCCTC	62
ISCS69	CACACACACACACACAA	52

B: G+T+C; D: G+A+T; H: A+C+T; V: G+C+A.

by electrophoresis in 2% agarose gel that contained 1 μg mL^{-1} of ethidium bromide and TAE buffer. Ten microliters of amplified DNA were mixed with 3 μL sample buffer (1.2 mg mL^{-1}; 125 mg mL^{-1} Ficoll), and 10 μL were applied in each well of the gel. DNA molecular weight markers (1 kb, Promega, Madison, WI, USA) were then added to each gel. Gels were run at a current of 50 mA until the bromophenol had migrated 10 cm from the well. Bands were then visualized under UV light and photographed. To ensure the reproducibility of the method, the procedure was repeated three times for each concentration of genomic DNA and primer (Table 2).

2.12. Statistical analysis

Germination data were summarized as cumulative percentages. The means and standard errors of both the germination data (n = 4) and embryo growth data (n = 25) were calculated. Using a multifactorial analysis of variance (ANOVA), we analyzed the photoperiod and the temperature effects during incubation on both the embryo growth and the germination percentages, as well as the effect attributable to the seeds' population origin.

In all cases, the factors responsible for the main effects were detected by a multiple comparison Tukey test (significance level = 0.05). Significant interactions were explored using contrasting confidence intervals. Previously, the normality (Cochran test) and homocedasticity (David test) of data had been checked. Percentages were arcsine square root-transformed to adjust their distribution to normality. However, the graphic representations of these percentages are shown without transformation.

We analyzed the ISSR data based on both allele and phenotypic frequencies. Polymorphic bands were selected at the 95% level (two-tailed test) to be used in further analyses. Data matrices were analyzed using POPGENE v. 1.32 with the assumption that populations were in Hardy–Weinberg equilibrium. The following parameters were determined: percentage of polymorphic loci (PPL), number of alleles per locus (na), effective number of alleles per locus (ne), genetic diversity (HE = expected heterozygocity), Shannon's index of phenotypic diversity (I). We examined the ahierarchical genetic variation of the populations studied using an analysis of molecular variance (AMOVA) determined with GenAlEx v. 6.41, and we determined the principal coordinates analysis (PCA) using GenAlEx v. 6.41 (Peakall and Smouse, 2006).

3. Results

3.1. Effects of temperature on embryo growth

In the IS population, the mean embryo length in fresh seeds was 1.07 mm, and the mean seed length was 8.03 mm. Critical embryo length for germination was 4.8 mm (critical E:S ratio = 0.59). The minimum embryo length found in a split seed was 3.0 mm (i.e., the threshold at which a seed could germinate) and the minimum E:S ratio value was 0.37. In the P population, these values were as follows: mean embryo length in fresh seeds = 1.08 mm; mean seed length = 8.04 mm; critical embryo length = 5.3 mm; critical E:S ratio = 0.66; minimum embryo length = 3.5 mm; minimum E:S ratio = 0.43.

In both populations, embryos grew when seeds were cold-stratified (5 °C in light), but not in the warm stratification treatment (28/14 °C in light). After 12 weeks of cold stratification, 31% of the seeds had germinated and 92% had surpassed the minimum E:S ratio in the IS-population seeds. In the P-population seed sample, these values were 11% for the germinated seeds, and 70% for seeds that surpassed the minimum E:S ratio, respectively (Figure 1).

3.2. Effects of stratification temperature and seed age on germination ability

Cold stratification (5 °C) was effective in breaking dormancy, as over 90% and around 50% of the seeds from the IS and P populations, respectively, germinated when incubated at 5 °C after being cold-stratified for 12 weeks. Similar results were recorded in the control test for the seeds incubated at 5 °C for 16 weeks (the P-population seeds needed 4 more weeks to manifest a notable increase in germination; Table 1). In contrast, seed germination was null at any tested incubation temperature when seeds had previously been warm-stratified (28/14 °C; data not shown).

