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Effect of temperature and cold stratification on seed germination of the Mediterranean wild aromatic *Clinopodium sandalioticum* (Lamiaceae)

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Abstract

A germination study was carried out on seeds of *Clinopodium sandalioticum* (Bacch. & Brullo) Bacch. & Brullo *ex* Peruzzi & Conti (Lamiaceae), a wild aromatic plant endemic to Sardinia. Seeds were incubated at a range of constant (5–25°C) and an alternating temperatures regime (25/10°C), with 12 hours of irradiance per day. The results achieved at 10°C were also compared with those obtained after a period of cold stratification at 5°C for three months. Final seed germination ranged from ca. 28% (5°C) to ca. 72% (25/10°C). A base temperature for germination (T_b) of ca. 5°C and a thermal constant for 50% germination (S) of 89.3°Cd were identified and an optimal temperature for germination (T_o) was estimated to be comprised between 20 and 25°C. Cold stratification negatively affected seed viability and germination at 10°C. Although a typical "Mediterranean germination syndrome", could not be detected for *C. sandalioticum* seeds, these results were coherent with those previously reported for other Mediterranean Lamiaceae species.

Keywords: *Base temperature, cold stratification, endemic, germination rate, Mediterranean climate*

Introduction

The "Mediterranean germination syndrome" is characterized by low optimal germination temperatures (mainly 5–15°C; Thanos et al. 1989) and low germination rate (Doussi & Thanos 2002). This germination behavior is considered an advantageous ecological adaptation of species to the unpredictable rainfall pattern under the Mediterranean climate, by limiting germination to winter and therefore maximizing the length of the growing season before the onset of summer drought (Thanos et al. 1995; Fenner & Thompson 2005). Coherently to this germination phenology, chilling is not supposed to enhance seed germination in Mediterranean species. Skordilis and Thanos (1995) detected a loss of viability for chilled seeds of *Pinus halepensis* Mill. However, cold stratification may enhance germination of species with physiologically dormant seeds, growing in the Mediterranean area also at relatively low altitudes (Mancuso et al. 2012).

In non-dormant seeds, the germination response to accumulated temperature can been modeled by a thermal time (θ) approach. In this model, seeds accumulate units of thermal time (°Cd) to germinate for a percentile (g) of the population. When seeds are subjected to temperatures (T) above a base temperature for germination (T_b), at which germination rate is zero, and below an optimum temperature (T_o), above which germination rate does not increase anymore (sub-optimal temperature range), germination rate increases linearly with temperature (Garcia-Huidobro et al. 1982). Thus, in this sub-optimal range, germination occurs in the time t_g, when the thermal time accumulated has reached the critical value (θ_g) for a percentile (g) of the population and can be described as $\theta_g = (T - T_b)t_g$.

Seeds of Lamiaceae species are non-dormant, or physiologically dormant (Baskin & Baskin 1998; Finch-Savage & Leubner-Metzeger 2006; Copete et al. 2009, 2015). Mediterranean species such as *Thymbra capitata* (L.) Cav., *Satureja thymbra* L. and *Origanum vulgare* L. subsp. *hirtum* (Link) Ietsw. showed an optimum temperature for germination of 15–20°C (Thanos et al. 1995). In their study on endemic aromatic Lamiaceae species of Crete, Thanos and Doussi (1995) detected no dormancy, low germination temperature requirement and a slow germination for *Origanum dictamnus* L., *Salvia pomifera* L. subsp. *pomifera* and *Salvia fruticosa* Mill., supporting early autumn seed germination. However, they found that seeds of *Sideritis syriaca* L. subsp. *syriaca* (a species growing up to 1500 m a.s.l.), germinat-

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ed at relatively warmer temperatures (>15°C) with a faster rate, in accordance with its high mountain distribution, favoring spring seedling emergence. Accordingly, Estrelles et al. (2010) found an optimal temperature for germination of 20°C for two Iberian endemic *Sideritis* L. species: the mountain *S. pungens* Benth. and the coastal psammophylous *S. chamaedryfolia* Cav. Kadis and Georghiou (2010) examined seed dispersal and germination in three endemic Lamiaceae of Cyprus: *Origanum cordifolium* (Montbret & Aucher ex Benth.) Vogel, *Phlomis brevibracteata* Turrill and *Phlomis cypria* Post subsp. *occidentalis* Meikle. These authors found that although seeds of these species ripe in summer, seed dispersal mainly occurs only at the beginning of the rainy season when the opening of the calyces seems to be caused by absorption of moisture and seed germination occurs at relatively low temperatures (≤20°C; Kadis & Georghiou 2010).

