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Original Article

Chemical Applications, Scarification and Stratification Effects on Seed Germination of Rare Endemic *Verbascum calycosum* Hausskn. ex Murb. (Scrophulariaceae)

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Abstract

Verbascum calycosum is an endemic plant species having considerable narrow distribution in Erzincan (Turkey) region. This species is known from only a single population and its habitats are highly threatened due to intensive human activities and soil erosion. In this study, the germination behavior of *V. calycosum* under different concentrations of NaCl, HCl, KNO_3, GA_3 (100 and 200 µM), hot-cold stratification and mechanical scarification were investigated. Seeds were exposed to a photoperiod of 8 h light/16 h dark with a 23/18 °C thermoperiod. Germination rates increased with GA_3-100 µM (39%), GA_3-200 µM (54.5%), mechanical scarification (34.5%) and cold stratification treatments (+4 °C, 23.25%; -20 °C, 18.25%) on the other hand, KNO_3, NaCl, HCl and stratification with hot water treatments have decreased germination rates significantly when compared to the control (12.25%). Increased germination after GA_3 application and mechanical scarification indicated that seeds of *V. calycosum* exhibited both non-deep and intermediate physiological dormancy as well as physical dormancy due to its hard seed coat. The highest speed of germination index was obtained at cold stratification of +4 °C and -20 °C (10.3). This study represents first report about seed dormancy and germination characteristics of *V. calycosum*. Overall, these results will provide valuable data for *ex situ* conservation of this rare endemic plant.

Keywords: dormancy; endemic; *ex situ*; germination; Scrophulariaceae; *Verbascum*

Introduction

Verbascum L. (Scrophulariaceae) is the largest genus of Turkish flora. This genus is represented by 234 species and 85% endemism rate in Turkey (Erik and Tarıkahya, 2004). Endemic *Verbascum calycosum* Hausskn. ex Murb. is a rare plant species that shows narrow distribution around Erzincan province (Turkey). The species was first collected by Sintenis from Kemaliye / Erzincan district in 1890 (Davis, 1978). In Turkey, many endemic plant species considered as a source of genetic diversity are faced with the risk of extinction due to various reasons such as rapid urbanization, industrialization and other anthropological factors. According to the IUCN criteria, *V. calycosum* was evaluated as EX (Extinction) for many years (Ekim *et al.*, 2000). However, it has been rediscovered from its original localities in 1992 and 2004 (Nydegger-Hügli, 2002; Kandemir and Makbul, 2004; Aytaç *et al.*, 2005). *V. calycosum* is represented by a single population located in Kemaliye, Erzincan. Currently, habitats of this species are highly threatened due to intensive human activities and soil erosion (Kandemir and Makbul, 2004) and recently evaluated as CR (Critically endangered) (Kandemir *et al.*, 2015).

Seed germination is a trait of primary significance for the reproductive success of plants (Bu *et al.*, 2008). Thus, detailed information on the different stages in the reproductive cycle of endemic, rare and threatened species may contribute to improved understanding of the phenomenon of rarity, and at the same time assist conservation management decisions for the species under study (Menges, 1986; Schemske *et al.*, 1994). Seed germination is vital for the long-term preservation of germplasm, and the maintenance of genetic diversity for the conservation of threatened plant species, which has become a global concern over recent decades (Cousins *et al.*, 2014).

It is known that some pre-treatments such as sanding, boiling or cold stratification and chemical applications such as potassium nitrate, acid and gibberellic acid are used in germination studies to break down seed dormancy (Jones *et*

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al., 2016; Peng *et al*., 2017). The aim of this study was to determine the effect of different chemical applications (NaCl, HCl, KNO_3, GA_3) and some pre-treatments techniques (hot/cold and mechanical) on seed germination for the rare endemic *V. calycosum* plant bearing the risk of extinction. The results obtained from this study could provide basic information to develop effective *ex situ* conservation strategies for this rare and endemic plant.

Materials and Methods

Plant material and seed collection

V. calycosum is only known from type locality (around Salihli Village in Kemaliye District). Its population is separated by Erzincan-Kemaliye highway and members of the species are sparsely scattered (roughly 8250 individuals) on 15.99 km^2 serpentine area. Plant samples of *V. calycosum* were collected from Erzincan (37 453742 E, 4356278 N, altitude 1370-1488 m), Turkey (Fig. 1) between July and September 2014.