Seed age did not affect germination: the 8-month seeds dry-stored in the laboratory obtained similar values to those recorded in the fresh (0-month) seeds, and required similar cold stratification duration to trigger germination (data not shown).

3.3. Effect of gibberellic acid on germination ability

GA_3 did not stimulate germination after 12 weeks of incubation at 20/7 °C. The range of germination percentages was 2%–3% in all cases, and the E:S ratio range was 0.23–0.26 (data not shown in tables or figures).

3.4. Phenology of embryo growth, radicle and seedling emergence, and dormancy break under seminatural field conditions

In both the IS and P seeds, embryos grew very little in September and October. Early in February, however, after 12 weeks of effective cold stratification (0–10 °C), the mean embryo length was 4.75 mm. In the IS population seeds, first radicle emergence occurred in early January, while it started early in February in the P seeds. In early April, cumulative radicle emergence was 99% and 70%, respectively, in each population (Figure 2).

Hardly 2 weeks elapsed between radicle and seedling emergence. In mid-April, 99% of the IS-population seedlings had emerged. In the P population, cumulative seedling emergence was 66% early in May (Figure 2), and this percentage increased very little from that date onward (68% in April 2012).

All of the seeds remained dormant in October and November. Dormancy break started early in December and was higher than 50% by early February. From this date, the number of seeds that presented an emergent radicle substantially increased, and this increase was higher in the IS than in the P population (Figure 3). As of May, only a germinated decomposed seed bulk filled the exhumed nylon bags.

