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Breaking of dormancy in the narrow endemic *Jasione supina* Sieber subsp. *supina* (Campanulaceae) with small seeds that do not need light to germinate

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Abstract – The germination properties of *Jasione supina* Sieber subsp. *supina* (Campanulaceae), which is endemic to Mount Uludağ, Bursa, Turkey, were determined. In this study, we investigated the effects of GA_3, the combination of hormone series and short-term moist chilling (1-month), and long-term moist chilling (4-month) on the germination percentage and mean germination time in relation to seed dormancy breaking. All treatment series were incubated under continuous dark (20 °C, 24 h) and light/dark (20/10 °C, 12/12 h) conditions. Seeds were collected from specimens widespread on the alpine and subalpine grasslands and dwarf shrubs of Mount Uludağ (1800–1900 m a.s.l.). Depending on the concentration, GA_3 and the combination of hormone and moist chilling treatments promoted seed germination in both dark and light/dark conditions. After 4-month-moist chilling treatment, seeds germinated 27% in a light/dark and 80% in a dark regime. Hormone and moist chilling treatments reduced the mean germination time. Our results showed that *J. supina* seeds have physiological dormancy, require prolonged times of moist chilling and preferentially complete germination in darkness.

Keywords: alpine belt, endemic, germination, *Jasione supina*, seed dormancy

Introduction

Alpine environments have high levels of endemic species, as a result of extreme environmental conditions including low temperatures, frost, and wind erosion (Körner 2003). The diversity ofmicrohabitats has resulted in a range of germination strategies and dormancy types among alpine species. Thus, it is difficult to address a common germination strategy for alpine plants (Körner 2003, Schwienbacher et al. 2011). Studies of the germination strategies and factors regulating seed dormancy of rare or endangered species are important for conservation efforts and establishing *ex situ* collections (Maunder et al. 2004, Havens et al. 2006). Dormancy is a process that helps seeds to adjust their germination timing to environmental conditions. The germination time is crucial for seedling survival in alpine environments (Baskin and Baskin 1998, Schwienbacher et al. 2011). Many alpine species evince deep physiological dormancy, though others can be non-dormant (Schwienbacher et al. 2011). Many researches have recently been focused on the effect of global climate change on alpine plant species as their phenology, seed germination, seedling establishment, and distribution areas are negatively affected (for details see Briceño et al. 2015, Giménez-Benavides et al. 2018).

Light requirement for seed germination especially small size seeds is an important parameter for evaluating the germination properties of plants (Fenner and Thompson 2005). Since small sized seeds probably have a more restricted burial capacity in soil than large sized seeds, they can remain on or close to the surface of the soil (Milberg et al. 2000). According to Milberg et al. (2000), there is a negative correlation between seed size and light requirement and this relationship is considered coevolved for adaptation. Besides, Bu et al. (2017) reported that the germination is more stimulated by light in smaller-seeded species than in larger-seeded ones. Although the germination of many genera belong-

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Fig. 1. Habit of *Jasione supina* Sieber subsp. *supina* (Campanulaceae).

ing to Campanulaceae has attracted well-informed discussion (Koutsovoulou et al. 2014), studies regarding the germination requirements on the genus *Jasione* are less worthy of attention. A consideration of seed germination requirements for *Jasione* shows that the seeds of *J. montana*, *J. heelreichii*, *J. montana* subsp. *montana*, and *J. orbiculata* are light-germinating and non-dormant (Pegtel 1988, Peco et al. 2006, Koutsovoulou et al. 2014). It has been found that *Jasione cavanilesii* has a low germination percentage in light and is considered to be dormant (Fernández-Pascual et al. 2017).

