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Effects of moist chilling, gibberellic acid, and scarification on seed dormancy in the rare endemic *Pedicularis olympica* (Scrophulariaceae)

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Abstract: We investigated the germination requirements of a rare endemic plant species, *Pedicularis olympica* Boiss. (Scrophulariaceae), which grows in wet habitats on Uludağ Mountain, Turkey. We studied the effects of scarification, moist chilling (+4 °C) for 15 days, different doses of gibberellic acid (GA_3; 100, 150, and 250 ppm) and combined hormone and moist chilling treatments under dark (20 °C) and photoperiod (20/10 °C; 12/12 h, respectively) conditions. GA_3 was able to break dormancy. The highest germination rate was found in the seeds treated with 250 ppm GA_3; 64% of these seeds germinated when treated with moist chilling and incubated in the dark, while 75% germinated under photoperiod conditions. Moreover, mean germination times were significantly lower in the seeds treated with 250 ppm GA_3. Significant differences in final germination percentages and mean germination times were found only for treatments under photoperiod conditions. Scarification also permitted germination; the highest germination rate (78%) was found with 15 min of scarification. Dormancy was not broken by moist chilling for up to 90 days. The germination requirements of dormant *P. olympica* seeds are found to be consistent with characteristics of its habitat. This can be considered an ecological advantage for the species' establishment and persistence.

Key words: Seed dormancy, *Pedicularis olympica,* endemic, stratification, gibberellic acid, scarification

Nadir ve endemik *Pedicularis olympica* (Scrophulariaceae) türünde nemli soğuklama, gibberellik asit ve skarifikasyonun tohum dormansisi üzerindeki etkileri

Özet: Bu çalışmada Uludağ'da nemli alanlarda yayılış gösteren nadir endemik *Pedicularis olympica* Boiss. (Scrophulariaceae) türünün çimlenme gereksinimleri araştırılmıştır. Çalışmada, skarifikasyon, 15 gün nemli soğuklama (+4 °C) ve farklı dozlarda gibberellik asit (GA_3; 100, 150 ve 250 ppm) ile hormon ve nemli üşütme kombinasyonlarının karanlık (20 °C) ve fotoperiyot (sırasıyla 20/10 °C; 12/12 s) şartlarında etkileri araştırılmıştır. GA_3 uygulamalarının dormansiyi kırmada etkili olduğu saptanmıştır. En yüksek çimlenme oranı 250 ppm GA_3 ile muamele edilmiş tohumlarda bulunmuştur; karanlık şartlarda nemli üşütmede % 64 iken fotoperiyotta % 75 çimlenme gerçekleşmiştir. Ayrıca, 250 ppm GA_3 ile muamele edilen tohumlarda ortalama çimlenme süresi de anlamlı derecede kısalmıştır. Final çimlenme oranları

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ve ortalama çimlenme süreleri arasında anlamlı fark grupları sadece fotoperiyot şartları altında bulunmuştur. Skarifikasyon çimlenmeyi uyarmıştır ve en yüksek çimlenme 15 dakika skarifikasyon sonucunda bulunmuştur (% 78). 90 güne kadar yapılan nemli üşütme muamelelerinde dormansi kırılmamıştır. *P. olympica* türünün çimlenme gereksinimlerinin bulunduğu habitatla uyum içinde olduğu bulunmuştur. Bu da türün yerleşme ve devamlılığında bir ekolojik avantaj olarak değerlendirilebilir.

Anahtar sözcükler: Tohum dormansisi, *Pedicularis olympica*, endemik, soğuklama, gibberellik asit, skarifikasyon

Introduction

Seedling establishment is a critical stage in the life history of any plant species that relies on sexual reproduction for the persistence of its populations (Grubb, 1977; Harper, 1979; Bu et al., 2008). Variations in seed dispersal efficacy or germination percentage are often interpreted as reflecting adaptations to specific ecological conditions (Grime et al., 1981; Nishitani & Masuzawa, 1996). A large number of endemic species are found in alpine habitats (Väre et al., 2003), and seeds of such species must possess elaborate dormancy mechanisms to survive the harsh climatic conditions that ensue immediately after their seed maturation and dispersal (Billings & Mooney, 1968). Previous studies on alpine endemic species suggest that no universal alpine germination strategy exists (Gimenez-Benavides et al., 2005), but these species generally exhibit innate or enforced dormancy that prevents germination during the unfavourable conditions of winter (Körner, 1999).

