

Germination ecology of the perennial *Centaurium somedanum*, a specialist species of mountain springs

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Abstract

To better understand how a rare endemic species of *Centaurium* adapts to a specialized ecological niche, we studied the germination ecology of the mountain spring specialist *C. somedanum*, a perennial species restricted to a habitat unusual in the genus. We conducted laboratory experiments with fresh seeds collected from two populations for three consecutive years, to investigate (1) the effect of temperature and light in germination, (2) the existence of seed dormancy and (3) inter-population and inter-annual variation in germinability. Germination occurred only in the light and at relatively low temperatures (15-22 °C) with no differences between constant and alternate regimes, and a significant decrease at high temperatures (25 °C and 30 °C). We found non-deep simple morphophysiological dormancy and variation in seed germinability depending on the year of seed collection. *C. somedanum* diverged from the common germination characteristics of the genus in (1) its germination at lower temperatures, which contrasts with what is generally expected in wetland species but could be adaptive in the spring habitat; and (2) its morphophysiological dormancy, which we report here for the first time in the genus and which could be an adaptation to its mountain habitat.

Introduction

Centaurium Hill (Gentianaceae), as defined by Mansion (2004), includes ca. 27 species of Mediterranean origin and Old World distribution. It is comprised of annual, biennial and rarely perennial herbs occurring in different habitats of the Mediterranean basin and surrounding areas (Mansion, 2005). Because of its wide distribution and pharmacological importance (Jensen and Schripsema, 2002; Sefi et al., 2011), certain aspects of *Centaurium* germination ecology have been studied in detail. The comparative germination study of Grime et al. (1981) reported germination of the biennial *C. erythraea* Rafn. over a wide range of temperatures (13 – 29 °C) and only in light. Thompson and Grime (1983) confirmed the light requirement for the same species and reported that germination was insensitive to temperature fluctuations, while Schat (1983) found very similar germination characteristics in another biennial, *C. littorale* (D. Turner) Gilmour. More recently, a series of studies focusing on the effects of salinity, manganese and plant growth regulators reported successful germination at 24-25 °C in light (Mijajlovic et al., 2005; Zivkovic et al., 2007; Todorovic et al., 2008, 2009; Mistic et al., 2009) for the same two species as well as for *C. maritimum* (L.) Fritsch, *C. pulchellum* (Swartz) Druce, *C. spicatum* (L.) Fritsch and *C. tenuiflorum* (Hoffmanns. & Link) Fritsch, all of them annual or

biennial. All these reports also indicated that germination takes place without previous dormancy-breaking treatments, in contrast with the requirement for cold-stratification found in other genera of Gentianaceae (Favarger, 1953) and the morphophysiological dormancy reported in *Frasera caroliniensis* Walt. (Threadgill et al., 1981) and *Sabatia angularis* (L.) Pursh (Baskin and Baskin, 2005). However, investigating dormancy was not the aim of any of the aforementioned *Centaurium* references, and they only studied lowland annual/biennial generalist species of broad geographical distribution. It remains to be seen if the same germination patterns apply in perennial *Centaurium* species, especially in those living in particular habitats departing from the common characteristics of the genus.

The rare *C. somedanum* M. Laínz shows several divergences from the general *Centaurium* traits, as it is a perennial chamaephyte and a specialist species of mountain calcareous springs (Jiménez-Alfaro et al., 2005). It is also a narrow endemic species, confined to a small geographical area (210 km²) located in a transitional region between Mediterranean and Oceanic climatic zones in the Cantabrian Mountain Range of NW Spain (Jiménez-Alfaro et al., 2010). Because of the ecological and geographical uniqueness of *C. somedanum*, we may expect divergences from the

general germination patterns of the annual/biennial, generalist and widely distributed *Centaureum* species. For example, temperate wetland species show a preference for relatively high (ca. 30 °C) or alternate germination temperatures (Grime et al., 1981; Thompson and Grime, 1983; Schütz, 2000). Another possible adaptation of *C. somedanum* could be seed dormancy, to avoid the risks of winter germination in a mountain environment (Baskin and Baskin, 1998), although this dormancy could vary among years and populations, as is usually the case in wild species (Andersson and Milberg, 1998; Giménez-Benavides et al., 2005).