The collected plants were air dried for seven days in room conditions. The mature capsules were carefully harvested. After counting the total number of seeds per fruit, healthy and fully developed seeds were selected under stereomicroscope. The seed weight was determined according to Bonner (1974).

Seed germination experiment

Seed germination experiments were carried out on filter paper in Petri dishes (9 mm diameter, sterilized) at constant a photoperiod of 8 h light/16 h dark with a 23/18 °C thermoperiod. The Petri dishes containing seeds were subjected to different treatments namely:

- Chemical applications; containing sodium chloride (NaCl), hydrochloric acid (HCl), potassium nitrate (KNO_3), gibberellic acid (GA_3) solution at 100 µM and 200 µM concentrations.
- Mechanical scarification; sanding was gently made with sandpaper (grade 150) about 30 sec or 1 min.
- Cold stratification; the Petri dishes were wrapped with aluminium foil and placed at +4 °C and -20 °C for seven days before transferring to growth chambers.
- Hot stratification; soaking in hot-boiling water for 30 sec, 1 min and 2 min.
- Control treatment; distilled water was used to germinate seeds.

Each treatment was replicated 4 times with at least 25 seeds per replication (ISTA, 1985). Seed germination was recorded daily, the radical emergence of 2-5 mm accepted as a successful germination (Asl *et al*., 2011).

Data analysis

The germination percentage (GP) using the equation: "GP = number of germinated seeds /total number of seeds x 100" and its angular transformation (arcsine$\sqrt{GP}$) were calculated. Speed of germination (SG) was defined as the number of seeds germinated during a limited period according to the formula described by Yücel (2000):

$$\text{Germination Speed (GS)} = \sum (G \times 100)/\sum H,$$

where

G = Daily germination percent,

H (Daily germination speed) = G × A

(A = Days since sowing).

Time to 50% germination (T50) was calculated according to the formula of Coolbear *et al*. (1984) modified by Farooq *et al*. (2005):

$$T50 = ti\ (N/2-ni)(tj-ti)/nj-ni,$$

where

N is the final number of seeds germinated and nj, ni are the cumulative number of seeds germinated by adjacent counts at times tj, ti when $ni < N/2 < nj$.

The data were analyzed using the SPSS (Version 15.0, SPSS Inc. Chicago, IL) software and the means were compared with Duncan's Multiple Range Test (DMRT) ($P < 0.05$).

Results

Seed structure

The seeds of *V. calycosum* develop in capsules that are usually few in number. Among 20 capsules were randomly examined, one was empty and one contained undeveloped five seeds. In the rest 18 capsules, the number of seeds ranged from 2-37 per capsule. About 47.46% of the total seeds were found to be healthy. Seed size is about 1 mm by 1.3 mm in dimension, ovoid in shape and its outer surface is indented (Fig. 2). Brown-black testa is very hard and seems impervious to water and gases. The weight of 1000 healthy seeds was found as 203 mg.

Fig. 1. *Verbascum calycosum* in its habitat

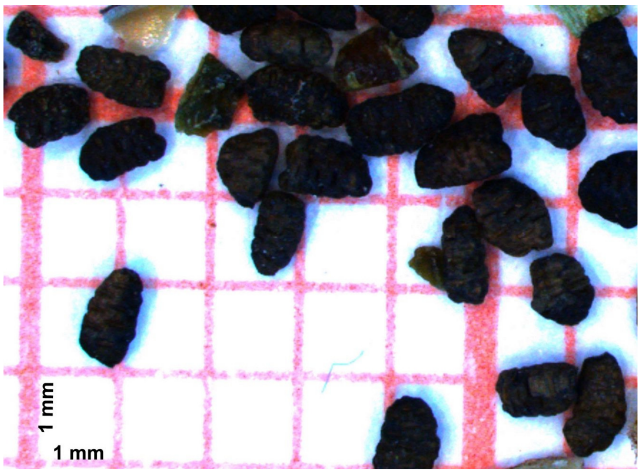

Fig. 2. Seeds of *Verbascum calycosum*

Table 1. Mean values of GP, SG, T50 for *Verbascum calycosum* under different treatments

