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Germination of *Hippophae rhamnoides* L. seed after 10 years of storage at ambient condition in cold arid trans-Himalayan Ladakh region

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The actinorhizal plant seabuckthorn (*Hippophae rhamnoides* L., Elaeagnaceae) is a wind-pollinated dioecious crop. In the present work we study two important aspects of germination of seabuckthorn seeds: (i) germination-related studies of aged seed stored up to 10 years under ambient condition in cold arid condition and (ii) the effect of seed pre-soaked treatment on germination-related parameters of aged seeds. Seed stored up to 6 years does not show any significant difference in germination percentage. However, seeds aged 9 and 10 years showed significant reduction in germination percentage, being 65.3 and 65.67 respectively, compared to 100 and 99 in one- and two-year-old seeds respectively. KNO_3 pre-soaking treatment showed negative effect on seed germination. Correlation studies showed that with advancement of age of seabuckthorn seed, the moisture content, germination percentage and seed vigour index decrease. It takes more time for seeds to germinate with ageing. Similarly, decrease in moisture content results in decrease in germination percentage and seed vigour index. Results showed that short- and medium-term storage of seeds could be achieved at ambient condition in cold arid region at lower cost without the limitation of space.

Keywords: Pre-soaking treatment, seabuckthorn, seed age, seed germination.

STUDY of behaviour of seed germination and the factors controlling the process in an environment is an important aspect not only for physiologists and ecologists, but also for seed technologists. Seed moisture content and storage temperature are the most important factors affecting seed longevity and vigour during storage[1]. Preferable conditions for long-term seed storage are 3–7% moisture content and –18°C temperature[2]. However, its use in developing countries has been greatly limited because of the high cost of building and operation[3]. Storage of seeds in cold

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arid region may serve as an alternate cheaper means for short and medium terms in view of the naturally prevailing low temperature and relative humidity in the region.

Seabuckthorn (*Hippophae rhamnoides* L., Elaeagnaceae) is an ecologically and economically important plant, which grows in marginally fertile soils and often serves as a pioneer plant species. Seabuckthorn berries are among the most nutritious of all fruits. Concentration of vitamins B_2, B_3, B_5, B_6, B_{12}, C and E is much higher than other fruits such as apricot, banana, mango, orange and peach[4]. Seabuckthorn pulp, seeds, leaves and stem bark contain high levels of phenolic content and antioxidants[5]. The shrub serves as a storehouse for researchers in the field of biotechnology, neutraceutical, pharmaceutical, cosmetic and environmental sciences[6].

Though few studies have been conducted on different aspects on seed germination of *H. rhamnoides*[7–9], no information is available on the effect of ageing on seed moisture, germination percentage, mean germination time, germination index, seed vigour index and synchronization index. In this study, two important aspects of seabuckthorn seeds: (i) germination-related studies of aged seed stored up to 10 years under ambient condition in cold arid condition and (ii) the effect of seed pre-soaked treatment on germination-related parameters of aged seeds have been addressed. The results provide knowledge on germination and effect of pre-soaking treatment on aged seabuckthorn seeds. Besides, feasibility of cost-effective seed storage for short and medium term in cold arid region has been addressed.

Seeds of three healthy *H. rhamnoides* subsp. *turkestanica* plants were collected from Leh valley (3235 m amsl, 37°05.5N, 077°35.8E) of Trans-Himalayan Ladakh region, India during October 2001, 2002, 2005, 2009, 2010 and 2011. The seeds were stored in three-ply aluminium-laminated (10 μm) pouches in a room with natural air and temperature. The outside mean maximum and minimum temperature in the experimental locality was 18.9 ± 9.5°C and –5.8 ± 9.8°C respectively, whereas the mean maximum and minimum relative humidity was 35.54 ± 7.3 and 25.0 ± 3.7 respectively, during the study period. The average annual precipitation was less than 200 mm, of which more than 70% was in the form of snowfall.

Seed moisture content was determined using the oven-drying method[10] and expressed as percentage fresh weight. Germination experiment was conducted in December 2011. Three sets of seeds from each plant were pre-treated using two cheap methods as follows: (a) submersion in distilled water for 48 h; (b) submersion in 0.1% KNO_3 for 48 h and (c) control without any treatment. Four replicates of 30 seeds each were placed on one sheet of filter paper (Whatman No. 1) in 50 mm diameter petri dishes and germinated at the predetermined optimal dark condition at 25°C. The filter paper was moistened as needed with distilled water and seedling counts were performed after every 24 h. Final germination rate (measured as percentage) was recorded after 35 days.

