Seed Production and in Situ Germination of Lamyropsis microcephala (Asteraceae), a Threatened Mediterranean Mountain Species

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Seed Production and *in situ* Germination of *Lamyropsis microcephala* (Asteraceae), a Threatened Mediterranean Mountain Species

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Abstract

Seed reproduction is considered a critical bottleneck of the plant life cycle, constraining population growth, especially in the Mediterranean area. In this study, we investigated seed reproduction of *Lamyropsis microcephala* (Asteraceae), a threatened species occurring only in the Gennargentu massif (CE Sardinia, Italy). Seed output was quantified in two of the four localities where the species occurs, which differed in population size. Germination of seeds from all the four localities was assessed, both in the field and under controlled conditions, and the annual trend of soil temperature recorded by data-loggers. Plants had ca. 60 cypselas (i.e. the fruits of Asteraceae) per capitulum in the larger Rio Aratu and ca. 30 in the smaller Pisargiu locality, with only ca. 1.7 and 0.3 germinating cypselas per capitulum, respectively. Under controlled conditions, seeds of the two large localities (Bau 'e Laccos and Rio Aratu) germinated above 80%, while those of the two small ones (Bruncu Spina and Pisargiu) did not reach 55%. All seeds sown in the field germinated in April–June, when diurnal fluctuations of temperatures were almost 10 times higher than in winter, limiting the length of the growing season before the onset of summer drought, and highlighting an increasing threat from global warming.

Introduction

High seed yield represents a successful trait for plant establishment and population growth. However, in the Mediterranean area, seed reproduction represents a lottery in colonizing open, even distant, suitable patches, and multiple demographic strategies, such as plant longevity and vegetative reproduction, enable persistence of threatened long-lived plants (García and Zamora, 2003), especially in high Mediterranean mountains whose vegetation usually consists of patchy communities (Escudero et al., 2004). A negative relationship has been reported between a narrow population size (or a low population density) and seed production, with the latter being reduced to small, isolated populations because of both increased inbreeding and reduced number of compatible mates (Vergeer et al., 2003; Campbell and Husband, 2007).

In seasonal climates, temperature is usually the main environmental factor governing seed germination in moist soil (Fenner and Thompson, 2005). In particular, diurnally fluctuating temperatures vary according to season, topography, and global climate, being lower in winter and under cloud cover (Dai et al., 1999). Since the amplitude of the diurnal temperature fluctuations vary among seasons, they may serve to detect different signals at different times of the year, such as moist conditions in winter or drought in spring (Saatkamp et al., 2011). Plants in environments that experience frost have been found to be more likely to possess some form of seed dormancy than species of milder environments (Allen and Meyer, 1998; Jurado and Flores, 2005), and populations from habitats with severe winters tend to have a higher fraction of dormant seeds and require a longer period of moist chilling to germinate (Allen and Meyer, 1998; Pendleton and Meyer, 2004). In alpine regions, spring emergence prevails due to temperatures being too low to stimulate germination following autumn dispersal or due to

a requirement of cold stratification over winter (Baskin and Baskin, 1998). Conversely, under Mediterranean climate, germination is limited to winter, thereby maximizing the length of the growing season before the onset of summer drought (Thanos et al., 1991, 1995; Doussi and Thanos, 2002). The Mediterranean-type alpine (or cryoromediterranean) zone, characterized by the presence of thorny cushion communities, is present in the most notable mountain areas that extend beyond the potential treeline, such as Sierra Nevada, High Atlas, Mount Etna, some peaks of the Hellenids, including the mountain in Crete, the Thaurus, and the Lebanese Mountains (Nagy and Grabherr, 2009). In these regions, even the high mountains may experience drought in the summer months, as a trend perceptibly increasing from north to south and from west to east (Nagy and Grabherr, 2009). However, high mountain Mediterranean plants do not seem to differ in germination characteristics from ''typical'' alpines, except for the high number of species ready to germinate without any pre-treatment (Giménez-Benavides et al., 2005). In addition, increases in temperature have been predicted and reported for the Mediterranean mountain ranges due to climate change (Peñuelas and Boada, 2003; Giménez-Benavides et al., 2007). As a consequence, seed germination of some species may be anticipated from summer to autumn, thereby determining seedling demise during winter, as predicted for *Nepeta sphaciotica* (Thanos and Fournaraki, 2010).