Jasione supina Sieber subsp. *supina* is an endemic perennial species, caespitose with numerous procumbent or ascending stems. Basal leaves are oblong, spathulate and obtuse, and ciliated at the base. Cauline leaves are ovate to lanceolate and sessile. Calyx lobes are linear to lanceolate. Flowers are blue (Fig. 1). Its flowering time is between June and August (Davis 1967). Seed maturation and dispersion periods of species are between July and August. This taxon is confined to Mount Uludağ and it has rare and narrow distribution from 1600 to 2300 m. In 1997, it was classified as a rare taxon by the International Union for Conservation of Nature (Walter and Gillett 1998). Mount Uludağ was established as a National Park in 1961 and is categorized as one of the Important Plant Areas (IPAs) of Turkey (Güleryüz et al. 2010). It also however, hosts important winter sport centres with hotels and other facilities whose activities have damaged plant diversity (Güleryüz et al. 2010, 2011). Nowadays, these activities continue to increase, threatening the habitats of many plants as apart from *J. supina*.

Plant conservation requires a deeper knowledge of a plant's life cycle, and seed germination is a critical stage. In this study, we aim to understand the germination properties of *J. supina* in order to provide information for possible *ex situ* conservation efforts.

Material and methods

Seed material

Seed sampling sites were selected from six different populations among grasslands, hard cushion and dwarf shrub communities to represent the subalpine and alpine belts of Mount Uludağ. Mature seeds were collected from at least 100 plants with dry fruits of each population between 1800–2000 m a.s.l. (40°05'54" N and 29°09'40" E) in August 2010. Then, seeds were extracted from the dry fruits with the help of forceps and stored under laboratory conditions (25 °C, 50% relative humidity). All experiments were started within 1 month of collection. Mean seed mass was determined to be 48.25 ± 0.01 μg seed^{-1} (n=1000).

Seed germination treatments

Sterile, plastic, 9-cm Petri dishes were used for seed germination. Surfaces were sterilised using a 5% sodium hypochlorite solution for 5 minutes, and then seeds were rinsed with tap water. Seeds were placed on sterilised filter paper moistened with 4 mL of distilled water (control) or GA_3 (potassium salt) solution. GA_3 at concentrations of 250, 500 and 1000 mg L^{-1} alone or in combination with short-term moist chilling (1 month, +4 °C in a refrigerator) was tested. Seeds were imbibed for 24 hours in GA_3 solutions before sowing. In addition, seeds were maintained in a fridge at +4 °C in Petri dishes with distilled water for 4 months for long-term moist chilling. To reduce evaporation, Petri dishes were wrapped with stretch film during photoperiods and with aluminium foil for dark incubations. We evaluated two different temperature regimes for the germination experiments: 20 °C (24 h dark), 20/10 °C (12 h / 12 h light/dark, approx. 30 μmol m^{-2} s^{-1} photosynthetic photon flux density during the light phase provided by Phillips TLD 30W/54-764 cool fluorescent tubes). The variable temperature regimes stimulated the expected diurnal changes in temperature in the natural habitat. Namely, the climate in Mount Uludağ is included in the first family of the Eastern Mediterranean climatic group (Akman 1990); the lower parts reflect a Mediterranean climate, and at higher altitudes, a rainy, micro-thermic, ice-filled winter climate. Annual mean temperature of Mount Uludağ is 4.6 °C, and average annual precipitation is 1483 mm, according to data of the Meteorological Station in Zirve (1877 m). Annual mean number of snowy days at the top of Mount Uludağ is 66.7, total number of snow covered days being 179.2, and maximum snow depth 430 cm (Güleryüz 1992). Four replications of 25 seeds per Petri dish were used. Seeds were considered to have germinated when the radicle emerged from the testa. Germinated seeds were counted and removed every day for up to 25 days. Seeds incubated in darkness were checked under filtered red light.

Germination parameters

The final percentage of germinated seeds was calculated as follows: (i) G (%) = (A/B) × 100, where A represents the total number of seeds that germinated within 25 days, and B represents the total number of seeds tested (25 seeds). For all experiments, the final germination percentage (mean ± standard error) and mean germination time (MGT, mean in days ± standard error) were calculated. The latter repre-

Tab. 1. Final germination percentages and mean germination time (MGT) of *Jasione supina* seeds subjected to different GA_3 concentrations in dark (20 °C) and light/dark conditions (20/10 °C; 12/12 h) with or without short-term moist chilling treatment for 25 days. Values are mean ± standard error (n = 4). Capital letters are used to compare results ($P < 0.05$) between hormone series and combination of short-term moist chilling and hormone series, while lower-case letters are used for comparison among different series (short-term moist chilling x hormone series, and hormone series), for two main treatments (light/dark and continuous dark-incubated seeds, separately. The variance analysis and Tukey HSD test were not applied to long-term moist chilling treatments.