Mean germination time (MGT) was determined using the formula[11]

$$\mathrm{MGT} = \frac{\sum_{i=l}^{k} n_i t_i}{\sum_{i=l}^{k} n_i},$$

where t_i is the day from the start of the experiment to the ith observation, n_i the number of seeds germinated on day i and k is the last day of germination.

Germination index (GI) was calculated as[12]

$$\mathrm{GI} = \sum_{i=l}^{n} \frac{|(36 - D_i)G_i|}{S},$$

where n is the number of germination counting (days), 36 the total number of days spent in the germination test plus 1, D_i the number of days until the ith reading, G_i the number of normal seeds germinated on the ith day and S is the total number of seeds used in the test.

Germination synchrony (GS) was calculated[13] as

$$\mathrm{GS} = -\sum_{i=l}^{k} f_i \log_2 f_i, \quad f_i = \frac{n_i}{\sum_{i-l}^{k} n_i},$$

where f_i is the relative frequency of germination, n_i the number of seeds germinated on day i and k is the last day of observation.

Seed vigour index (SVI) was calculated at the final count by measuring average seedling length of 20 seedlings[14] as

$$\mathrm{SVI} = \frac{\text{Germination (\%)} \times \text{Seedling length (cm)}}{100}.$$

All experiments were conducted in a completely randomized block design. Germination data were arcsine transformed before analysis of variance. One-way ANOVA was performed with the help of 2-sided Tukey's HSD at $P \leq 0.05$ and 2-tailed Pearson correlation using SPSS for Windows version 17.0. Two-way ANOVA was used to test the effect of the main factors and their interactions (age and treatment) on seed germination percentage, mean time to germinate, germination index, seed vigour index and synchronization index.

No significant decline in germination percentage of seeds stored up to six years was observed (Table 1). Therefore, seabuckthorn seeds can be kept satisfactorily up to six years without significant loss of viability at room temperature in cold desert condition. However, seeds

Table 1. Moisture content and effect of seed age and pre-soaking treatments on seed germination percentage and mean germination time

		Germination (%)			Mean germination time (day)		
		Treatment			Treatment		
Age of seed (year)	Moisture (%)	KNO_3	Water	Control	KNO_3	Water	Control
0	8.25 ± 0.57[b]	83.00 ± 5.57[c]	98.67 ± 1.15[b]	92.00 ± 13.86[b]	9.97 ± 1.29[a]	9.35 ± 1.60[ab]	10.95 ± 2.13[a]
1	8.01 ± 0.36[b]	91.00 ± 11.27[c]	98.33 ± 2.89[b]	100.00 ± 0.00[b]	9.39 ± 3.14[a]	6.73 ± 1.33[a]	9.79 ± 1.38[a]
2	8.01 ± 0.62[b]	77.67 ± 16.74[c]	99.33 ± 1.15[b]	99.00 ± 1.73[b]	10.26 ± 1.16[a]	6.90 ± 1.62[a]	10.57 ± 1.58[a]
6	5.78 ± 0.88[a]	68.67 ± 1.53[bc]	95.00 ± 5.00[b]	94.33 ± 4.04[b]	9.93 ± 0.61[a]	8.28 ± 0.47[a]	11.70 ± 1.40[a]
9	5.56 ± 0.70[a]	44.00 ± 2.00[ab]	55.33 ± 3.06[a]	65.33 ± 3.06[a]	10.53 ± 0.26[a]	12.24 ± 1.94[b]	12.77 ± 0.61[a]
10	4.09 ± 0.69[a]	26.33 ± 15.37[a]	44.00 ± 23.07[a]	65.67 ± 10.69[a]	7.68 ± 1.98[a]	12.97 ± 0.65[b]	11.96 ± 1.74[a]

Values represented as mean ± SD; for each column, different lowercase letters indicate significantly different at $p < 0.05$, as measured by two-sided Tukey's HSD between seed age.