Clinopodium sandalioticum (Bacch. & Brullo) Bacch. & Brullo *ex* Peruzzi & Conti (Lamiaceae) is a wild aromatic small half-shrub, localized in a narrow gorge in SW Sardinia. Recently, this species has been evaluated as Critically Endangered, according to IUCN criteria (Fenu et al. 2012). No information is available in literature on seed germination of this narrow endemic species.

The aims of this study were to: (1) characterize the thermal requirements and (2) assess the effect of cold stratification on seed germination of the narrow endemic *C. sandalioticum*.

Materials and methods

Study species and seed lot details

Clinopodium sandalioticum is a chasmophyte, occurring in rocky habitats of Paleozoic and dolomitic limestones, with a Mediterranean pluviseasonal-oceanic bioclimate. This species is very rare and localized in a small gorge near Buggerru, in the Iglesiente subsector (SW Sardinia), which is included in the Important Plant Area (IPA) SAR07 (Blasi et al. 2011; Fenu et al. 2012). Flowering occurs from May to September, while fruiting from July to December (Bacchetta & Brullo 2005).

Calyces containing nutlets (hereafter, seeds) were collected in September 2011 from the original population (Is Lisandrus) near Buggerru in SW Sardinia (altitude of 80–260 m a.s.l), at the time of natural dispersal.

Germination tests

For this study, three replicates of 20 seeds each per treatment were sown on the surface of 1% water agar in 60 mm plastic Petri dishes and incubated in growth chambers (SANYO MLR-351, Japan) at a range of constant (5, 10, 15, 20, and 25°C) and an alternating temperatures regime (25/10°C) in December 2011. The relatively low number of replicates and of seeds per replicate used in all experiments were due to a limited seed availability, resulting from this species being threatened with a small population, and were chosen in order to allow testing a wide range of germination conditions. In all treatments, seeds were exposed to irradiance for 12 hours per day. In the alternating temperature regime, the light interval coincided with the elevated temperature period. To investigate the effect of chilling on germination, the results achieved at 10°C were also compared with those obtained after a stratification at 5°C for three months.

Germination was considered to have occurred at the time of visible radicle emergence. Germination was scored three times per week and any seeds that had germinated were removed. When no additional germination occurred for two weeks, after a maximum of four months, the viability of any remaining seeds was checked by a cut test.

Data analysis

Final germination percentage was calculated on the basis of the total number of filled seeds as the mean of the three replicates ± standard deviation (SD). Effect of temperatures on germination percentages and of cold stratification on seed mortality were determined by fitting generalized linear models (GLMs), with a logit link function and a binomial error structure, using R v. 2.14.1 (R Development Core Team 2011).

Theoretical cardinal temperatures were evaluated, by determining the seed germination rate, defined as the reciprocal of time to reach 50% of germination for the tests carried out at constant temperatures (5–25°C). The data were regressed using a linear model to estimate the base temperature (T_b) at which the germination rate is equal to zero, by the *x*-intercept for the suboptimal temperature range (Ellis et al. 1986). According to Trudgill et al. (2000), the thermal constant for 50% germination (*S*, °Cd), given by the reciprocal of the slope of the regression (Garcia-Huidobro et al. 1982; Trudgill et al. 2000) was also calculated. Regression analyses were carried out using SigmaPlot 2002 for Windows version 8.0 (SPSS, Chicago, IL, USA).

Results

Seed germination varied from ca. 60 to ca. 70% in the range of constant temperatures between 10 and 25°C, with a minimum at 10°C (62.02 ± 18.5%)

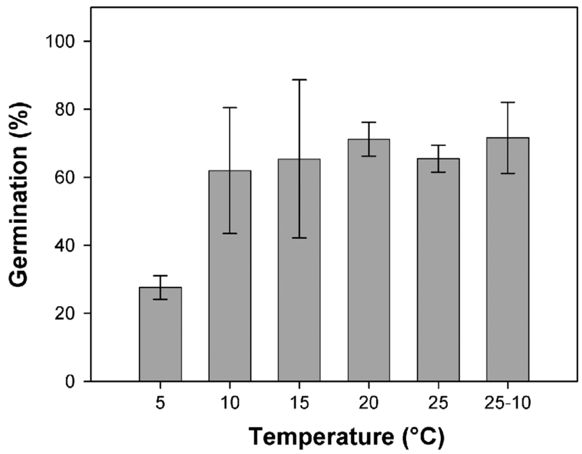

Figure 1. Final germination percentages of *Clinopodium sandalioticum*. Data are the mean of three replicates ± SD.