The Mediterranean basin has been recognized as one of the 34 most important global biodiversity hotspots (Myers et al., 2000; Conservation International, 2007). Médail and Quézel (1997) defined 10 regional hotspots of plant biodiversity within this area and, more recently, Médail and Diadema (2009) identified 52 putative glacial refugia, with 26 of them (i.e. 50%) occurring within the regional hotspots. Sardinia, situated in the West Mediterranean

basin, has been included in the ''Tyrrhenian Islands'' regional hot-spot (Médail and Quézel, 1999) and NE Sardinia, including the Gennargentu massif, is considered a glacial refugium *sensu* Médail and Diadema (2009).

Lamyropsis microcephala (Moris) Dittrich et Greuter (Asteraceae) is a Sardinian endemic, growing exclusively in the Gennargentu massif, and has been reported to be mainly vegetatively propagated, by virtue of both low seed set and low seed germination (Diana Corrias, 1977). More recently, Mattana et al. (2009) reported that germination success for *L. microcephala* was higher than 60% at temperatures ≥20 °C, after a cold stratification of 3 months at 5 °C. These authors concluded that seeds of this species showed a type 2 of non-deep physiological dormancy (i.e. seeds germinate also at low temperatures after dormancy is released), following the dormancy classification system (Baskin and Baskin, 2004). Seed dispersal takes place in late summer (Diana Corrias, 1977), and *L. microcephala* seeds, having a transient soil seed bank, presumably germinate in the following late spring, after experiencing the low winter temperatures that accelerate germination (Mattana et al., 2009). However, germination data reported by Mattana et al. (2009) were calculated for viable filled seeds and no quantitative data are available on the production of viable seeds for this species and on the environmental factors that promote germination in the wild.

The aim of this study was to assess the effectiveness of seed reproduction of *L. microcephala* by (1) quantifying its seed output, (2) assessing its seed germination both in the field and under controlled conditions, and (3) characterizing the annual trend of soil temperature in its localities.

Materials and Methods

STUDY SPECIES

Lamyropsis (Charadze) Dittrich (Asteraceae) belongs to the tribe Cardueae Cass., subtribe Carduinae Dumort, and comprises eight perennial species distributed from S Europe eastwards to SW Asia (Mabberley, 2008) and, in particular, in the Caucasus region, where temperate continental bioclimate prevails (Rivas-Martínez et al., 2004). *Lamyropsis microcephala* is the westernmost and the most threatened species of the genus, being the only one included under the Critically Endangered IUCN category (Fenu et al., 2011), listed as a priority species in the Annex II of the European Habitats Directive (DIR. 92/43/EEC), and in the European threatened plant list (Bilz et al., 2011).

STUDY AREA AND SEED COLLECTION

The Gennargentu massif (CE Sardinia) is characterized by a maximum elevation of 1834 m a.s.l. and by several ridges higher than 1500 m. The *L. microcephala* population is situated in small catchment areas on the northern and western slopes of this massif (Fenu et al., 2011). Vegetation is characterized by open grassland, where hemicryptophytes and cushion chamaephytes prevail (*Carici-Genistetea lobelioidis* vegetation class; Fenu et al., 2011), included in the ''Endemic Oro-Mediterranean heaths with gorse (code 4090) and the subtype Cyrno-Sardinian hedgehog-heaths (code 31.75)'' habitat (Biondi et al., 2009). Tourism and other outdoor activities represent the main threats to the population of this species, followed by expansion of pastoral activities and nomadic grazing of sheep, cattle, horses, and pigs (Fenu et al., 2011). The population is constituted by four localities which differ in their population size and area (Fenu et al., 2011; Table 1). For this study, eight experimental sites were identified: three in each of the two large localities (Rio Aratu and Bau 'e Laccos), in order to cover the elevation range (L: low elevation, M: medium, and H: high; see Table 1), while for the two small ones (Bruncu Spina and Pisargiu), the experimental sites coincided with their central points (Table 1).

TABLE 1

Data of the four localities and experimental sites (BA, BS, PG, and RA).