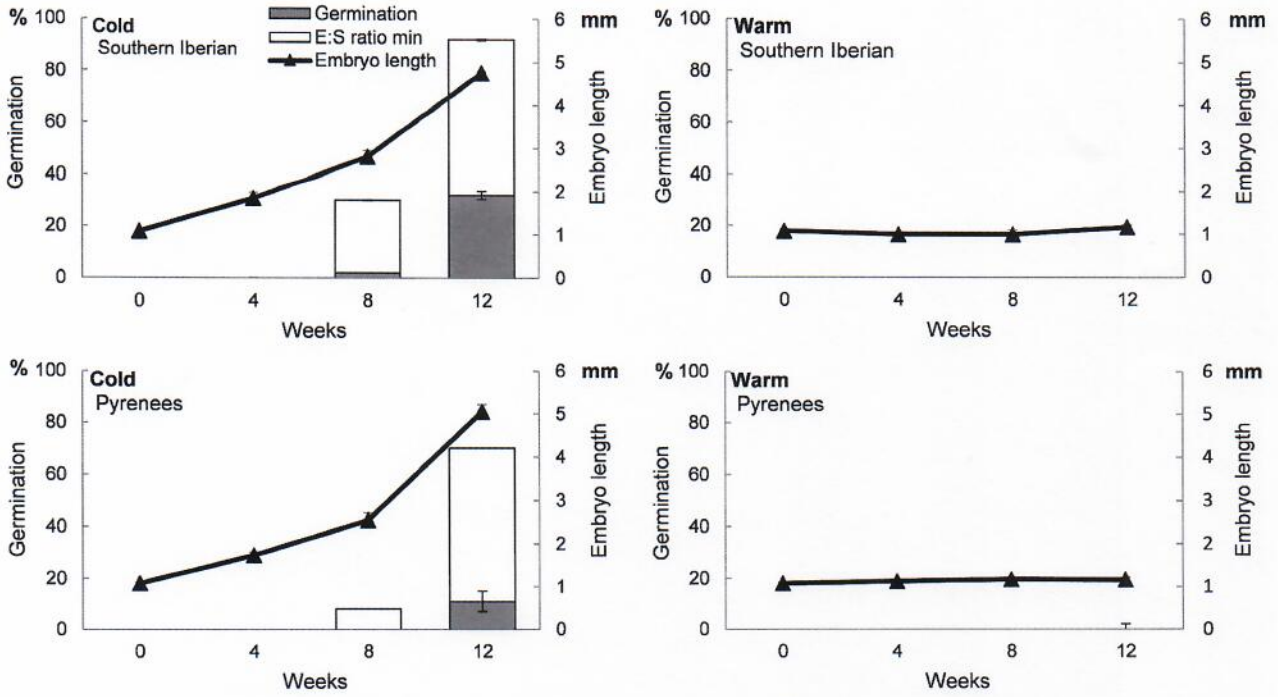

Figure 1. Embryo growth (mm) in freshly matured seeds of *C. aureum* (mean ± SE; n = 25) submitted to treatments of cold (5 °C) and warm (28/14 °C) stratifications over 12 weeks. The dark portion in the histograms represents the percentage of germinated seeds (mean ± SE), and the white one the percentage of embryos whose E:S ratio surpassed the minimum E:S ratio (0.4; i.e. the value from which germination is possible).

3.5. Genetic population analysis

Two *C. aureum* samples were randomly selected for the preliminary experiments to determine the optimal amplification reaction conditions and primer screening for ISSR. Ten primers did not result in amplification at all, 12 gave unclear profiles in some samples, and only 13 out of the 35 tested primers resulted in well-separated bands. Based on these data, the 109 samples included in the study were analyzed with 13 primers (Table 2). Most loci were polymorphic within each population compared with the presence and absence of bands. The percentage of polymorphic loci was 60.53% in the IS population, and 89.47% and 76.32% in Pyrenees subpopulations 1 and 2, respectively. The highest Shannon information index (0.256) was found in Pyrenees subpopulation 1, followed by IS (0.199), while the lowest one (0.168) corresponded to Pyrenees subpopulation 2. An intrapopulation genetic diversity examination revealed the highest values of Nei's genetic diversity for Pyrenees subpopulation 1 (0.256), whereas the lowest one (0.168) belonged to Pyrenees subpopulation 2.

Pairwise Nei's distances were calculated for all population groups. The longest interpopulation average distance (0.311) was between the IS and Pyrenees subpopulation 2, while the shortest one (0.109) was between Pyrenees subpopulation 1 and Pyrenees subpopulation 2. The AMOVA analysis showed that only 20% of total variation lay among the *C. aureum* populations, while 61% was attributable to differences among individuals within populations, and 19% among regions. The fact that the highest percentage of variation was due to intrapopulation genetic diversity indicates the existence of high outcrossing rates among the members of this species.

The principal component analysis (PCA) revealed the presence of two major groups (Figure 4). Group 1 contained Pyrenees subpopulations 1 and 2 along with four IS population members, while Group 2 contained only IS population members, which agreed with the data obtained in the pairwise Nei's distances.

4. Discussion

Chaerophyllum aureum seeds have underdeveloped embryos at dispersal (length = 1.07–1.08 mm) which need to grow to at least 3.0–3.5 mm to be able to germinate. To this end, they require longer exposure than 8 weeks at the most favorable temperature (5 °C) for embryo growth. In light of these results, we conclude that they have morphophysiological dormancy (MPD). However, nondeep simple MPD, identified in other close taxa (*C. tainturieri* and *C. procumbens*), can be rejected because (i) the embryo growth in the *C. aureum* seeds did not occur at a high temperature (28/14 °C), or (ii) seeds did