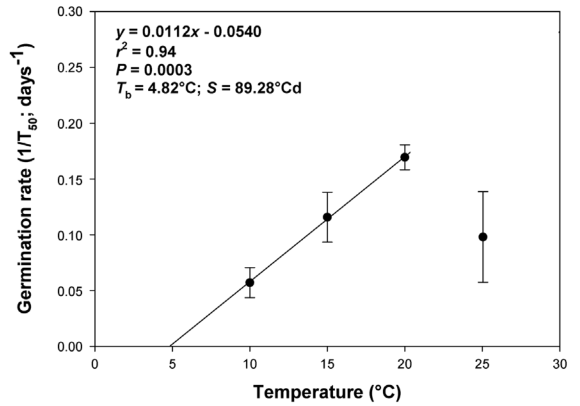

Figure 2. Germination rates calculated on the basis of the reciprocal of the times to reach 50% germination. Points correspond to the actual data and solid lines indicate the fitted lines from the linear regressions. Data are the means of three replicates (±SD).

Table I. Results of the GLMs, with binomial distribution and logit link function, on (A) the effect of the incubation temperature on seed germination and (B) the effect of cold stratification on seed mortality).

Coefficients	Estimate	Std. error	*z* value	*P* (>|*z*|)
(A)				
(Intercept)	0.4754	0.1187	4.004	<0.001
Temperature: 15°C	0.1731	0.1700	1.018	>0.05
Temperature: 20°C	0.4362	0.1744	2.502	<0.05
Temperature: 25°C	0.1731	0.1700	1.018	>0.05
Temperature: 25/10°C	0.4526	0.1747	2.591	<0.01
Temperature: 5°C	−1.4365	0.1754	−8.191	<0.001
(B)				
(Intercept)	−0.4895	0.1189	−4.116	<0.001
Cold stratification	0.5162	0.1658	3.114	<0.01

and a maximum at 20°C (71.2 ± 4.9%). Germination was reduced by more than a half when seeds were incubated at 5°C (27.6 ± 3.5%), while they reached 71.7 ± 10.4% of final germination when sown in the alternating temperature regime of 25/10°C (Figure 1). GLM results statistically confirmed the negative effect on final germination of the incubation at 5°C and the positive effect of constant 20°C and the alternating 25/10°C temperature regimes (Table I(A)).

The cut test carried out at the end of the germination tests highlighted that 12.5 ± 8.7% of the sown seeds were empty, while the great majority of filled non germinated seeds died (29.7 ± 17.5%), with very few seeds (<5% of the seeds sown at the start of the experiments) being still viable at the end of the experiments.

Time to reach 50% germination decreased with increasing temperatures from 10°C (18 days) to 20°C (6 days), whereas at 25°C it increased to 11 days. It was not possible to calculate this value for 5°C, due to the low final germination (ca. 28°C, i.e. <50%) achieved at this incubation temperature (see Figure 1). Consequently, germination rate increased from 0.06 to 0.17 d^{-1}, for seeds incubated between 10 and 20°C, respectively and decreased again to 0.08 d^{-1} for seed incubated at 25°C (Figure 2).

The germination rates at the range of the tested constant temperatures allowed a suboptimal range to be identified from 5 to 20°C, with 25°C being already in the supra-optimal range (Figure 2). In this suboptimal model, germination rate increased linearly with temperature and base temperature (T_b) of ca. 5°C was identified, according to the fitted regression line. The thermal constant for 50% germination (*S*) was 89.29°Cd (Figure 2). In addition, considering that 25°C was already in the supraoptimal range, the optimal temperature for germination can be estimated to range between 20 and 25°C (Figure 2).

The applied cold stratification period (three months at 5°C) negatively affected the subsequent seed germination at 10°C (<2%). Seeds mainly germinated during the incubation at 5°C, reaching 38.9 ± 21% (Figure 3). A stop on the cumulative germination was visible when seeds were moved to 10°C, with seeds germinating till final percentages of ca. 40.6 ± 17.8% (Figure 3). The cut test carried out at the end of the germination test highlighted a percentage of non viable seeds of 50.8 ± 17.7% against 37.9 ± 18.5% for seeds incubated directly at 10°C, without cold stratification. This increment on seed mortality was statistically significant, as highlighted by the GLM analysis (Table I(B)).