Dormancy can be defined in multiple ways. Seed dormancy can be defined simply as inhibited germination of an intact viable seed to optimise the distribution of germination over time (Bewley & Black, 1983; Hilhorst, 1995). This inhibition of germination has evolved differently across species for adaptation to the prevailing environment so that germination occurs when conditions are likely to be suitable for establishment of a new generation (Finch-Savage & Leubner-Metzger, 2006). Baskin and Baskin (1998) have reported that among 300 temperate herbaceous species, dormancy breaking and germination requirements are not phylogenetically constrained. According to the classification system proposed by Baskin and Baskin (1998, 2004), physiological dormancy is the most common form, found in seeds of gymnosperms and of all angiosperm clades. Physiological dormancy is the most prevalent form of dormancy in temperate seed banks. It may be deep, intermediate, or non-deep (Finch-Savage & Leubner-Metzger, 2006). Dormancy may be strong to weak, and the extent of dormancy present at any particular moment is referred to as the degree of dormancy. Dormancy patterns are similar for closely related taxa but may differ within a family, even between co-occurring species with similar life histories (Karlsson et al., 2006). The germination requirements may represent extreme conditions that do not normally exist, thus inhibiting complete germination.

Soil moisture and soil temperature are the most important factors regulating seed behaviour under natural conditions (Bewley & Black, 1982). In some wetland species, germination is not limited by secondary dormancy; once seeds have come out of primary dormancy, germination can occur at any time during the growing season if conditions are favourable (Baskin & Baskin, 1993a, 1993b; Milberg, 1994; Jensen, 2004). It has been shown that seed germination in wet meadows is negatively affected by the thickness of the litter layer (Facelli & Pickett, 1991; Diemer et al., 2001).

Wetland species require high temperatures for germination and respond to large daily temperature fluctuations (Grime et al., 1981). These characteristics are regarded as adaptations to delay germination until the water table recedes and the soil surface becomes exposed in the spring, thus creating oxygenated conditions with warmer soil temperatures and larger temperature fluctuations (Grime et al., 1981; Thompson & Grime, 1983). Many studies have examined the influence of various treatments, including gibberellins (Li et al, 2007; Ren & Guan, 2008) and stratification (Schütz & Rave, 1999; Brändel & Jensen, 2005; Brändel, 2006; Kettenring & Galatowisch, 2007) on seed germination in wetlands.

These studies have pointed out the important role of dormancy in the reproductive success of wetland plants.

Germination requirements for native species, particularly for rare and/or endemic species, are important in conservation biology (Cerabolini et al., 2004). Investigation of germination requirements may show how a species' germination process is adapted to habitat conditions, how it is regulated by environmental factors (Van Assche et al., 2002), and how it influences subsequent seedling establishment in a particular habitat (Schütz & Rave, 1999). *Pedicularis olympica* Boiss. (Scrophulariaceae) is an endemic species from Uludağ Mountain, Turkey. The species' habitat has been damaged by heavy recreational and winter sports activity. The status of the species is Vulnerable (VU) according to the IUCN classification (Güleryüz, 1998). The germination requirements of the species were not previously studied. In this study, we evaluated the germination requirements of *P. olympica* by testing its responses to GA_3, moist chilling, and scarification treatments in relation to the breaking of dormancy.