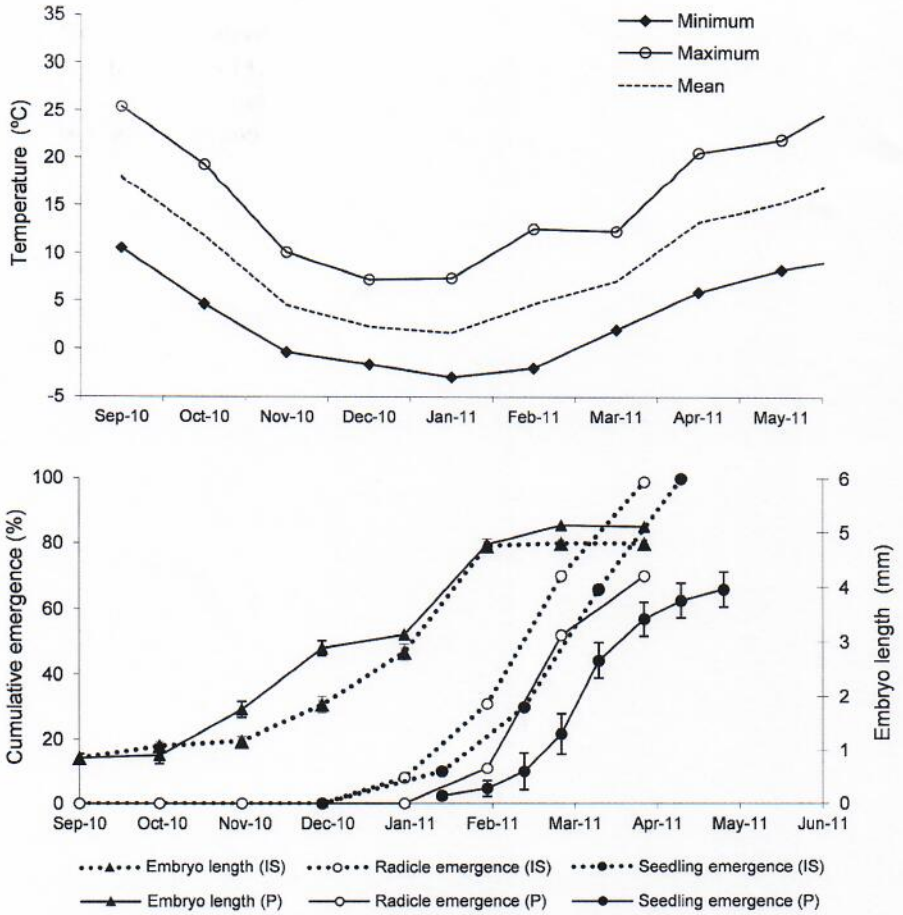

Figure 2. Phenology of embryo growth (mean ± SE, if SE > 0.1; n = 25), seed germination and seedling emergence (mean ± SE; n = 3) in the seeds of *C. aureum* from Pyrenees (P) and southern Iberian System populations (IS). Tests started in September 2010 (see Materials and methods). The upper graph shows changes in temperature (mean of maximum, mean of minimum, and mean of month temperatures) recorded in the shadehouse throughout the study.

not germinate when incubated at 5, 15/4, and 20/7 °C after warm (28/14 °C) stratification for 4, 8, and 12 weeks, respectively. In contrast, the fact that embryo growth and radicle emergence took place during a long seed stratification period at 5 °C shows that *C. aureum* seeds have one of the three complex MPD levels described in the literature (Baskin and Baskin, 2014). The nondeep complex level can be ruled out because embryo growth during cold stratification did not require previous warm stratification. This study also provides strong arguments for rejecting the intermediate complex level: neither gibberellic acid nor dry seed storage was effective in promoting embryo growth or seed germination. Indeed, the *C. aureum* seeds have deep complex MPD, like other previously studied Eurasian representatives *C. bulbosum* (Augé et al., 1989) and *C. temulum* (Vandelook et al., 2007). Both species are also perennial herbs that inhabit cold-winter regions. The need for long cold-stratification periods to achieve high germination percentages, as detected in the seeds of both IS and P populations (16 and 20 weeks, respectively), confirms the existence of this MPD level (Baskin and Baskin, 2014).

The results in the phenology study were in line with those obtained in the laboratory tests: the more marked increase in embryo length took place in November, December, and January, coinciding with the period of lower temperatures, and determined seed dormancy break and the massive emergence of radicles and seedlings in the following February, March, and April. This phenological model is similar to that described in other Apiaceae species with deep complex MPD: *Osmorhiza aristata* (Thunb. ex Murray) Rydb. (Walck et al., 2002), *C. temulum* (Vandelook et al., 2007), and *Aegopodium podagraria* L. (Vandelook et al., 2009).