In the present study, we investigate the germination ecology of *C. somedanum* as a contribution to understanding germination in *Centaureum* and the adaptation of rare endemic species to an ecological niche unusual in this genus. Specifically, we want to determine the (1) temperature and light requirements for its germination, (2) possible existence of physiological and/or morphological dormancy and (3) existence of inter-population and inter-annual variation in seed germinability.

Materials and methods

Plant material

Discrete populations of *C. somedanum* occur from 600 to 1700 m a.s.l. in the edges of calcareous mountain springs. In these habitats, alkaline spring waters (pH > 7.7) flow from aquifers continuously through the year and are relatively cold even in summer (mean day temperature in late summer = 14.1 ± 0.2 °C; data from 160 measures covering the entire altitudinal gradient of the species). Depending on the slope and species composition of the spring edges, two habitat types harbour the populations of *C. somedanum*: vertical travertines and flat calcareous fens (Jiménez-Alfaro et al., 2005).

Although little information is available regarding its reproductive biology, *C. somedanum* is assumed to be a facultative outcrosser as other species in the genus (Brys and Jacquemyn, 2011). Flowering begins in early July, proceeds during summer and ripe seeds are dispersed in September and October (Jiménez-Alfaro et al., 2010). We observed abundant seedlings in the field in August, so we assume that emergence occurs in summer (mean summer temperature = 15 °C, min = 10 °C, max = 22 °C), after the cold season has ended (mean winter temperature = 4 °C, min = 0 °C, max = 8 °C) (data extrapolated from neighbouring climate stations, Spanish National Meteorological Agency). Seed set is relatively high in wild populations, with ca. 140 seeds per fruit. The seeds are roughly spherical, extremely small (ca. 390 µm wide) and show a reticulate pattern which is common in the *Centaureum* genus (Bouman et al., 2002). Seed mass (ca. 20 µg) also is similar to that of other *Centaureum* species (Royal Botanic Gardens

Kew, 2008). The embryo belongs to Martin's dwarf category (Martin, 1946).

For three consecutive years, we harvested fruits from the two largest populations of *C. somedanum*, which are representative of the two associated habitat types and the altitude gradient of the species (Table 1). In both sites, we sampled all individuals bearing ripe fruits (dry and brownish). The fruits spent a 3-wk period in our laboratory (ca. 22 °C, 50 % RH) to ensure homogeneous after-ripening. Afterwards, we removed the seeds from the fruits; cleaned them using sieves and by hand sorting; and immediately used them in the experiments.

Embryo measurements

We sowed 2009 seeds from both populations on 1 % distilled water agar in Petri dishes sealed with parafilm to prevent desiccation (4 dishes with 25 seeds each per population) and kept them 24 h in continuous darkness at 20 °C. Afterwards, we excised and measured embryos from 15 seeds per population using a dissecting microscope (MZ6, Leica Microsystems GmbH, Wetzlar, Germany) equipped with a micrometer. The remaining seeds were cold stratified for 12 wks (3 °C, darkness) and then 15 embryos per population were measured. After cold stratification, seeds were incubated in a growth chamber (Grow-S 360, Ing. Climas, Barcelona, Spain) with a 12/12 h photoperiod (ca. 20 µmol m⁻² s⁻¹ provided by six Philips TLD30W/54-765 cool fluorescent tubes) at 22/12 °C, the summer temperature expected by us to be optimal. We examined them daily and when the seed coat began to split, i.e. when the embryo had reached its critical length for radicle emergence, we measured another 15 embryos per population. To analyze the embryo measurements, we performed a main effects ANOVA with stratification/incubation stage and population as fixed effects using SPSS for Windows 15.0.1 (SPSS Inc., Chicago, USA).