Table 2. Two-way ANOVA for pre-treatment, age of seed and their interactions on germination percentage and mean germination time

	Germination percentage					Mean germination time				
Independent variable	Sum of squares	*df*	Mean square	*F*	$P \leq 0.05$	Sum of squares	*df*	Mean square	*F*	$P \leq 0.05$
Treatment	0.441	2	0.220	24.901	0.000	38.010	2	19.005	7.978	0.001
Age of seed	2.138	5	0.428	48.305	0.000	58.779	5	11.756	4.935	0.002
Treatments × age of seed	0.139	10	0.014	1.570	0.156	80.357	10	8.036	3.373	0.003

aged 9 and 10 years showed significant reduction in germination percentage – 65.3 and 65.67 respectively, compared to 100 and 99 in one- and two-year-old seeds respectively. In contrast, it has been reported that dry seeds can be kept satisfactorily for 1–2 years at room temperature[7] and 60% viability has been reported for seeds stored for 4–5 years[8]. Higher seed germination of aged seeds in our study could be due to lower temperature and relative humidity in the storage condition. Seed storage stability and the kinetics of seed viability loss are largely dependent upon seed water content and storage temperature[1]. High temperature during storage enhances seed deterioration as does high seed moisture content. A drop of 5°C in storage temperature doubles seed longevity[15,16]. Relative effects of seed moisture content and temperature on longevity differ with species and the structural and biochemical composition of seeds. A complete pattern of loss in viability could be understood on the basis of seed moisture and storage temperature[17]. In the present study, moisture content of seeds stored at ambient condition ranges from 4.09% to 8.25% and the outside mean maximum and minimum temperature in the experimental locality was 18.9 ± 9.5°C and –5.8 ± 9.8°C respectively. The phenomenon may have ecological importance as seabuckthorn is a pioneer plant species and it is selectively advantageous to maintain high germination rate stretched over a period of time to offset unfavourable conditions for germination prevailing in cold arid environment.

Two-way ANOVA for pre-soaking treatment, age of seed and their interaction showed that pre-soaking treatment has significant influence on seed germination percentage (Table 2). KNO_3 treatment has a negative effect on germination of seabuckthorn seeds of all ages. KNO_3 is a growth-regulating and germination-stimulating substance that can either stimulate or inhibit seed germination depending on the plant species. Adverse effect of KNO_3 has also been observed in *Terminalia sericea*[18]. However, significant reduction in seed germination was observed in 9- and 10-year-old seeds when treated with water. The aged seeds have lower moisture content (4.09–5.56%) and significant reduction in germination could be due to imbibition injury during soaking. It is established that when dry seeds are tested for germination, the rapid uptake of water which ensues on contact with water can lead to imbibition injury and decreased germination[19].

Influence of age and pre-soaking treatment on seed mean time to germinate is given in Table 1. No significant difference in mean germination time with respect to age of seed was observed. However, 9- and 10-year-old seeds treated with water showed significant difference compared to seeds stored for six years or less. Two-way ANOVA for pre-soaking treatment, age of seed and their interaction showed that pre-soaking treatment and age of seed have significant influence on seed mean time to germinate (Table 2). The combined effect of seed pre-soaking treatment and age of seed is also significant.

Table 3. Effect of seed age and pre-soaking treatments on germination index, vigour index and synchronization index

Age of seed (year)	Germination index (seed day^{-1}) Treatment KNO$_3$	Water	Control	Seed vigour index Treatment KNO$_3$	Water	Control	Synchronization index Treatment KNO$_3$	Water	Control
0	2.37 ± 0.16^{c}	2.82 ± 0.03^{b}	2.63 ± 0.40^{b}	2.12 ± 0.47^{bc}	2.44 ± 0.43^{b}	2.15 ± 0.19^{a}	0.190 ± 0.009^{a}	0.207 ± 0.003^{a}	0.248 ± 0.085^{a}
1	2.60 ± 0.32^{c}	2.81 ± 0.09^{b}	2.86 ± 0.00^{b}	2.55 ± 0.62^{c}	2.63 ± 0.43^{b}	2.30 ± 0.58^{a}	0.154 ± 0.015^{a}	0.221 ± 0.019^{ab}	0.256 ± 0.030^{a}
2	2.22 ± 0.48^{c}	2.90 ± 0.12^{b}	2.83 ± 0.05^{b}	2.08 ± 0.16^{bc}	2.15 ± 0.22^{ab}	2.10 ± 0.58^{a}	0.174 ± 0.023^{a}	0.220 ± 0.012^{ab}	0.191 ± 0.021^{a}
6	1.96 ± 0.05^{bc}	2.95 ± 0.15^{b}	2.88 ± 0.08^{b}	1.92 ± 0.30^{bc}	2.23 ± 0.73^{ab}	1.31 ± 0.89^{a}	0.186 ± 0.006^{a}	0.232 ± 0.000^{ab}	0.201 ± 0.008^{a}
9	1.24 ± 0.06^{ab}	1.74 ± 0.08^{a}	2.01 ± 0.09^{a}	1.13 ± 0.37^{ab}	1.53 ± 0.20^{ab}	1.02 ± 0.63^{a}	0.184 ± 0.009^{a}	0.209 ± 0.011^{ab}	0.244 ± 0.030^{a}
10	0.75 ± 0.44^{a}	1.26 ± 0.66^{a}	1.88 ± 0.31^{a}	0.56 ± 0.37^{a}	1.07 ± 0.46^{a}	1.41 ± 0.36^{a}	0.267 ± 0.037^{b}	0.257 ± 0.036^{b}	0.213 ± 0.011^{a}

Values represented as mean ± SD; for each column, different lowercase letters indicate significantly different at $p < 0.05$, as measured by two-sided Tukey's HSD between seed age.