Locality	Code	Elevation range (m a.s.l.)[a]	Mean aspect (°)[a]	Mean slope (°)[a]	Area (m²)[a]	Estimated N of reproductive ramets[a]	Mean N of capitula per reproductive ramet[a]	Experimental site code (L: low, M: medium, and H: high altitude)	Elevation (m a.s.l.) (°)	Aspect (°)	Temperature Logger
Bau 'e Laccos (Fonni, NU)	BA	1450–1590	N (10)	15	12,500	66,268	2.69 ± 1.8 (N = 188)	BA L	1455	NW (305)	
								BA M	1520	NW (335)	X
								BA H	1555	NW (335)	
Bruncu Spina (Fonni, NU)	BS	1625–1637	N (355)	45	600	3076	2.46 ± 1.8 (N = 100)	BS	1630	N (355)	
Pisargiu (Fonni, NU)	PG	1580–1590	N (5)	35	200	1080	1.98 ± 1.5 (N = 100)	PG	1585	N (5)	
Rio Aratu (Desulo, NU)	RA	1450–1820	W (280)	20	240,000	1,170,120	2.59 ± 1.7 (N = 144)	RA L	1565	W (270)	
								RA M	1665	SW (235)	X
								RA H	1705	W (280)	

[a] Data from Fenu et al. (2011).

Cypselas (the achene fruits of the Asteraceae) were collected after obtaining the permits required by European and national laws for the species listed in the Habitats Directive (DIR. 92/43/EEC). Harvesting was conducted when seed ripening was complete in August 2009 for two localities (Rio Aratu and Pisargiu) and in August 2010 for all of them (see Table 1), as specified below.

SEED OUTPUT ASSESSMENT

In 2009, a capitulum was collected from 30 reproductive ramets in the Rio Aratu and Pisargiu localities, chosen as representative of two different population sizes (see Table 1). Each capitulum was analyzed and cypselas were counted and evaluated according to the following categories: ''filled'': cypselas containing well-developed seeds; ''aborted'': cypselas containing aborted seeds; and ''empty'': cypselas with no seeds. Filled cypselas differed in size from the empty ones or those containing aborted seeds, allowing evaluation by external visual analysis. Filled cypselas of each capitulum were sown, after removing the pappus, on the surface of 1% solid water agar in plastic Petri dishes and incubated at 25 °C in the light (12 h of irradiance per day) after a pre-chilling period of three months at 5 °C. These conditions were chosen on the basis of the results achieved for this species in a previous work (Mattana et al., 2009). At the end of the experiment a cut test (ISTA, 2006) was carried out to check the viability of any remaining non-germinated seeds.

GERMINATION EXPERIMENTS

Cypselas (hereafter seeds) were collected from all localities in 2010. A subsample of filled seeds was taken in the laboratory and four plastic Petri dishes of 25 seeds each were sown at the above-reported conditions. At the end of the experiment, a cut test was carried out to check the viability of any remaining non-germinated seeds.

At the time of the harvesting, three sets of 20 filled seeds each were also placed in envelopes made by plastic mesh and sown in their field, after removing the pappus, at a depth of ca. 2 cm in the eight experimental sites (Table 1). This depth was chosen considering a maximum depth of seed burial for seedling emergence of 3.6 cm, as calculated following the formula: maximum depth (mm) $= 27.3 \times$ seed weight $(mg)^{0.334}$ (Bond et al., 1999) for seeds with a fresh seed mass of 2.32 $\pm$ 0.69 mg. Envelopes were checked for emerged seedlings in April 2011, when emergence was checked by gently removing the soil over the envelopes to avoid damaging seedlings, while they were exhumed and analyzed in the laboratory in June 2011.

In the medium elevation sites of the two large localities, a temperature logger (TidbiT® v2 Temp logger, Onset Computer Corporation, Cape Cod, Massachusetts, U.S.A.) was also sown in order to record soil temperature at 90-min intervals during a year (Table 1).

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA), followed by a *post hoc* Fisher least significant difference test (LSD) was carried out, when the ANOVA assumptions were met, otherwise a non-para-

metric Wilcoxon test was applied. ANOVA was used, for seed collected in 2010, to test whether localities differed in final germination percentages under controlled conditions and if seasons differed in mean values of diurnally fluctuating temperatures (square-root transformed data; Saatkamp et al., 2011), calculated as differences between daily maximum and minimum temperatures (Thompson et al., 1977). The Wilcoxon test was carried out to verify differences between localities, for seeds collected in 2009, in the mean percentage of filled seeds and the number of germinated seeds per capitulum. All the statistical analyses were carried out by using R v. 2.13.0 (R Development Core Team, 2010).