Germination experiments

We carried out laboratory germination experiments on 1 % distilled water agar in 6-cm-diameter Petri dishes sealed with parafilm. For each treatment, we sowed four dishes with 25 seeds each. To assess the effect of incubation temperature on germination, we incubated 2009 seeds from both populations in growth chambers under a 12/12 h photoperiod at 22/12, 15, 20, 25 and 30 °C. To take into account the inter-annual variation on seed germinability, we also incubated 2008 and 2010 seeds at 22/12 °C. Finally, to assess the effect of light on germination, we incubated 2008 seeds at 22/12 °C in continuous darkness (achieved by wrapping the dishes in two foils of aluminium paper). In all cases, we incubated seeds both after 0 (= fresh, i.e. after 3 wk after-ripening in the laboratory) and 12 (= stratified) wks of cold stratification in 1% agar at 3 °C in darkness, to check for the existence of physiological dormancy. We did not consider warm stratification as it would be

ecologically meaningless according to available knowledge on the species habitat and dispersion timing.

We counted and discarded germinated seeds each Monday, Wednesday and Friday (with the exception of the dark-incubated seeds, which we only checked at the end of the experiment). Radicle emergence was the criterion for germination. After 4 wks, we terminated all germination tests and opened the non-germinated seeds with a scalpel, classifying them as normal, empty and fungi infected. We excluded the empty and infected seeds from the statistical analyses and the calculation of germination percentages (pooling all the dishes, empty seeds = 3 ± 1 %; infected seeds = 3 ± 1 %). We analyzed the results by fitting main effects Generalized Linear Models (GLM, binomial error distribution, logit link function) with the test conditions as fixed factors using SPSS.

Results

Embryo growth

We found significant differences in embryo length depending on the stage of the stratification/incubation process ($F = 279.592$; $p < 0.0001$). While after cold stratification embryo length was similar to that of fresh embryos, at the point of radicle emergence embryos had undergone an increase of 86 % in their length (Fig. 3). We did not detect significant differences in embryo growth between the populations ($F = 0.029$; $p = 0.866$).

Germination temperature

Higher seed germination occurred at lower temperatures (15-22 °C) with a marked decrease at warmer temperatures (Fig. 1). No seeds germinated at 30 °C and only cold-stratified seeds germinated to a low percentage at 25 °C. These two temperature regimes were qualitatively different and thus we did not include them in the statistical analysis, as their lack of variance would alter the GLM procedure. Analyzing the results for the other three temperatures, we detected a significant positive effect of cold stratification on germination (Wald's $\chi^2 = 186.720$; $p < 0.0001$) but neither an effect of incubation temperature (Wald's $\chi^2 = 5.195$; $p = 0.074$) nor of population (Wald's $\chi^2 = 0.352$; $p = 0.553$). Thus, in the range most favorable for seed germination, we did not find differences between constant (15, 20 °C) and alternate (22/12 °C) regimes and no differences between the germination temperature range of seeds from the two populations.

Germinability variation

We found differences in seed germinability among our collections (Fig. 2). Cold stratification produced a significant germination increase (Wald's $\chi^2 = 222.293$; $p < 0.0001$) across years and populations, but we only obtained almost complete germination in 2008. In the other two years, a percentage (47-80 %) of the seeds did not germinate after 12 wks of cold stratification. Analyzing together all years and populations, we found

a significant effect of year (Wald's $\chi^2 = 173.515$; $p < 0.0001$) but not of population (Wald's $\chi^2 = 0.647$; $p = 0.421$).

Effect of light

Darkness had a clear negative effect on germination. When incubated in darkness, the fresh seeds did not germinate at all, and only 1 ± 1 % cold-stratified seeds from one population (La Malva) did germinate. This clear-cut effect of darkness made it impossible as well as unnecessary to apply any statistical test.

Discussion

C. somedanum shows two significant divergences from the previously reported *Centaureum* germination patterns, namely (1) a lower germination temperature range and (2) seed dormancy. Furthermore, its germination requirements are in contrast to the typical responses of wetland species (Grime et al., 1981; Thompson and Grime, 1983; Schütz, 2000) as germination not only occurs at low temperatures, but also shows no increase in response to alternating temperatures. Among the tested conditions, significant higher germination occurred between 15 and 22 °C. As we did not test colder temperatures, it is not possible to establish the lower temperature limit of the species. In addition, the low germination percentages achieved at 22/12 °C in 2009 and 2010 could indicate that these are suboptimal conditions and that the optimal germination temperature is even lower. However, since the germination of the 2008 stratified seeds was almost complete at 22/12 °C, the lower germination found in the following years seems more related to a deeper dormancy in those years, suggesting that the optimal germination temperature should not be too far from the 15-22 °C range.