The ecological consequence of cold stratification requirements for seed dormancy break and embryo growth is that seeds cannot germinate until late in winter, when embryos culminate their development, and thus

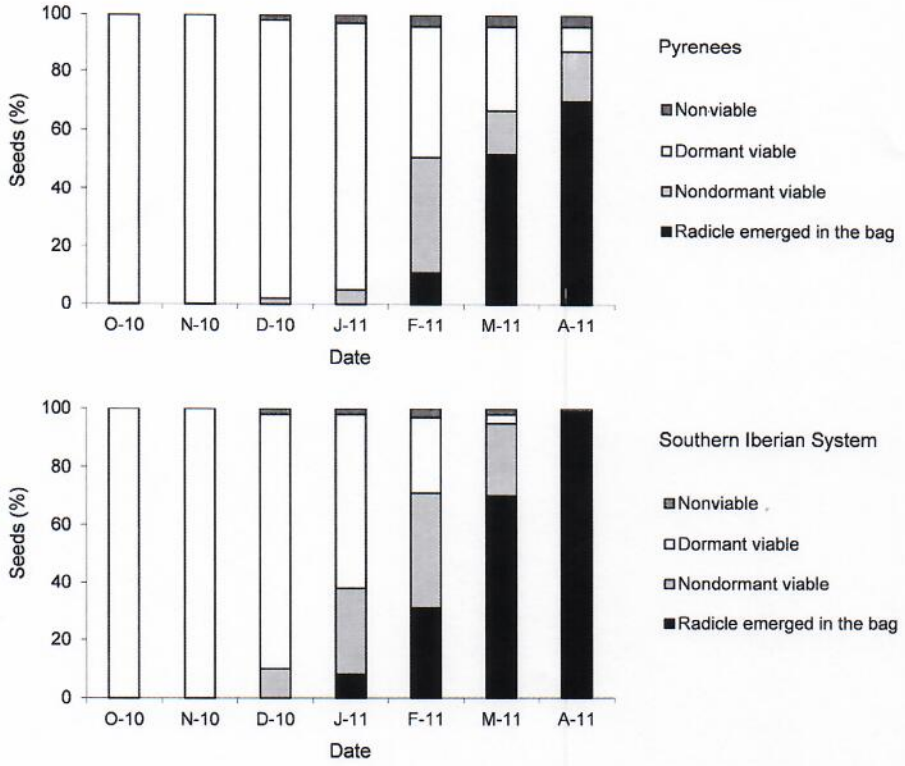

Figure 3. Stage of the *C. aureum* seeds exhumed monthly from 1 October 2010. Seed classes: radicle emerged in the bag, nondormant viable, dormant viable, and nonviable seeds.

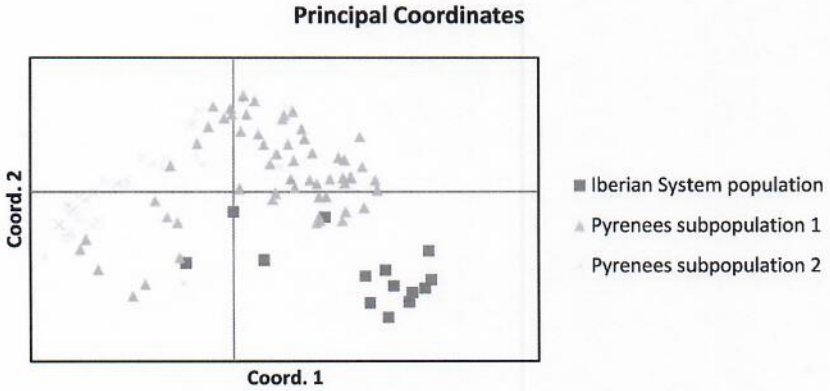

Figure 4. Principal component analysis (PCA) plot of the three population units based on the first two principal axes (first axis = 47.20%; second axis = 16.59%). Seeds from Pyrenees subpopulation 1 and Pyrenees subpopulation 2 required 12 and 16 weeks of cold stratification, plus 4 weeks of incubation at 5 °C, respectively.