Materials and methods

Study area

Uludağ is the highest mountain in the Marmara region, including Thrace and the north-western side of the Anatolian peninsula. The climate of the mountain varies with elevation, resulting in high biological diversity. Because of its high plant diversity, Uludağ Mountain is one of the important plant areas (IPAs) in Turkey (Güleryüz et al., 2005). To preserve the unique plant communities and various geomorphological features, an area of 11,338 ha was designated as a National Park in 1961. This area was enlarged to 12,762 ha in 1998 (Arslan et al., 1999). A Mediterranean-type climate is found at lower elevations, near the city of Bursa on the north-western side of the mountain. Rainy, partially mild, micro-thermic climates with icy winters are found at higher altitudes (Güleryüz, 1992).

Study species and habitat characteristics

Pedicularis olympica (Scrophulariaceae) is a perennial herb with erect, unbranched, eglandular-pubescent, or almost glabrous stems. Its leaves are linear-oblong, simply pinnatisect or pinnatifid, and crenate-serrate. The basal leaves are petiolate; the cauline leaves are several and alternate. The inflorescence is a many-flowered, oblong, sublanate spike that elongates in fruit. In general, seeds of *Pedicularis* species are very small, containing a minimal embryo with a linear shape, and are protected by a thin brown-coloured seed coat. The seeds are laterally compressed and elliptic in outline. The seed surface is reticulate, and the embryo is linear and axile (Ellis, 1985).

P. olympica is known only from Uludağ Mountain, where it is found in wet habitats and on stream banks, especially near springs. The species grows on granite substrates. It is one of the first species to flower in spring after snow melts in the subalpine zone, forming an attractive landscape of rose-red flowers in meadows. The flowering period is from May to June (Davis, 1975; Güleryüz, 1998).

Germination tests

Mature seeds of *P. olympica* were collected from the subalpine belt of Uludağ Mountain between 1800 and 1850 m in elevation during June 2008. Germination experiments were started immediately after drying and cleaning the seeds. Sterile plastic 9-mm petri dishes were used for germination experiments. Seeds were surface-sterilised for 3 min with 5% sodium hypochlorite and then rinsed with tap water. Some preliminary treatments were made to determine the moist chilling duration and GA_3 doses. These were 30, 60, and 90 days of moist chilling with 250, 500, and 1000 ppm GA_3, and control without GA_3 (distilled water). After the preliminary treatments the test solutions were chosen as 100, 150, and 250 ppm GA_3 and distilled water as a control. All of the hormone solutions were analytical grade. The hormone solutions were applied as a pre-treatment for 24 h of imbibition, after which the seeds were rinsed with distilled water. Four replicates of 25 seeds per petri dish were made. Half of the replicates were incubated under photoperiod conditions: 20/10 °C (12/12 h). The other half of the replicates were incubated at 20 °C in the dark. For dark treatments, the petri dishes were wrapped with aluminium foil. Moist chilling was achieved by incubating seeds under wet and cold (+4 °C) conditions for 15 days. The number of germinated seeds was counted and the germinated seeds were removed every day for up to 25 days. Seeds were recorded as having germinated when the radicle emerged from the testa. Mean germination time (MGT) was calculated from the germination counts and used to determine the speed of germination. Final germination percentages and mean germination times were determined at the end of the 25-day incubation period. For scarification, seeds were treated with 80% sulphuric acid and then rinsed with tap water.

Statistical Analyses

Data were analysed using one-way analysis of variance (ANOVA). Differences among treatment means were tested by Tukey's honest significance difference (HSD) test. All tests were performed at the significance level of $\alpha = 0.05$ using the Statistica 6.0 (StatSoft, 1984-1995) software package.