In any case, the upper germination temperature limit of *C. somedanum* is below what it was expected. Germination at low temperatures is a common trait of lowland Mediterranean species (Escudero et al., 1997; Doussi and Thanos, 2002), and consequently the temperature requirement for *C. somedanum* germination would seem to be related to the Mediterranean origin of the genus. However, lower germination temperature ranges are usually interpreted as an adaptation to the seasonality of Mediterranean climates that prevents germination during the dry season (Doussi and Thanos, 2002), and this is obviously not the case in the extremely wet environment of *C. somedanum*. The traditional understanding of germination in wetland habitats, as proposed by Thompson and Grime (1983), indicates that the fall of the water table during the spring season produces rising soil temperatures and an increase in diurnal temperature fluctuations. Wetland seeds perceive these signals as marking the optimal season for germination, but the conditions may be very different in mountain spring habitats where water flow is continuous and even more intense during the spring

season, when snowmelt recharges aquifers. In our study case, seed germination and seedling establishment in *C. somedanum* take place in the soil of spring edges, where the constantly cold running waters heavily influence the temperature. According to data obtained by a soil data-logger in La Malva population (M-Log5W, GeoPrecision GmbH, Ettlingen, Germany; data from September 2010 to September 2011), the diurnal thermal amplitude in the soil of the spring edges is relatively low through the year (winter = 1.9 ± 0.1 °C, summer = 3.0 ± 0.1 °C) and the summer temperature is considerably less variable than expected (mean = 16.9 °C, min = 15.7 °C, max = 18.8 °C). In this environment, germination at low and constant temperatures is probably the only option for the species. However, since we could not establish the lower germination temperature limit, it cannot be excluded that seed germination begins earlier in the year, as from April onwards the soil mean temperature exceeds 10 °C.

The inability of *C. somedanum* to germinate at 25 °C and above also differs from the successful germination achieved at 25 °C (Mijajlovic et al., 2005; Zivkovic et al., 2007; Todorovic et al., 2008, 2009) and the 29 °C upper germination limit found by Grime et al. (1981) in generalist *Centaurium* species of broad European distribution. It is still necessary to determine whether the lower range in *C. somedanum* is an ancestral Mediterranean character of the genus, which was conserved in this rare endemic and allowed it to colonize the spring habitat; or if it is rather a recent adaptation acquired in the course of such colonization. Although the phylogenetic origin of *C. somedanum* is unclear, the molecular study of Mansion et al. (2005) suggests an allopolyploid origin from the perennial *C. scilloides* (L. fil.) Samp and the biennial Spanish endemic *C. gypsicola* (Boiss & Reut.) Ronniger. *C. scilloides* is a generalist species widely distributed in the Atlantic coasts of Europe, while *C. gypsicola* is a specialist species of Mediterranean semi-arid gypsum communities of the central Iberian Peninsula. Thus, investigating the germination response of these species would provide some light to whether the low germination temperature trait of *C. somedanum* has a phylogenetic origin or is a new character.

The second divergence from the *Centaurium* germination pattern regards the seed dormancy we found in the three years analyzed, contrasting with the non-dormant behaviour reported in the literature for other *Centaurium* species. Our results show that *C. somedanum* seeds have non-deep simple morphophysiological dormancy (MPD) according to the classification system of Baskin and Baskin (2004) and that the embryo belongs to the underdeveloped linear embryo category proposed by Baskin and Baskin (2007). Fresh seeds have a non-deep physiological dormancy (PD) which prevents embryo growth and which is overcome by a period of cold stratification. As we did not test warm stratification it is not possible to