Treatment	Concentration (µM)	GP (%)	AT	SG	T50 (Day)
Control	-	12.25^{e} (±1.1)	20.48	8.6^{b} (±1.4)	10^{b} (±0.5)
KNO_3	100	1^{f} (±0.2)	5.73	-	-
KNO_3	200	2^{f} (±0.2)	8.13	-	-
GA-3	100	39^{b} (±2.4)	38.64	7.6^{b} (±0.7)	12^{a} (±1.0)
GA-3	200	54.5^{a} (±2.9)	47.58	7.5^{b} (±0.9)	13^{a} (±0.8)
NaCl	100-200	-	-	-	-
HCl	100-200	-	-	-	-
Sandpaper	-	34.5^{c} (±1.8)	35.97	9.5^{ab} (±1.0)	11.5^{ab} (±0.9)
Hot water (30sec)	-	1.25^{f} (±0.3)	6.41	-	-
Hot water (1 min)	-	-	-	-	-
Hot water (2 min)	-	-	-	-	-
Stratification (+4°C)	-	23.25^{d} (±1.5)	28.82	10.3^{a} (±1.1)	8.5^{c} (±0.6)
Stratification (-20°C)	-	18.25^{d} (±1.4)	25.29	10.3^{a} (±0.9)	8.7^{c} (±0.7)

In each column, means with different letter(s) differ significantly according to DMRT at $p < 0.05$. GP: Seed germination percentage, AT: Angular transformation of GP, SG: Speed of germination, T50: Time to 50% germination (Standard deviation, in brackets)

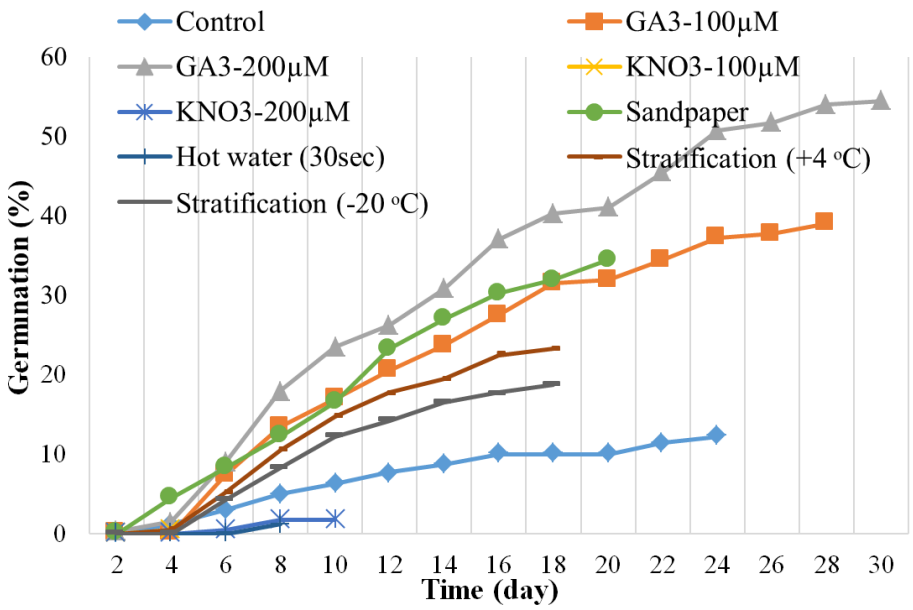

Fig. 3. Diagram of germination percentage and time for *V. calycosum* seeds under different treatments

In vitro seed germination

Different pre-treatments were used *in vitro* seed germination experiments. The effect of all pre-treatment of breaking dormancy was shown in Table 1.

Values of seed germination percentage speed of germination and time to 50% germination (T50) varied as 0-54.5%, 7.5-10.3 and 8.5-13, respectively. The influence of treatments on germination showed that GA_3 (39 and 54.5%), mechanical scarification with sandpaper (34.5%) and cold stratification 18.25% and 23.25% have increased germination percentage in *V. calycosum* seeds, while the KNO_3, NaCl, HCl and stratification with hot water treatments have significant decrease in germination percentage compared with control seeds (12.25%) according to Duncan test ($P < 0.05$). The speed of germination in the present study is low or insignificant in GA_3 when compared the control ($P < 0.05$). On the other hand, the SGs are higher than that of the control in scarification with sandpaper and cold stratification treatments ($P < 0.05$). The time to 50% germination (T50) increased (T50=11.5-13) in GA_3 and sandpaper treatments whereas decreased in cold stratification treatments (T50 = 8.5-8.7) when compared to control (Fig. 3).