Results and discussion

Results for pre-treatments with GA_3 and moist chilling for seeds of *P. olympica* under photoperiod conditions are given in Table 1. Seeds failed to germinate in control group, in which neither GA_3 nor moist chilling was applied. Seeds also failed to germinate in control moist chilling treatments with distilled water, except after 60 and 90 days of chilling, which produced 8.3% and 7.8% germination, respectively (Table 1). The highest germination rate after 21 days of moist chilling was found in seeds treated with 250 ppm GA_3 (93.2%). However, germination rate decreased with increasing GA_3 above 250 ppm. Ren and Guan (2008) have found similar results for different *Pedicularis* species; longer durations of stratification with GA_3 lowered germination. Mean germination times decreased in moist-chilled compared to non-chilled ones. After the preliminary treatments, GA_3 doses of 100, 150, and 250 ppm and combinations of GA_3 with 15 days of moist chilling were chosen as treatments for the main experiment.

Seeds failed to germinate in control treatments with distilled water under both dark and photoperiod conditions (Figure). There was a clear difference among GA_3 doses of 100, 150, and 250 ppm. Germination increased with increasing GA_3 dose under both dark and photoperiod conditions. The highest germination rates were found in the seeds treated with 250 ppm GA_3: 64% for dark conditions with moist chilling and 75% for photoperiod conditions (Figure). Final germination percentages and MGTs are presented in Table 2. Significant differences in the final germination percentages and MGTs were found only for treatments under photoperiod conditions ($P < 0.005$).

Table 1. Preliminary treatments results for determination of GA_3 doses and duration of moist chilling on seeds of *Pedicularis olympica* (Mean ± Standard Error, nd: not determined).

Moist Chilling Duration (days)	GA_3 concentration (ppm)			
	0	250	500	1000
Final Germination (%)				
0 (control)	0 ± 0	52.0 ± 6.7	96.8 ± 1.8	97.6 ± 3.3
21	0 ± 0	93.2 ± 1.8	88.4 ± 3.8	87.2 ± 7.8
30	0 ± 0	nd	nd	nd
60	8.8 ± 5.6	nd	nd	nd
90	7.3 ± 1.9	nd	nd	nd
Mean germination time (days)				
0 (control)	0 ± 0	16.0 ± 0.8	7.4 ± 0.8	7.2 ± 0.1
21	0 ± 0	4.4 ± 0.3	2.5 ± 0.8	2.3 ± 0.4
30	0 ± 0	nd	nd	nd
60	9.4 ± 0.5	nd	nd	nd
90	7.4 ± 2.1	nd	nd	nd

