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Original Contribution

SEED GERMINATION AND DORMANCY BREAKING IN *DOREMA AMMONIACUM* D., AN ENDANGERED MEDICINAL PLANT

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ABSTRACT

Dorema ammoniacum D. Don. (Apiaceae) which is native to Iran is classified as a vulnerable medicinal species. The aim of this study was to enhance the germination of *Dorema ammoniacum* seeds, which have a low percentage under normal laboratory conditions. Different pretreatments were carried out to obtain efficacy in breaking dormancy and speeding germination, using of scarification, cold stratification, Washing and chilling treatments, gibberellic acid (GA_3) and N6-benzyladenine (BA) treatments. The rate and percentage of seed germination varied with duration and medium of stratification. The highest germination percentage was obtained in the moist sand cold stratification for 45 days (68.00 ± 1.7). Both germination rate and percentage of *Dorema ammoniacum* increased by increasing the number of exposure days at 4 ˚C. However, when the chilling temperature was decreased from 4 to -5 and -10 ˚C, a negative response was monitored for both germination rate and percentage. Addition of GA did not promote germination. Regardless of applied treatment to seeds, germination rate was positively correlated with germination percentage. Therefore, fast germination was associated with high germination percentage. For the first time, these results indicated that *Dorema ammoniacum* seeds which have deep morphophysiological dormancy need a chilling treatment for reliable germination.

Key words: germination rate; endangered medicinal plant; cold stratification; *Dorema ammoniacum.*

INTRODUCTION

Dorema ammoniacum D. belongs to the family Apiaceae, and grows to 1-2 m in height. It is one of the important medicinal plants endemic in many arid and semi-arid regions of Central Asia including Iran, Afghanistan and Pakistan altitudes of 900-2500 m (1). The best growth of this species is reported in arid regions with an average annual precipitation of 140-170 mm and a thermal range of -5 ºC to 38 ºC. A medicinal gum resin commonly known as Ammoniacum or gum ammoniac is found in cavities in stems, roots, and petioles (2-3). The gum resin commercially produces in two forms of tear ammoniacum and lumps or block ammoniacum. The resin serves as a carminative, diaphoretic, mildly diuretic, expectorant, poultice, stimulant, Antimicrobial, and vasodilator (4-2). It is still used in Indian and Western Medicine and is listed in the British pharmacopoeia as an antispasmodic and expectorant. It is occasionally used for chronic bronchitis and persistent coughs (3-1).

Large amounts of *Dorema ammoniacum* gum resins secreted by incising of the root for medical demands (2). The genetic diversity of *D. ammoniacum* in the wild populations is getting endangered at an alarming rate because of ruinous harvesting practices and over-

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harvesting for production of medicines, with little or no regard to the future. Thus, high trade has threatened its natural populations. Further, the methods of extraction employed are almost invariably crude and unsystematic (1). Consequently, *D. ammoniacum* is recorded as a vulnerable species in the Red Data Book of Iran (5). On the other hand, this species readily germinate within the native environment, but fail to show good germination under laboratory conditions or when cultivation is attempted. Therefore, there is an urgent need to perform a conservation strategy for taking advantages of this plant. Currently, *Dorema ammoniacum* can only be propagated from seeds but is hampered by the seed dormancy.

It seems there is any information concerning the capacity of seed dormancy problems for *Dorema ammoniacum*. Therefore, all test conditions were based on the phenology of embryo growth and seedling appearance in natural conditions. Several endogenous factors can determine seed dormancy and, as a result, the systems capable of removing it are different (6). Stratification (moist-prechilling) has been one of the methods customarily used to break seed dormancy (7-8). It is well known that environmental conditions are changed along altitudinal gradients (9). One of the most obvious changes is the increasing duration of snow cover with elevation. Meyer and Mosen (1991) suggest that populations normally exposing long periods with snow cover and hard winter conditions would require longer periods of cold stratification for germination than those encountered to milder winters (10). On the other hand, Representatives of many plant families typically have seeds containing an underdeveloped embryo at the time of dispersal. Seeds are termed morphophysiologically dormant if an additional physiological mechanism, inhibiting germination, has to be overcome. Deep complex morphophysiologically dormancy has previously been observed in a number of other Apiaceae species (11, 12-13).

The role of plant growth regulators, especially gibberellins and N6-benzyladenine (BA) pretreatment on seeds, in the breaking of seed dormancy has been studied extensively (14). On the other hand, for some species, treated gibberellic acid did not promote germination (15). Seed coats have been shown to enforce dormancy because they may limit the supply of oxygen to the seed or may contain growth inhibitors or may prevent the removing of inhibitors from the embryo (14).