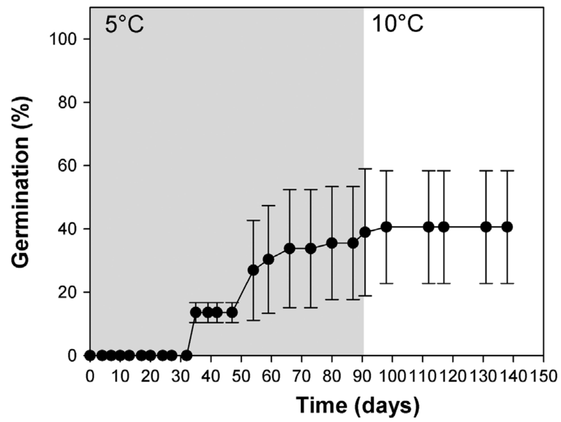

Figure 3. Germination progress curve during the cold stratification and the subsequent incubation at 10°C. Data are the mean of three replicates ± SD.

Discussion

Seeds of *Clinopodium sandalioticum* were not dormant *sensu* Baskin and Baskin (1998, 2004) at the time when the experiments were carried out, as they germinated to high percentages (>60%) in a wide range of temperatures (10–25°C), without any pretreatment. However, further studies on freshly collected seeds should be carried out, before excluding any types of physiological dormancy at the time of dispersal on seeds of this species (Baskin et al. 2006). Low germination was detected at 5°C (<30%) while the highest germination percentages were detected at temperatures >10°C. These results are similar to those results previously reported for other Mediterranean Lamiaceae (i.e. Thanos & Doussi 1995; Thanos et al. 1995; Kadis & Georghiou 2010).

The germination rate analysis highlighted an optimum temperature for seed germination (T_o) between 20 and 25°C as the latter was found to be in the supra-optimal range. Previous studies on Mediterranean Lamiaceae highlighted a highest germination rate at 15–20°C (Thanos & Doussi 1995; Thanos et al. 1995; Estrelles et al. 2010; Kadis & Georghiou 2010), with the exception of *S. syriaca* subsp. *syriaca*, growing on the high mountain of Crete at altitudes higher than 1000 m a.s.l., whose highest germination rate was detected at 30°C (Thanos & Doussi 1995).

Non-dormant seeds of Mediterranean species such as *Dianthus morisianus* Vals. (T_b = −0.04°C; Cogoni et al. 2012) and *Centranthus ruber* L. (DC.) (T_b = −0.5°C; Mattana et al. 2010) showed lower values of T_b, allowing seed germination at low temperatures. *Clinopodium sandalioticum* seeds showed a base temperature for germination (T_b) of approximately 5°C, therefore higher than "typical" Mediterranean species, but similar to the value previously reported for *Centranthus amazonum* Fridlender & A. Raynal, a critically endangered narrow endemic to Central–Eastern Sardinia (Italy), which occurs in rocky habitat (T_b = 3.75°C; Mattana et al. 2010). However, as previously pointed out by other authors, cardinal temperatures are theoretical values that should be treated with caution as they were obtained from extrapolation (e.g. Galiè et al. 2015).

The detected detrimental effect of cold stratification on germination confirmed the findings previously reported for other Mediterranean species. In particular, a loss of viability was detected for chilled seeds of *P. halepensis* (Skordilis & Thanos 1995) and a secondary dormancy induced by cold stratification reported for seeds of other Lamiaceae such as *Ziziphora aragonensis* Pau (Copete et al. 2009) and *Sideritis serrata* Cav. ex Lag. (Copete et al. 2015).

Although further studies carried out on freshly collected seeds are needed in order to better understand the seed germination ecology of *C. sandalioticum*, the results of this study allowed thermal requirement for seed germination of this species to be characterized in terms of base temperature for germination and thermal requirement for 50% of germination. In addition, a detrimental effect of cold stratification on seed viability and germination was identified. Although a typical "Mediterranean germination syndrome", could not be detected, the results achieved in this study were coherent with those previously reported for other Mediterranean Lamiaceae, suggesting a germination in autumn for seeds of *C. sandalioticum*, maximizing therefore the length of the growing season before the onset of summer drought.

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