Results

SEED OUTPUT

Lamyropsis microcephala plants at the two localities investigated in 2009 showed significant differences in their seed production. While, in the Rio Aratu, capitula had an average of 57.42 $\pm$ 17.64 (mean $\pm$ SD) cypselas, in Pisargiu the mean number of cypselas per capitulum was 29.90 $\pm$ 9.79 ($N = 30$; $P < 0.001$ by Wilcoxon test). However, only 7.74 $\pm$ 13.40% (mean $\pm$ SD) and 2.82 $\pm$ 3.99% of the cypselas per capitulum were filled for Rio Aratu and Pisargiu, respectively ($N = 30$; $P < 0.05$ by Wilcoxon test; Fig. 1). The great majority of the cypselas collected in both localities were not filled, with 61.74 $\pm$ 17.00% and 80.39 $\pm$ 14.33% having aborted seed and 30.37 $\pm$ 15.20% and 16.79 $\pm$ 13.30% being empty for Rio Aratu and Pisargiu, respectively (Fig. 1). After incubation of the filled cypselas for germination under controlled conditions, a mean number of 1.77 $\pm$ 1.80 and 0.27 $\pm$ 0.47 seeds per capitulum germinated for Rio Aratu and Pisargiu, respectively ($N = 30$; $P < 0.001$ by Wilcoxon test). All non-germinated seeds died during the experiment.

SEED GERMINATION AND PHENOLOGY OF SEEDLING EMERGENCE

Localities sampled in 2010 differed for final germination percentages under controlled conditions. While seeds belonging to the two main localities reached germination percentages above 80% (86.45 $\pm$ 10.42% and 80.74 $\pm$ 3.29%, for Bau 'e Laccos and Rio Aratu, respectively), those of the two small localities did not germinate more than 55% (52.38 $\pm$ 18.75% and 47.48 $\pm$ 11.46%, for Bruncu Spina and Pisargiu, respectively). The difference on final germination among populations was statistically significant ($P < 0.01$ by one-way ANOVA; Table 2), and all non-germinated seeds died during the experiment.

During the first measurement period of the envelopes buried in the field in April 2011, few emerged seedlings were found in the medium and high elevation experimental sites of the Bau 'e Laccos locality (ca. 12% and 7%, for BA M and BA H, respectively), while no emerged seedlings were found in the lower elevation site (BA L) as well as in all the other localities (Table 2). At the end of the experiment in June 2011, emerged seedlings were found only for Rio Aratu locality (95% and ca. 42% for RA L and RA H, respectively; see Table 2). After opening the envelopes, only empty split seed coats were found, indicating that all the sown seeds germinated before exhumation. The presence of emerged seedlings in the lowest locality (Bau 'e Laccos) in April and in the highest (Rio Aratu) in June, as well as the fact that all sown seed
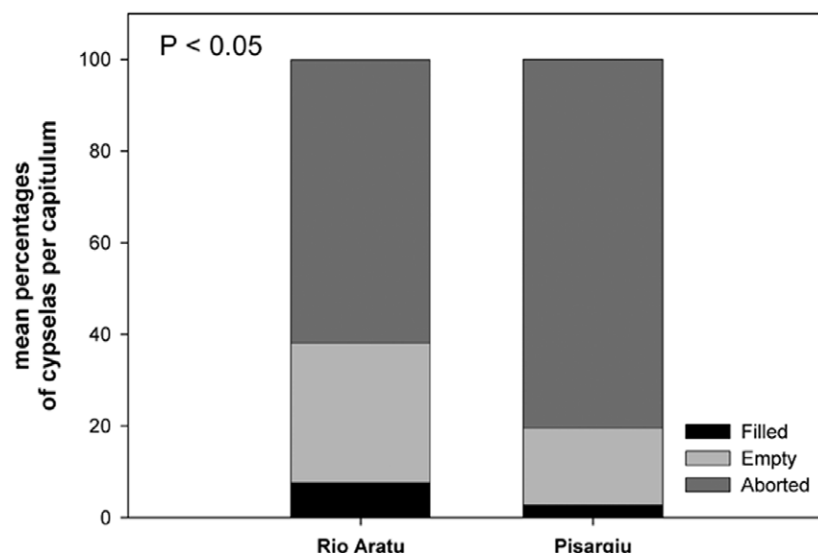
germinated before exhumation, indicate that *L. microcephala* seed germination occurs from April to June (Fig. 2).