So far, there are no reports on an efficient culture system for seed germination of this species. This work was, for the first time, undertaken to study the effect of different pretreatments on the seed germination and dormancy breaking in *D. ammoniacum*.

MATERIALS AND METHODS

1. Seed materials

The mature seeds of *Dorema ammoniacum* were received from research station of Fozveh in July 2010 (Lat: 361 270 N; Lon: 591 630 E). Seeds have been collected from altitude ranges between 900 and 1400 m in which the climate is typically arid, with an annual mean temperature of 11.5 ℃ and an annual total rainfall of 400–600 mm. Seeds were dried in sunlight and then sifted to obtain desirable seeds and kept into a paper bags in the laboratory until used.

The seeds were treated with 70% ethyl alcohol for 1 min, and then rinsed with sterile water for three times. The seeds were then surface sterilized in 2.5% sodium hypochlorite solution for 15 min and was followed by three washes in succession with sterile water prior to applying any treatment.

2. Treatments and experimental design

Prior to germination test process, the seeds were subjected to chemical scarification, as well as different concentrations of GA_3 and BA and varied stratification medium treatments.

2.1. Chemical scarification: the seeds were soaked in H_2SO_4 (50% v/v) for 2 and 3 min and then washed thoroughly by distilled water, before germination tests.
2.2. Cold stratification: Surface-sterilized seeds were transferred to sealed glass jar containing moistened sand with 10 ml distillated water in one treatment and also transferred to Petri dishes containing treble layered filter papers moistened with 5 ml of double sterilized water in another treatment. All containers were sealed with Parafilm to prevent desiccating of the seeds. These containers were placed in a laboratory refrigerator set at 4 ˚C for 15, 30 and 45 days.
2.3. Washing and chilling: Seeds were washed every day thoroughly in running water and kept at -5 ˚C and -10 ˚C for periods of 15 and 30 days.

2.4. Exogenous GA_3 treatments: seeds were soaked in, 500 and 1000 ppm GA_3 for 48 and 72 h.
2.5. Effect of BA treatments: Seeds were soaked in 10 and 20 mg/l BA for 24 h.

Due to seed limitation, nineteen treatments were designed in total and 25 seeds were used for each treatment with four replicates. After any treatment, seeds were placed on 15 cm sterilized Petri dishes containing double layered Wathman No.1 filter papers moistened with 10 ml of double sterilized water and incubated at 24 ℃ with a 16/8 h (light/dark) photoperiod in a growth room fitted with a light intensity of 6000 lx provided by cool white fluorescent lamps. Germinated seeds and rotted seeds were counted every other day and removed for 60 days. Seeds were regarded as germinated when the radicle was at least equal to half of the seed length (6). A batch of untreated seeds was used as control.

3. Germination and data analysis
The germination percentage was calculated according to the following formula:

$$\text{Germination percentage} = \frac{\text{number of germinating seeds}}{\text{number of viable seeds initiated}} \times 100$$

The germination rate was calculated as follows (16):

$$\text{Germination rate} = \sum_{n-1}^{60} \frac{Gt}{Dt}$$

Where Gt is the number of germinated seeds after t days (Dt).

The results were arcsine transformed and statistically analyzed by a one-way analysis of variance (ANOVA) using SAS version 9.1 (CoHort Software). The data were analyzed using a Completely Randomized Design (CRD) and LSD test was used to detect significant differences among the treatments with a probability of 95% ($P< 0.05$).

RESULT AND DISCUSSION
The relative effectiveness of different physico-chemical and plant growth regulator treatments in causing dormancy removal and germination improvement is summarized in the Table 1. In general, *D. ammoniacum* seed germination was low in all the treatments, although chilling had a very positive effect on germination percentage and rate.

Our results indicated that none of the seeds germinated after 2 and 3 min of scarification, indicating that no mechanical or inhibitory constraint was posed by the covering structure **(Table 1).** It did not exert any beneficial effect upon germination of seeds. Because, the acid damaged any seed, since no swollen seed was observed. This might be because the H_2SO_4 has a very strong causticity and then damage to embryo. Thus, acid should not be used to treat the scarified dormant *D.ammoniacoum* seeds.