determine if cold is a requirement per se, but in nature this dormancy release takes place during the cold winter season. Once the PD is broken, embryo growth must take place before the seed germinates (morphological dormancy, MD). Since embryo growth did not occur during cold stratification, but did occur at warmer temperatures after the seeds were moved to 22/12 °C, they have non-deep simple MPD. To our knowledge, this is the first time that a MPD is found in the *Centaurium* genus, although it has been reported in New World Gentianaceae genera (Threadgill et al., 1981; Baskin and Baskin, 2005). As we have already explained, the thermal regime in the spring habitat is stable during the year in comparison to other, more seasonal, wetland environments. In this context, seed dormancy becomes of foremost importance to ensure that germination takes place in the correct period of the year, i.e. after the colder months of winter.

It is interesting to note that, contrary to what happened with the temperature and light requirements, we found a relatively large variation among seed collections in their germinability. Such variation, depending on year and population, is a well-known phenomenon (Andersson and Milberg, 1998; Giménez-Benavides et al., 2005), which has been usually related to genetic adaptation to local conditions or to the parental environment effect on seed maturation (Fenner, 1991; Baskin and Baskin, 1998). In our case study, the significant effect of year rather than location suggests that it is the environment in each maturation season what determines seed germinability. Nevertheless, further studies either controlling the seed maturation environment (common garden studies) or the genetic background of the populations are necessary to elucidate the relative contribution of the genotype versus the environment.

Besides the commented divergences, *C. somedanum* germination agrees with previous *Centaurium* studies regarding the lack of response to alternating temperatures (Thompson and Grime, 1983) and the light requirement for germination (Grime et al., 1981; Schat, 1983). The incapacity to germinate in darkness is a trait generally associated with the ability to form a soil seed bank (Pons, 1991; Milberg, 1994) especially in small seeds (Milberg et al., 2000). Other traits of *C. somedanum* seeds tend to be related to persistence in the soil bank, namely their small size and rounded shape (Thompson et al., 1993; Cerabolini et al., 2003). Thus, it is possible that *C. somedanum* forms a persistent soil seed bank, as has been reported for other *Centaurium* species (Thompson, 1997).

In conclusion, our study shows that a rare perennial species of *Centaurium* living in an unusual habitat presents divergences from the general germination patterns of the genus. The lower germination temperature range in *C. somedanum* could be of adaptive significance in the very particular conditions of mountain springs, where water flow is continuous and

relatively cold during all the year. It would be of great interest to study the germination temperature ranges of those species phylogenetically linked to *C. somedanum*, i.e. *C. scilloides* and *C. gypsicola*, to better understand the evolutionary history of this trait in the genus. Additionally, it remains to be seen if the MPD, a possible adaptation to mountain spring habitats, is shared by more *Centaurium* species, especially those with a similar ecology such as *C. littorale* subsp. *uliginosum* (Waldst. & Kit.) Melderis, which lives in travertines and calcareous fens of Central Europe. Our findings also suggest that the germination traits of species living in temperate mountain springs could differ from those living in other wetland types, although more studies focused on spring specialists are needed to clarify germination ecology in these habitats.

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Table 1 Description of the two populations included in this study

Collection site	Altitude (m a.s.l.)	Habitat, mean annual temperature	Coordinates	Collection dates
La Malva	600	Travertine, 10.4 °C	43° 07' 05'' N 06° 15' 05'' W	17/09/2008
				24/09/2009
				02/09/2010
El Valle	1280	Flat fen, 8.3 °C	43° 04' 19'' N 06° 11' 49'' W	17/09/2008
				09/10/2009
				02/09/2010