The soil temperatures recorded by the two data loggers were very similar for the two localities with an annual mean temperature of ca. 10 °C, with mean values of ca. 1.5 °C in winter and ca. 20 °C in summer. In Bau 'e Laccos, temperatures ranged from a minimum of -1.7 °C (14 December 2010) to a maximum of 43.5 °C (29 August 2010), while in Rio Aratu, they ranged from 0.1 °C (13 March 2011) to 35.2 °C (21 August 2011). The snow continuously covered the soil from the end of January to the middle of March 2011 in both localities for a total of ca. 50 days, with mean temperatures close to 0°C, while mean daily temperatures were lower than 5 °C (cold stratification period) from November 2010 to March 2011 for a total of ca. 135 days. In April/June, when seed germination occurred, mean soil temperature was ca. 11 °C,

ranging from minimum temperatures of ca. 8 °C to maximum temperatures of ca. 17 °C (70 days). The analysis of the mean diurnally fluctuating temperatures recorded during a year period highlighted an average ΔT value of 4.6 ± 3.4 °C in autumn (from equinox to solstice), a minimum ΔT in winter (from solstice to equinox) with 1.1 ± 1.4 °C and then it increased in spring ($\Delta T = 8.2 \pm 4.2$ °C) and in summer when reached its maximum of 12.7 ± 3.6 °C ($P < 0.001$ by one-way ANOVA, followed by *post hoc* Fisher's LSD test; Fig. 3).

Discussion

SEED PRODUCTION

The number of germinated seeds per capitulum of *L. microcephala* reported in this study confirmed the observations of Diana

TABLE 2

Final germination percentages under controlled conditions of seeds collected in 2010 from each locality and values of seedling emergence in the field. Seeds were sown in the laboratory at 25°C, with a photoperiod of 12 h of irradiance per day, after a pre-chilling of 3 months at 5°C. Data are the mean of 4 replicates of 25 seeds each; values with the same letters are not different at $P > 0.05$ by *post hoc* Fisher least significant difference test. Values of seedling emergence in the field are expressed as percentages of three replicates of 20 seeds each. Seeds were sown on 26 August 2010. At the first monitoring (18 April 2011) envelopes were not exhumed, but emergence was checked by gently removing the upper soil to avoid damaging seedlings. The experiment was ended on 12 June 2011, when envelopes were exhumed and remaining seeds analyzed in the laboratory.

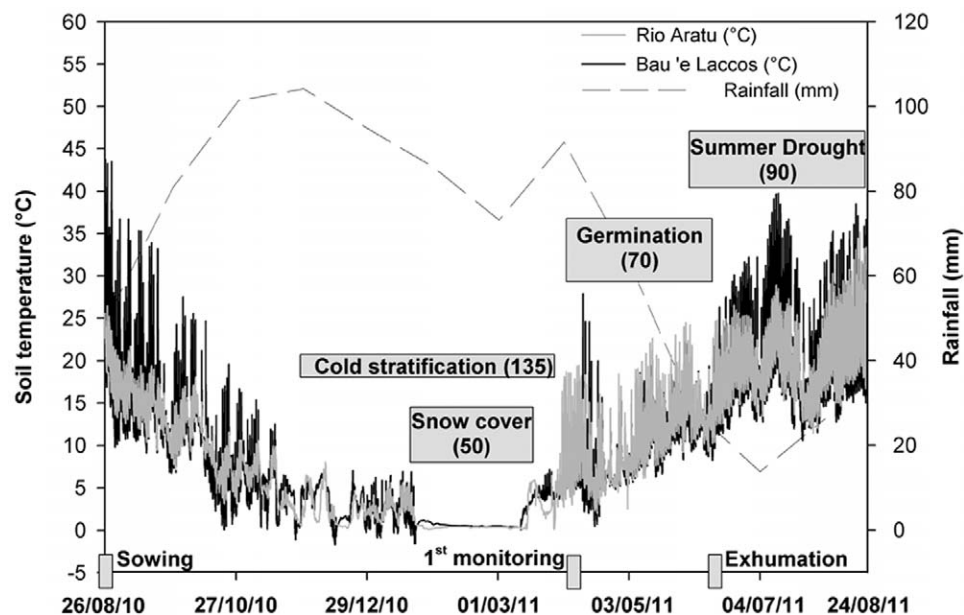| Locality	Germination under controlled conditions (%; mean ± SD) ($P < 0.01$)	Site code	18 April 2011 Emerged (%; mean ± SD)	12 June 2011 Seedlings (%; mean ± SD)	Empty split seed coats (%; mean ± SD)
BA	86.45 ± 10.42[a]	BA L	0	0	100.0 ± 0
		BA M	11.7 ± 20.2	0	100.0 ± 0
		BA H	6.7 ± 7.6	0	100.0 ± 0
BS	52.38 ± 18.75[b]	BS	0	0	100.0 ± 0
PG	47.48 ± 11.46[b]	PG	0	0	100.0 ± 0
RA	80.74 ± 3.29[a]	RA L	0	95.0 ± 5.0	5.0 ± 5.0
		RA M	0	0	100.0 ± 0
		RA H	0	41.7 ± 30.5	58.3 ± 30.5