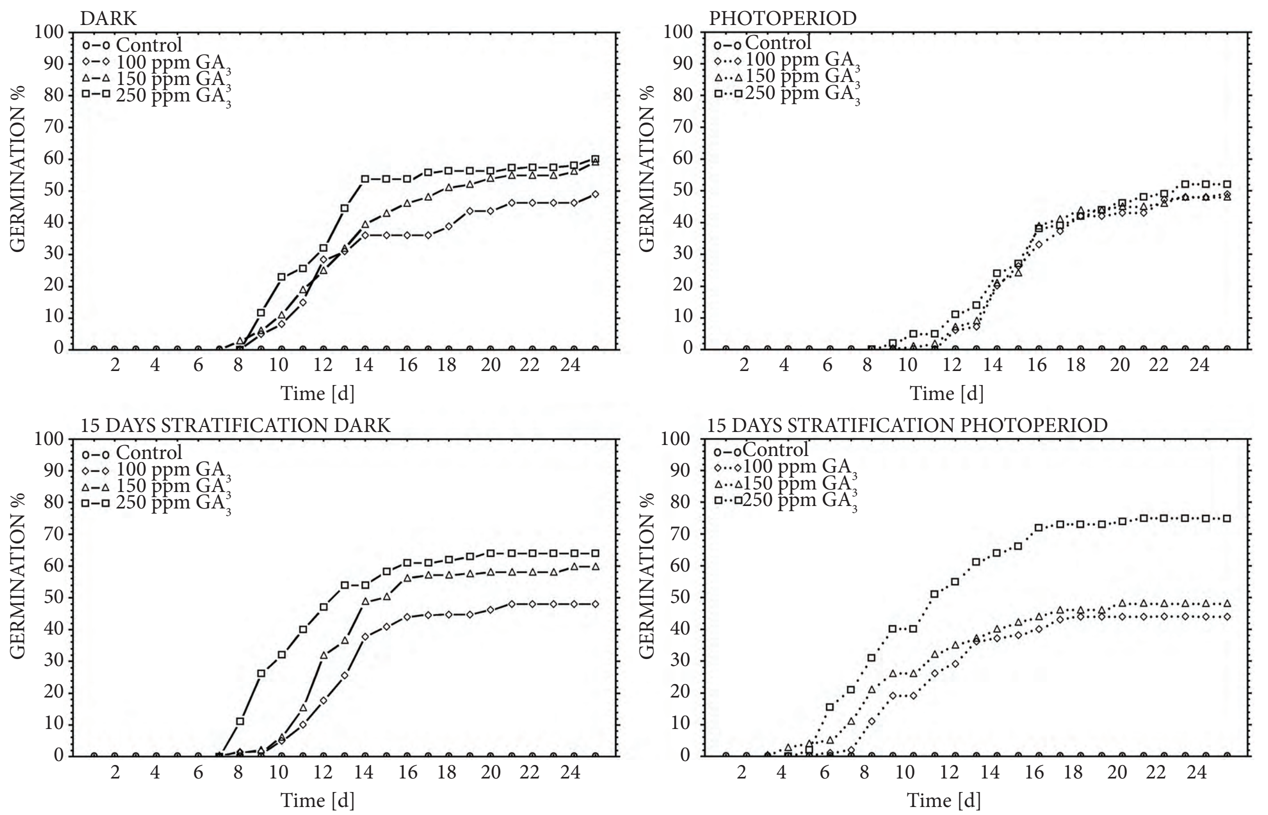

Figure. Cumulative germination percentage diagrams for *Pedicularis olympica* seeds in 4 different treatments under dark (20 °C) and photoperiod (20/10 °C; 12/12 h, respectively) conditions.

Scarification with sulphuric acid promoted germination (Table 2). Since the scarification with sand paper would be destructive for small seeds, we treated the seeds with 80% sulphuric acid for 5, 10, or 15 min. The highest germination rate was found with 15 min of scarification (78%). Scarification was also found to be the most effective method for promoting germination in 7 of 8 *Pedicularis* species from Yunnan, China (Li et al., 2007). The authors of that study reported that the germination behaviours of the 8 *Pedicularis* species varied considerably. It is thought that wetland species usually require light for germination (Grime et al., 1981). However, our results suggest that *P. olympica* does not require light for germination. This may be due to its habitat; *P. olympica* prefers to grow near stream banks, especially near springs.

According to the results found in this study, *P. olympica* species probably exhibits a combination of physical and physiological dormancy (PY+PD). GA_3 was able to break dormancy; scarification also permitted germination.

The germination rate resulting from 15 min of scarification was similar to the results of 250 ppm GA_3 with moist chilling and photoperiod germination conditions. While MGT was 16 days for seeds treated with 250 ppm GA_3 and moist chilling that were germinated under photoperiod conditions, it decreased to 8 days with scarification. *Pedicularis* species do not have thick seed coats, but endosperm can act as a mechanical inhibitor of seed germination in several angiosperms (Finch-Savage & Leubner-Metzger, 2006). A decline in the mechanical resistance of the micropylar endosperm may be a prerequisite for radicle protrusion during seed germination (Hilhorst, 1995; Leubner-Metzger, 2003; Kucera et al., 2005). The weakening of endosperm can be promoted by gibberellic acid and is inhibited (at least in part) by

Table 2. Final germination (%) and mean germination times (MGT; days) of *P. olympica* seeds for different treatment series. [Values are means followed by standard error (n = 4); the values followed by the same letter were not statistically significant at the α = 0.05 level].