Seed germination was significantly different among cold stratification treatments. As a whole, cold stratification treatments obviously increased germination in different stratification periods, but the difference was greatest when seeds were arranged to the moist sand media. This response also was dependent on the duration of cold stratification treatment. At lower duration, germination was lower. Germination percentage was significantly different between cold stratification within sand and Petri dishes treatments. In the Petri dishes, germination reached from 14.25 ± 0.8 % after 15 days to 31.50 ± 1.1 % after 30 days, and then changed little with additional time (32.00 ± 1.4% after 45 days). In contrast, when seeds were placed in moist sand, the germination percentage rose to 53.00 ± 1.8 % with 15 days of stratification, and 67.75 ± 1.4 % with 30 days of stratification (Table 1). Germination rate also varied with different stratification medium. Seeds stratified in the medium with moist sand tended to germinate sooner than seeds stratified in the medium with water. At moist sand for 15, 30 and 45 days, germination rate were 0.16 ± 0.01, 1.00 ± 0.02 and 0.90 ± 0.19, and it was 0.13 ± 0.01, 0.30 ± 0.07 and 0.27 ± 0.01at Petri dishes, respectively **(Table 1).**

Table 1. *Germination percentage and germination rate in Dorema ammoniacum under different treatments.*

Dormancy breaking treatments	Germination (%)	Germination rate (seeds per day)
control	0.00 ± 0.0^{h}	0.00 ± 0.00^{g}
H_2SO_4 (50%) 2min	0.00 ± 0.0^{h}	0.00 ± 0.00^{g}
H_2SO_4 (50%) 3min	0.00 ± 0.0^{h}	0.00 ± 0.00^{g}
cold stratification in petri dishes (15 d)	14.25 ± 0.8^{d}	0.13 ± 0.01^{de}
cold stratification in petri dishes (30 d)	31.50 ± 1.1^{c}	0.30 ± 0.07^{c}
cold stratification in petri dishes (45 d)	32.00 ± 1.4^{c}	0.27 ± 0.01^{c}
cold stratification within moist sand (15 d)	53.00 ± 1.8^{b}	0.16 ± 0.01^{d}
cold stratification within moist sand (30 d)	67.75 ± 1.4^{a}	1.00 ± 0.02^{a}
cold stratification within moist sand (45 d)	68.00 ± 1.7^{a}	0.90 ± 0.19^{b}
Washing and chilling (15 days, -5 ?C)	11.5 ± 0.5^{e}	$0.10 \pm 0.03^{d-f}$
Washing and chilling (30 days, -5 ?C)	13.00 ± 0.7^{d}	$0.10 \pm 0.01^{d-f}$
Washing and chilling (15 days, -10 ?C)	8.75 ± 0.4^{f}	0.04 ± 0.02^{fg}
Washing and chilling (30 days, -10 ?C)	11.13 ± 0.7^{e}	0.05 ± 0.00^{fg}
GA_3 500 ppm (48 h)	0.00 ± 0.0^{h}	0.00 ± 0.00^{g}
GA_3 1000 ppm (48 h)	0.00 ± 0.0^{h}	0.00 ± 0.00^{g}
GA_3 500 ppm (72h)	0.00 ± 0.0^{h}	0.00 ± 0.00^{g}
GA_3 1000 ppm (72 h)	0.00 ± 0.0^{h}	0.00 ± 0.00^{g}
BA 10 mg/l (24h)	6.25 ± 0.4^{g}	$0.06 \pm 0.01^{e-g}$
BA 20 mg/l (24h)	7.25 ± 0.4^{g}	$0.07 \pm 0.01^{e-g}$

Means within a column followed by the same letter are not significantly different by LSD test.

The seeds of *D. ammoniacum* maybe require a period of after-ripening to aid in overcoming embryo dormancy, when stratified at -10-4 ˚C, they can complete the process of after-ripening. Stratification at 4 ˚C was more effective than at -5 or -10˚C to induce germination of *D .ammoniacum* since at 4 ˚C germination was generally faster and finished sooner. Moist chilling has successfully diminished endogenous dormancy for various dormant seeds (6). These results agree with those obtained for *Cyclocarya paliurus* (6) and *Ferula gummosa* (17). In general, chilling for 60 days is well enough to remove the embryo dormancy in many plants (6). The current study also indicated that stratification at 4 ˚C for 45 days yielded fastest germination of all stratification treatments in *D.ammoniacum*.

Seed germination patterns should vary depending on the environmental characteristics. Geophytes native to temperate zone often have dormancy, which require exposure to low temperature to break dormancy (18). In Iran, this herb shows a wide

latitudinal range. Its habitats locate at altitudes of 900-2500 m. The best growth of this species is reported in arid regions with an average annual precipitation of 140-170 mm and a thermal range of -5 ºC to 38 ºC (2).Seed germination under a range of environmental conditions is essential for plant species inhabiting wide altitudinal ranges and this combination of conditions is powerful for detecting selective effects on seed germination patterns. The stratification periods necessary for germination has also been found to increase with elevation of seed source in other plant species growing along altitudinal gradients. Hence, this may be a typical pattern for species with wide altitudinal distribution, and an adaptation for the increase of duration of snow cover with elevation (9). Our results support this conclusion and also show that the effects of stratification are temperature and duration dependent. Also, the embryos of this species are underdeveloped at maturity and must elongate before radicle emergence can occur (morphological dormancy). Moreover, cold temperatures are required to overcome the physiological block present in the embryos of seeds when freshly ripe (13). Thus, the low temperature requirement for germination in seeds, indicate that seeds of *D. ammoniacum* have a deep complex type of morphophysiological dormancy.