FIGURE 2. Soil temperature (°C) recorded by the dataloggers with a 90-min interval in the two investigated localities (Bau 'e Laccos, 1520 m and Rio Aratu, 1665 m a.s.l.). Dates are expressed as DD/MM/YY. The times of the sowing of the seeds in the field, their first monitoring, and their exhumation are indicated, as well as the period of snow cover and, according to results achieved in the field experiments, seed germination time. Length of the gray bars correspond to the duration of the phases, and they are expressed in days (numbers in parentheses). Historical annual trend of rainfalls (mm) in the study area is also reported (data from the nearby weather station of Fonni at ca. 1000 m a.s.l.).

Corrias (1977), who suggested a low seed set occurred in this species. However, it should be taken into account that multiplying the number of germinated seeds per capitulum calculated in this study per the estimated number of reproductive ramets per locality by the mean number of capitula per ramet reported in Fenu et al. (2011), gives ca. 5,000,000 and 600 germinable seeds for Rio Aratu and Pisargiu, respectively.

Localities of *L. microcephala* with different population size differed significantly also in their seed viability, with a higher percentage of filled cypselas per capitulum in the larger locality (Rio Aratu, ca. 1,000,000 reproductive ramets) than in the smaller one (Pisargiu, ca. 1000 reproductive ramets). These results are consistent with several previous studies investigating the significance of population size for the reproductive success of rare (e.g. Fischer and

Matthies, 1998; Leimu et al., 2006) and endangered plant species (Mattana et al., 2010). Small populations may fail to attract sufficient pollinators, leading to pollen limitation and reduced seed set (Ågren, 1996; Colling et al., 2004). This process may be the cause of reduced fecundity in small populations of several insect-pollinated species such as *Senecio integrifolius* (Widén, 1993), *Gentianella germanica* (Fischer and Matthies, 1998), and *Primula elatior* (Jacquemyn et al., 2002). Although self-incompatibility is found within Asteraceae (Matton et al., 1994), mating system and pollination strategy of *L. microcephala* are still not investigated. Thus, the low seed output reported for the species in general, and particularly for the small locality of Pisargiu, may also be due to inbreeding depression (Ågren, 1996; Fischer and Matthies, 1998; Campbell and Husband, 2007).