	Treatment	GA_3 (mg L^{1})	Germination (%)	MGT (days)
Continuous dark (20 °C)	Hormone series	0	19.0 ± 6.0^{cE}	8.2 ± 1.8^{aB}
		250	92.0 ± 6.5^{bB}	4.7 ± 1.8^{bC}
		500	100.0 ± 0.0^{aA}	5.8 ± 1.2^{bC}
		1000	100.0 ± 0.0^{aA}	4.9 ± 0.3^{bC}
	Short-term moist chilling (1-month, +4 °C) and hormone series	0	30.0 ± 2.3^{dD}	11.8 ± 1.6^{aA}
		250	54.0 ± 8.3^{cC}	9.1 ± 1.7^{bB}
		500	80.0 ± 0.0^{bB}	2.5 ± 0.2^{cD}
		1000	100.0 ± 0.0^{aA}	2.2 ± 0.9^{cD}
Light/dark (20/10 °C;12/12 h)	Hormone series	0	10.0 ± 5.1^{cE}	15.1 ± 4.5^{aA}
		250	69.0 ± 6.8^{bB}	8.4 ± 0.3^{bB}
		500	79.0 ± 15.1^{bB}	8.1 ± 0.4^{bB}
		1000	100.0 ± 0.0^{aB}	7.6 ± 0.5^{bB}
	Short-term moist chilling (1-month, +4 °C) and hormone series	0	17.0 ± 3.8^{cD}	11.8 ± 1.6^{aA}
		250	54.0 ± 11.5^{bC}	9.5 ± 2.6^{bB}
		500	55.0 ± 7.5^{bC}	6.3 ± 1.7^{bB}
		1000	92.0 ± 16.0^{aA}	3.9 ± 1.0^{cCD}
Long-term moist chilling (4-month, +4 °C)	Continuous dark (20 °C)	0	80.0 ± 3.2	7.5 ± 0.4
	Light/dark (20/10 °C; 12/12 h)	0	27.0 ± 13.6	11.6 ± 1.3

sented the average length of time in days it took seeds to germinate, calculated by the formula: $MGT= \Sigma DN/\Sigma N$; where D is the number of days counted from the date of sowing and N is the number of seeds germinated on day D. The final germination percentages were arcsine square-root transformed.

Statistical analysis

Final germination (arcsine-transformed) and MGT were analysed by two-way ANOVA and differences between groups were performed by Tukey HSD test using SPSS Ver. 22 for Windows. Independent factors were GA_3 concentration, moist chilling, and their interaction (moist chilling × GA_3). Non-transformed data (percentage values) are presented in Table 1. All tests were analysed at a significance level of α= 0.05.

Results

Germination significantly increased in the GA_3 and moist chilling treatments at both darkness and photoperiod conditions (Tab. 1). Our results indicated that seed germination was possible in dark conditions. Moreover, germination was enhanced in dark conditions compared to light/darkness conditions (Tab. 1). The germination percentages of *J. supina* seeds in distilled water were 19% under darkness and 10% under light/darkness conditions. Short-term moist chilling provided increasing rates of germination: 30% in dark and 17% at light/dark in distilled water-treated seeds (Tab. 1). Long-term moist chilling increased the germination to the level of 80% in the dark and 27% in light/dark conditions in distilled water treatments (Tab. 1, Fig. 2). GA_3 treatment resulted in full germination at a concentration of 1000 mg L^{-1} for all treatments except that of short-term moist chilling incubated under light/dark conditions.