Washing and chilling treatments partially removed dormancy in *D. ammoniacum* (Table 1). Our results indicated that the mean germination of seeds stratified in -10 ˚C was increased to 8.75 ± 0.4 % and 11.13 ± 0.7% after 15 and 30 days of Washing and chilling treatments, respectively. However, the mean germination of -5 ˚C was significantly increased to 11.5 ± 0.5 % and 13.00 ± 0.7 % after 15 and 30 days of Washing and chilling treatments, respectively. These results show that although Washing and chilling treatments significantly increased germination, these treatments were not as effective as moist sand-chilling (stratified in the medium with moist sand). The germination speed improved as well as germination percentage, but there was no significant difference ($p<0.05$) among Washing and chilling durations. The germination rate ranging from 0.04 ± 0.02 and 0.05 ± 0.00 (at -10 ˚C for 15 and 30days, respectively) to 0.10 ± 0.01(at -5 ˚C for15 and 30 days) seeds per day. The response to the washing and chilling treatments is in accordance with results by Nadjafi et al (2006), who found that, in the natural habitats of *F. gummosa*, higher seed germination percentage occurred in colder regions with higher precipitation (17).

In seeds of *D. ammoniacum*, GA_3 did not substitute for cold stratification when seeds were incubated at 24 ˚C for 60 days (Table 1). Therefore, it can be concluded that seeds of *D. ammoniacum* have deep morphophysiological dormancy. This type of dormancy is also very common among other temperate Apiaceae species that germinate in late winter or spring (13-12). Classification of the type of morphophysiological dormancy first requires an understanding of environmental conditions necessary for overcoming morphological dormancy: seeds with simple types of morphophysiological dormancy require high temperatures (≥15°C) for embryo growth, and those with complex types of morphophysiological dormancy need low temperatures (0–10°C) (12). Because embryo growth in *D. ammoniacum* seeds occurred at low temperatures, they must have one of the three types of complex morphophysiological dormancy. Division of complex morphophysiological dormancy depends on the temperature regime and the effects of GA_3 for overcoming physiological dormancy (11). Seeds of *D. ammoniacum* required only cold stratification for dormancy break, and GA_3 did not substitute for it (Table 1). The type of morphophysiological dormancy in *D. ammoniacum* seeds would be classified as deep complex. The ineffectiveness of GA_3 has also been reported (19). Nadeem et al (2000) showed a remarkable promotion by GA_3 in seeds stored for 5 months at 4˚C, but not in freshly harvested seeds. This is apparently attributable to the storage-dependent changes in seed sensitivity towards GA_3 (20).

Increasing BA concentration rose both germination rate and percentage but there was no significant difference among BA concentrations. Exogenous application of 10 and 20 mg/l BA for 24 h increased the germination rate to a level comparable to that achieved with stratification in Petri dish for 15 days.

BA has low activity in dormancy and germination control compared with GA_3, but it is involved in breaking seed dormancy and stimulating germination of some plants. It has been suggested that BA may not be directly

involved in breaking dormancy but play a ‘‘permissive’’ role by decreasing level of germination inhibitors and making seeds more sensitive to gibberellins (16).

In all treatments the germination rate was positively correlated with germination percentage. Therefore, fast germination was associated with high germination percentage. It can be concluded that, cold stratification in moist sand stimulates seed germination and has a larger effect than the other treatments applied in this study.

CONCLUSION

In conclusion, the present work has established an effective strategy for breaking seed dormancy and enhancing seed germination of *D. ammoniacum* through chemical scarification, application of gibberellins, BA treatments and various stratification treatments. For *D. ammoniacum* seeds, the alternating scarification and GA_3 treatments used did not promote seed germination. Although, these seeds showed a chilly requirement for germination, they are able to germinate under BA treatments at lower percentages than those obtained under stratification. The study suggests cold stratification treatments are economic and easily applicable by nursery workers and poor farmers in developing mass planting stock, over costly plant growth regulators and associated technicalities. Further studies are required to elucidate the cultivation requirements of this endemic plant. Particularly, further investigation needed to identify the responsible inhibition mechanisms and studies on the hormonal balance in the seeds such as ABA and GA that are believed to be important. This laboratory germination results can be applied to propagation projects that would support conservation programs within the study site.

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