prevent seedlings from emerging in autumn, which would increase the likelihood of dying due to frost sensitivity in cold winter months (Santiago et al., 2012). Germination in late winter–early spring provides cold-intolerant seedlings with a 3-month period of optimal light and soil moisture conditions to grow before the onset of summer drought, which is common in mesic woodland herbaceous habitats of the inland mountains of the Iberian Peninsula (Copete et al., 2014).

The germination tests performed in the laboratory (Table 1) showed that the P-population seeds required 4 more weeks of exposure to 5 °C than the IS-population seeds in order to achieve germination percentages over 90%. The phenological study (Figures 2 and 3) also showed that late-winter radicle and seedling emergence is delayed by nearly 1 month in the P population when compared to the IS population, because the seeds of the former need longer cold-stratification exposure, which is one of the most

novel findings of this study. The higher seedling emergence recorded in the IS population compared to the P population (99% vs. 68%) can be explained by the climate conditions in the experimental shadehouse in Albacete, which come closer to those in the IS-population habitat. Thus, our results show an adaptation of the IS population to shorter winters, which characterize the southern geographical range of *C. aureum* (around 500 km south of the P population). In the IS population isolated from any other by more than 200 km (Jury and Southam, 2003), selection has probably occurred for individuals that produce seeds with embryos capable of germinating at a lower E:S ratio (0.37 vs. 0.43; Figure 1), which thus require shorter cold stratification periods, as evidenced in some *Artemisia tridentata* Nutt. populations (Meyer and Monsen, 1991), and in several species of the *Penstemon* genus (Meyer and Kitchen, 1994).

The present results confirm the null hypothesis that the fragmentation of the geographical distribution range of *C. aureum* has resulted in differentiation in physiological mechanisms of breaking seed dormancy among unconnected isolated populations. Although the maternal environment effect may have contributed to the variation responses among populations, the genetic study also confirms inter- and intrapopulation differences; i.e., Pyrenees subpopulation 2 obtained the lowest Shannon information index value (0.168), followed by the IS population (0.199), and Pyrenees subpopulation 1 (0.256). These data suggest that Pyrenees subpopulation 1 individuals possess greater genetic plasticity for adapting to climate changes than those of Pyrenees subpopulation 2. The data also indicate that the IS population still conserves a substantial amount of the original genetic background, despite its relictic condition and its geographical isolation from the species' main distributional range. For this population, the genetic pairwise Nei's distance to Pyrenees subpopulation 2 is much longer than that to Pyrenees subpopulation 1, which agrees with the germinative ecology results, particularly those that refer to the time required for breaking morphophysiological dormancy. Hence, there is evidence for a consistent parallelism between germination physiology and genetic identity, as postulated in our hypothesis.

The PCA also shows greater genetic similarity among the individuals from the relictic IS population with Pyrenees subpopulation 1 than with the Pyrenees subpopulation 2 plants. Our study suggests that the selection of individuals in the IS population which produce seeds that require shorter cold-stratification periods to germinate may occur from plants with a Pyrenees subpopulation 1 genetic pool, a component of a more diverse genetic pool that is currently well-represented in the species' main geographical range in the Iberian Peninsula (Pyrenees).

To conclude, differences in the availability of cold months promoting seed dormancy breaking between the Pyrenees and the more southern populations in the meridional Iberian System may have acted as a selective pressure on the genetic background of *C. aureum*. Over the course of time, the relictic southern Iberian System population, although coming from a similar genetic background as the population in the Pyrenees, has undergone selection leading to individuals which produce seeds capable of germinating during substantially shorter cold stratification periods. Selection on germination may act as a strong biased filter on juvenile recruitment, and may thus be decisive for modulating the genetic identity of the populations.

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