SEED GERMINATION AND SEEDLING FATE

The high germination percentages (>80%) reached under controlled conditions by seeds from the two main localities (Rio Aratu and Bau 'e Laccos) and the significantly lower germination (ca. 50%) detected for seeds of the two small localities (Bruncu Spina and Pisargiu), suggests that the size of the localities may have an effect also on seed germination. Menges (1991) found that germination of *Silene regia* was relatively low in small populations and uniformly high in populations with more than 150 plants, and suggested that this trend could have been determined by the presence of inbreeding depression and a reduced pollinator visitation in small populations. However, all seeds sown in the field germinated before exhumation, without differences among localities (see Table 2). In laboratory experiments, seeds were exposed to constant temperatures, which do not represent natural conditions (Baskin and Baskin, 1998), while seed germination is often increased by both the number and amplitude of temperature alternations (Thompson et al., 1977; Probert, 1992; Saatkamp et al., 2011). Therefore, the higher germination percentages observed in the seeds from the two larger localities under these controlled (un-natural) conditions are likely due to a difference in their seed vigor (Menges, 1991).
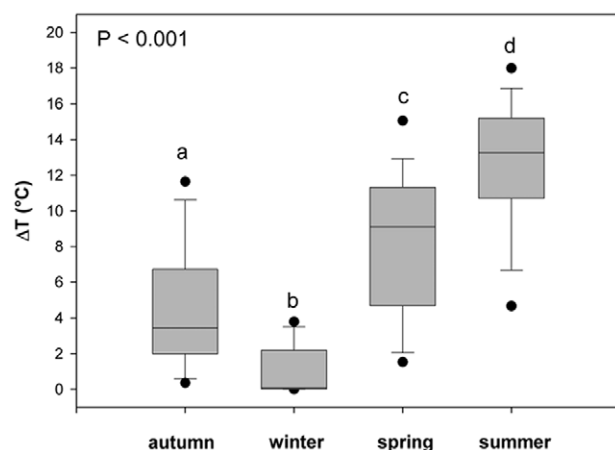

FIGURE 3. Diurnally fluctuating soil temperatures (ΔT) in the study area, recorded by the dataloggers with a 90-min interval. Data are the mean of the values ($N = 90$) recorded in each of the two investigated localities (Bau 'e Laccos, 1520 m and Rio Aratu, 1665 m a.s.l.). $P < 0.001$ by one-way ANOVA; boxes with different letters are different at $P < 0.05$ by *post hoc* Fisher's LSD test.

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The results of the field experiments highlighted a spring germination for this species. However, while in a previous study under controlled conditions, seeds germinated better at constant temperatures >20 °C or at the alternating temperature regime of 30/15 °C, suggesting a germination in late spring/early summer (Mattana et al., 2009), in the natural localities seeds germinated between April and June, with mean temperatures ranging around 10 °C. All the seeds germinated as soon as snow melted, when diurnal fluctuations of temperatures achieved ΔT values almost 10 times higher than in winter, confirming the role of high temperature fluctuations on the germination enhancement (Thompson et al., 1977; Probert, 1992; Saatkamp et al., 2011). Alternating diurnal temperatures are reported to promote germination in many alpine species, leading to early spring germination in order to ensure enough time for plant establishment (Bliss, 1971). This adaptation is essential for Mediterranean high mountain species, where topsoil remains moistened for only few weeks after snowmelt and an intense water deficit characterizes the summer when temperatures are high enough to enable growth (Giménez-Benavides et al., 2005). The difference in times of germination recorded between the lowest (Bau 'e Laccos) and the highest locality (Rio Aratu) is therefore likely to be due to a different time of snowmelt determined by their altitudes.

The soil temperatures recorded in the two main *L. microcephala* localities highlighted that although seeds germinate in early spring when soil is still moistened by the snowmelt and rainfall, in the subsequent summer seedlings face very harsh environmental conditions, with soil temperatures rising to values of ca. 20 °C with ΔT of ca. 15 °C. Considering that from the end of June to the end of August rainfall is drastically reduced, it is not surprising that no seedling recruitment was observed (Diana Corrias, 1977), as newly established seedlings might survive only in rare moist microsites, where water is still available during the main part of the summer (Mattana et al., 2009).

CONCLUSIONS

The results of this study confirmed that low seed set occurs in the endangered plant *Lamyropsis microcephala* (Diana Corrias, 1977) and identified spring as the season of seed germination in the field for this species, when soil is still moistened by snowmelt and rainfall. These results suggest that reproduction by seeds is limited by both a low seed production and the current Mediterranean climate, which reduces the number and quality of available sites for seedling establishment. These results are in agreement with the findings of García-Camacho et al. (2010), who suggested that species of high altitude Mediterranean communities may be both seed and microsite limited, and of García and Zamora (2003) who considered plant longevity and vegetative reproduction as major traits enabling persistence of threatened long-lived plants in the Mediterranean area. However, more studies are needed to verify if vegetative propagation alone may guarantee long term *in situ* persistence of this species. In addition, the high increases in temperature predicted for the Mediterranean mountain ranges (Peñuelas and Boada, 2003) are likely to exacerbate the environmental conditions, making still more difficult population recruitment by seeds of this endangered species.

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