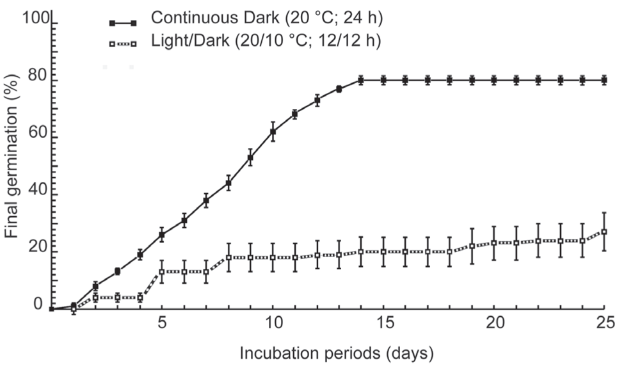

Fig. 2. Cumulative germination percentage of *Jasione supina* subsp. *supina* seeds after 4-months-chilling of seeds at 4 °C. The moist chilled seeds were then incubated under dark (20 °C) and photoperiod (20/10 °C, 12 h dark/12 h light, respectively) conditions in distilled water during 25 days. Vertical lines indicate standard error (n=4).

Tab. 2. Two-way ANOVA results of main effects (short-term moist chilling treatment and GA_3 treatments) for the arcsine-transformed germination percentage and mean germination time under dark and light/dark conditions for *Jasione supina* seeds. Values are two-way ANOVA F-ratios. Values in bold indicate significant ($P < 0.05$) differences.

Factor	Germination percentage			Mean germination time		
	df	F	*P*	*df*	F	*P*
Continuous dark (20 °C)						
Short-term moist chilling	1	159.57	<0.0001	1	1.20	0.283
GA_3	3	292.10	<0.0001	2	37.15	<0.0001
Short-term moist chilling × GA_3	2	40.59	<0.0001	2	17.29	<0.0001
Error	21			18		
Light/dark (20/10 °C; 12/12 h)						
Short-term moist chilling	1	3.83	0.062	1	1.52	0.231
GA_3	2	129.52	<0.0001	2	10.50	<0.0001
Short-term moist chilling × GA_3	2	6.04	0.003	2	9.08	<0.0001
Error	18			18		

The mean germination time (MGT) was delayed from 8.2 days to 11.8 days after a short-term moist chilling of seeds in distilled water in darkness. However, the MGTs of short-term moist chilling and light/darkness-incubated seeds in distilled water were reduced from 15.1 days to 11.8 days (Table 1). MGT values were decreased depending on hormone concentrations. In darkness as well as light/dark conditions, the combination of hormone and short-term moist chilling treatment was more efficient in reducing MGT than treatment with hormone alone. For example, the most striking decrease of MGT was found at 1000 mg L^{-1} GA_3 and short-term moist chilling combination treatment under darkness conditions (2.2 days). The 4-month-moist chilling also decreased MGT; it was 7.47 days for darkness and 11.6 days at light/darkness compared to distilled water controls (Tab. 1).

With respect to germination percentage, two-way ANOVA showed significant differences among all treatment series ($P < 0.05$) except short-term moist chilling treatment under light/dark conditions (Tab. 2). The effects of moist chilling on MGT were not significant under both dark and light/dark conditions ($P > 0.05$) (Tab. 2). However, GA_3 alone and GA_3 × moist chilling combination interaction effects were significant under both dark and light/dark conditions (Tab. 2).