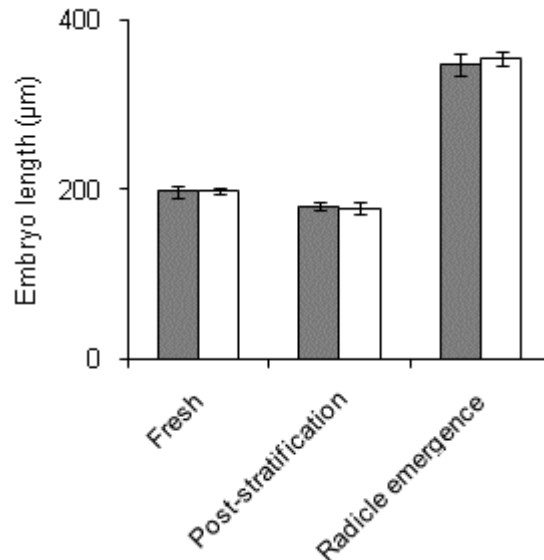


Figure 1 Final germination percentages of fresh (top panel) and stratified (bottom panel) seeds from two populations (grey = La Malva; white = El Valle) after 4 wks of incubation at different temperatures. Percentages are the mean \pm SE of four dishes.

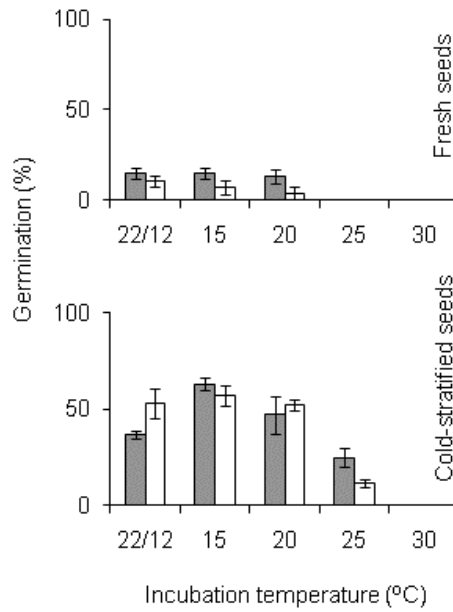


Figure 2 Cumulative germination of fresh (black circles) and stratified (open circles) seeds from each population and year of collection after 4 wks of incubation at 22/12 °C. The lines represent the Gompertz function fitted to the germination data using SigmaPlot 11.0.

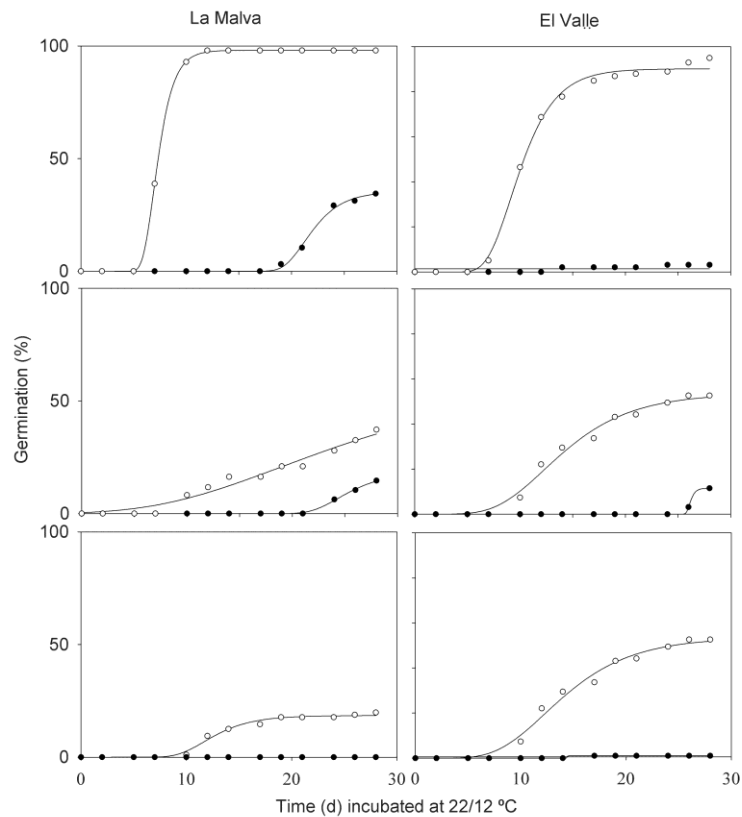


Figure 3 Embryo length in two populations (grey = La Malva; white = El Valle) at three different stages of the seed stratification-incubation process