Discussion

Seed dormancy is being incompletely understood, and therefore experimentation is important to choose treatments that improve germination (Zhou and Bao, 2010). Seed dormancy, dormancy breaking and germination responses in endemic species are generally unknown. This study describes the seed germination characteristics of endemic species *V. calycosum*. The highest germination percentages (54.5% and 39%) were obtained with different concentrations of GA_3 (100-200 µM), indicating that it was the most efficient treatment. Levels of

endogenous plant growth regulators such as GA_3 are believed to play a primary role in breaking dormancy (Zheng and Sun, 2009; Yücel and Yılmaz, 2009; Necajeva and Ievinsh, 2013) and endogenous GA_3 deficiency can be recovered with exogenous GA_3 application (Hilhorst and Karssen, 1992). In this study, exogenous application of gibberellic acid (GA3) at 100 and 200 µM concentrations increased germination speed of *V. calycosum* seeds significantly ($P < 0.05$). Dormancy break by GA_3 is typical in seeds with non-deep and intermediate physiological dormancy (Schwienbacher *et al*., 2011). GA_3 supplementation appears to be the most successful treatment for stimulating germination in *V. calycosum* and this may be related to its non-deep or intermediate physiological dormancy characteristics.

The second highest germination percentage (34.5%) was obtained with seeds mechanically scarified with sandpaper. Yildiztugay and Kucukoduk (2012) reported that scarification of lignified palisade cell layer of seeds with sandpapering triggers seed germination due to increase in water penetration.

Similarly, removing the cellular layer below the seed coat by mechanical scarification with sandpaper was reported to be the best method to overcome seed coat impermeability in many plant species (Sfairi *et al*., 2012; Asaadi *et al*., 2015). Therefore, our results indicated that seeds of *V. calycosum* possess physical dormancy due to the hard seed coat that can be broken down with mechanical scarification to get successful germination.

In our study, germination percentage was affected positively by cold stratifications (both in +4 °C and -20 °C for 7 days before starting the treatments), when compared with control groups. Also, highest speed of germination and shortened time for 50% germination ($P < 0.05$) were observed by cold stratifications (+4 °C and -20 °C). Likewise, RBG Kew (2008) reported that high levels of seed germination after cold stratification at 2 °C were observed for *Verbascum thapu*s L. and *V. nigrum* L. In our results, there were no significant differences between 4 °C and -20 °C in terms of GP, SG and T50 ($p < 0.05$).

The germination percentages decreased to 2%, 1%, and 0% after KNO_3, NaCl, HCl applications and hot stratification treatments, respectively. These results implied negative effect of salinity and acidity on seed germination in *V. calycosum*. It has been known that increased salinity levels affect water uptake by seed negatively, thereby inhibiting germination (Werner and Finkelstein, 1995). NaCl also was proposed as an inhibitory factor for the activities of some enzymes that may play crucial roles in seed germination (Katembe *et al*., 1998). On the other hand, different concentrations of HCl were applied to stimulate seed germination to soften thick seed coats directly or indirectly by stimulating fungi development on seed coats (Vleeshouwers *et al*., 1995).

However, in our study no germination with HCl applications was observed. These results may imply that this species is salt sensitive and cannot develop at lower pH. Indeed, *V. calycosum* grows on saltless (EC: 0,251 mS/cm) and slightly alkaline soil (Hilooğlu *et al*., 2016). A negative correlation between both salinity and acidity with germination was also reported in endemic *Hymenocrater platystegius* Recf.f (Teimouri and Mahallati, 2013). Hot-water treatment has been shown to increase germination by affecting factors such as seed coat permeability (Longer and Degago, 1996) and gaseous exchange (Corbineau *et al*., 1990). Furthermore, boiling seeds in water removes the cuticle and sometimes part of the pallisade layers of the seedcoat and can effectively break dormancy (FAO, 1983). It was also successfully used in seed germination of several endemic plant species (Luna *et al*., 2007; Choudhury *et al*., 2009). In our study, hot water treatment significantly decreased germination after 30 sec incubation, and no germination observed after 1 min and 2 min incubation, possibly due to embryo damage. In addition, similar to our hot stratification results, Luna *et al*. (2007) reported no germination in Mediterranean endemic *Verbascum rotundifolium* Ten. seeds after heat-shock treatments at 80 °C, 100 °C and 120 °C.

Conclusions

In conclusion, the results of our experiments showed that giberellic acid, scarification with sandpaper and cold stratification pre-treatments increased germination rates in the rare endemic *Verbascum calycosum*. Breaking dormancy is known as a critical step to develop effective *ex situ* conservation strategies for rare and endemic plants. Therefore, the data obtained from this study will provide basic information to establish effective, low cost and quick *ex situ* conservation management for *V. calycosum*.

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