Discussion

Our results indicate that the seeds of *J. supina* were dormant since we could not determine the germination above 50 percentage in any distilled water treatments under dark and light/dark conditions except for four-month-moist chilling treatments. Interestingly, long-term moist chilling that extended to four-month clearly increased the germination in darkness. This can be related to the role of long-term moist chilling in seed germination behaviour of *J. supina* seeds in natural conditions under the snow cover along the mean 179.2 days in a year (Güleryüz 1992). Several studies have reported that cold stratification is effective at breaking dormancy (Baskin et al. 2001, Kırmızı et al. 2010, García-Fernández et al. 2015, Schütz and Rave 1999). However, other studies have found no cold stratification requirement in some Campanulaceae members such as *Lobelia boninensis* (Marikoco and Kachi 1995) and some lobelioid shrubs from this family (Baskin et al. 2005). Fernández-Pascual et al. (2017) suggested that alpine plant species have two types of germination niche (warm and cold stratification) depending on habitat characteristics. Among *Jasione* species, *J. cavanilesii* was found to be dormant among 22 species of subalpine and alpine grasslands of the Cantabrian Mountains of Spain and germinated after warm stratification (at 25 °C) (Fernández-Pascual et al. 2017). Seeds of *J. crispa*, which is a high mountain species from the Mediterranean region, germinate at an average of 59% at warm temperatures and thus are not considered to be dormant (Giménez-Benavides et al. 2005). In addition, *J. montana* seeds germinate easily over a relatively wide range of temperatures (10–25 °C), including relatively low temperatures (Pegtel 1988).

Treatments with GA_3 and the combination of GA_3 and one-month-moist chilling stimulated the germination percentage up to 100% and reduced MGT (Tab. 1). These results indicate that GA_3 can substitute for long-term moist chilling and that *J. supina* seeds have physiological dormancy (PD). GA_3 is known as a germination stimulator that helps to overcome seed dormancy. GA_3 has been shown to induce the germination of many dormant species such as some *Allium* (Kırmızı et al. 2017), *Campanula glomerata* subsp. *hispida* (Gülbağ and Özzambak 2017) and *Allium stracheyi* (Payal et al. 2014).

Light is thought to induce germination in plant species with small seeds (Pérez-García and Gonzales-Benito 2006, Probert 2000). Given the size, the seeds of *J. supina* can be counted as small seed mass with a weight of 48 µg $seed^{-1}$ (Jaganathan et al. 2015). However, our results revealed that *J. supina* seeds can germinate in dark conditions (Tab. 1, Fig. 2). Wu et al. (2013) found that there was a negative correlation between seed mass and light requirement in small-seeded alpine meadow species of the Qinghai-Tibetan Plateau. Moreover, Koutsovoulou et al. (2014) studied the germination of 131 species with small mass seeds from Campanulaceae, including *J. helreichii, J. montana* subsp. *montana* and *J. orbiculata,* finding that seeds require light for germination. They argued that plant species with small seeds can germinate only if found on the soil surface or at shallow depths. In other studies, the seeds of *J. montana* have also been found to require light for germination and do not have dormancy (Peco et al. 2006, Pegtel 1988). Some researchers have reported that requirements for darkness and light in seeds can play an important role in delaying germination and contribute to seed persistence (Fenner and Thompson 2005, Koutsovoulou et al. 2014). There have been only two studies reporting darkness germination in Campanulaceae. One was concerned with *Wahlenbergia stricta* (Willis and Groves 1991), whereas the other was focused on *Howellia aquatilis* (Lesica 1992). However, Koutsovoulou et al. (2014) hypothesised that Willis and Groves (1991) examined dark incubations under short periods of light, which was sufficient for seed light requirements.

MGT is a parameter that is used for monitoring the speed of germination. A shortened MGT may provide support for the establishment of seedlings at the beginning of the next vegetation period. In our study, under both dark and light/dark conditions, moist chilling and GA_3 combination reduced the MGT of the seeds. In this way, species can survive by maintaining themselves in accordance with the vegetation period in an alpine environment.

Generally, Campanulaceae members that have small seed mass are considered to have a light requirement for germination. However, *J. supina* with small seeds did not have a light requirement for germination (Fig. 2). This characteristic seems to be consistent with its habitat characteristics. It is spread among *Juniperus communis* dwarf shrub and *Festuca cyllenica*, *F. punctoria*, and *Acantholimon ulicinum* hard-cushion plant communities that provide shade to soil surfaces and dark environmental conditions to germinate. Moreover, the breaking of dormancy with long-term moist chilling in this taxon may be evidence that its seeds are germinated in the spring after staying under snow cover during the winter. Our results can be important for possible *ex situ* or *in situ* conservation efforts.

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