Treatment Series			Germination (%)	MGT
Dark (20 °C)		Control	0	0
		100 ppm GA_3	$49^{a} \pm 3.5$	$14.2^{a} \pm 1.0$
		150 ppm GA_3	$59^{a} \pm 3.0$	$14.0^{a} \pm 0.2$
		250 ppm GA_3	$60^{a} \pm 4.9$	$12.7^{a} \pm 0.9$
	Stratification 15 days (+4 °C)	Control	0	0
		100 ppm GA_3	$48^{a} \pm 8.6$	$13.7^{a} \pm 0.5$
		150 ppm GA_3	$60^{a} \pm 4.8$	$14.2^{a} \pm 0.8$
		250 ppm GA_3	$64^{a} \pm 3.3$	$12.9^{a} \pm 0.9$
Fotoperiod (20/10 °C; 12/12 h)		Control	0	0
		100 ppm GA_3	$49^{a} \pm 2.5$	$16.0^{a} \pm 0.3$
		150 ppm GA_3	$48^{a} \pm 2.8$	$15.4^{ab} \pm 0.5$
		250 ppm GA_3	$52^{a} \pm 4.7$	$16.2^{a} \pm 0.4$
	Stratification 15 days (+4 °C)	Control	0	0
		100 ppm GA_3	$44^{b} \pm 8.2$	$9.4^{bc} \pm 1.8$
		150 ppm GA_3	$48^{b} \pm 9.2$	$11.9^{b} \pm 7.7$
		250 ppm GA_3	$75^{a} \pm 4.1$	$10.6^{bc} \pm 0.7$
Scarification (80% H_2SO_4)		5 min	$26^{c} \pm 2.6$	$2.3^{b} \pm 0.1$
		10 min	$47^{b} \pm 5.5$	$2.5^{b} \pm 0.1$
		15 min	$78^{a} \pm 2.5$	$3.4^{a\,\pm}\,0.2$

abscisic acid (ABA) (Finch-Savage & Leubner-Metzger, 2006). The germination of tomato is inhibited by ABA, but surgical removal of the micropylar cap permits germination even in the presence of ABA (Liptay & Schopfer, 1983). Endosperm weakening is also known to be required for radicle protrusion in Solanaceae species (Finch-Savage & Leubner-Metzger, 2006). GA_3 might enhance the growth potential of the embryo (Karssen & Lacka, 1986; Karssen et al., 1989) or induce degradation of food reserves in endosperm endosperm by stimulating hydrolytic enzyme activity (Da Silva et al., 2005) of *P. olympica* seeds. In nature, some of the ungerminated seeds may remain alive and serve as a soil seed bank, waiting for more favourable conditions. This strategy may be common among high-elevation wetland plants (Leck & Simpson, 1987; Kaye, 1997). Li et al. (2007) reported that GA_3, scarification and surface sowing promoted germination in the 8 *Pedicularis* species they studied and suggested that allowing sufficient time for germination may be useful for germination under natural conditions.

Alpine and subalpine plants provide good opportunities to determine differences between species in germination responses. Investigations of the germination ecology of alpine plants are rare; therefore, the factors and mechanisms that regulate germination in alpine habitats are poorly known (Baskin & Baskin, 1998). *P. olympica* seeds exhibit dormancy characteristics that are consistent with environmental conditions in its habitat. This can be considered an ecological advantage for the species' establishment and persistence.

The attractive rare endemic plant *Pedicularis olympica* is vulnerable to habitat degradation on Uludağ Mountain, its only known locality. It spreads in wet meadows within the subalpine belt of Uludağ Mountain and has very attractive rose red flowers that can be used for ornamental purposes. Conservation biologists must develop new strategies for protecting species and ecosystems because of habitat losses, landscape alteration, and extinction of species, communities and even ecosystems (Hamilton, 1994). Ex situ conservation from seed is regarded as a viable and inexpensive method for threatened plant species, but the germination requirements of alpine species are poorly known, with a few exceptions (Cerabolini et al., 2004; Gimenez-Benavides et al., 2005). The germination requirements found in our study will be useful for future ex situ conservation of *P. olympica*.

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