Table 4. Two-way ANOVA for pre-treatment, age of seed and their interactions on germination index and seed vigour index

Independent variable	Germination index Sum of squares	*df*	Mean square	*F*	$P \leq 0.05$	Seed vigour index Sum of squares	*df*	Mean square	*F*	$P \leq 0.05$
Treatment	4.521	2	2.260	30.932	0.000	990047.683	2	495023.841	2.385	0.106
Age of seed	17.244	5	3.449	47.194	0.000	1.546E+07	5	3.093E+06	14.902	0.000
Treatments × age of seed	1.398	10	0.140	1.913	0.076	2.205E+06	10	220527.834	1.063	0.415

Table 5. Two-way ANOVA for pre-treatment, age of seed and their interactions on synchronization index

Independent variable	Synchronization index Sum of squares	*df*	Mean square	*F*	$P \leq 0.05$
Treatment	0.013	2	0.006	8.244	0.001
Age of seed	0.013	5	0.003	3.374	0.013
Treatments × age of seed	0.026	10	0.003	3.341	0.004

Significant difference in germination index was observed for seeds stored for 9 and 10 years compared to others (Table 3). Two-way ANOVA for pre-soaking treatment, age of seed and their interaction showed that pre-soaking treatment and age of seed have significant influence on seed germination index (Table 4). However, the combined effect of seed pre-soaking treatment and age of seed is not significant.

Vigour index of untreated seeds did not show any significant decline with respect to age of seed. However, loss of vigour was significantly high in KNO_3 pretreated seeds with storage period (Table 3). Two-way ANOVA for pre-soaking treatment, age of seed and their interaction showed that age of seed had significant influence on seed vigour index (Table 4). However, pre-soaking treatment and the combined effect of pre-soaking treatment and age of seed are not significant.

Untreated seeds did not show any significant difference in terms of synchronization index with respect to age of seed (Table 3). However, significant difference in synchronization index was observed for 10-year-old seeds when pre-soaked treatment was given with water and KNO_3. Two-way ANOVA for pre-soaking treatment, age of seed and their interaction showed that pre-soaked treatment and the combined interaction of age of seed and pre-soaking treatment had significant influence on synchronization index (Table 5).

Table 6 shows the correlation among seed age, moisture content, germination, vigour index and synchronization index. Correlation studies showed that with advancement of age of seabuckthorn seeds, the moisture content, germination percentage and seed vigour index decrease. It takes more time for seeds to germinate with ageing. Similarly, decrease in moisture content results in decrease in germination percentage and seed vigour index.

On the basis of present results, it can be concluded that short- and medium-term storage of seabuckthorn seeds could be achieved at ambient condition in cold arid region. This will significantly reduce the cost of storage. Long-term storage in seed banks with controlled temperature

Table 6. Pearson's correlation for seed age, moisture content, germination performance, vigour index and synchronization index

	Age	Moisture %	G%[1]	MGT[2]	GI[3]	SVI[4]	SI[5]
Age	1	–0.970**	–0.870*	0.857*	–0.800	–0.912*	–0.247
Moisture %		1	0.817*	–0.791	0.757	0.853*	0.272
G %[1]			1	–0.847*	0.983**	0.765	–0.099
MGT[2]				1	–0.749	–0.950**	–0.135
GI[3]					1	0.639	–0.131
SVI[4]						1	0.209
SI[5]							1

*Correlation is significant at 0.05 level (two-tailed).
**Correlation is significant at 0.01 level (two-tailed). G%[1]: Germination %. MGT[2]: Mean germination time (days). GI[3]: Germination index. SVI[4]: Seed vigour index. SI[5]: Synchronization index.

and moisture is not always possible, especially in the developing countries due to high cost of building and operations. Besides, seed banks are public institutions whose management is influenced by political decisions, shortage of personnel and economic limitations[20]. Seeds developed by research institutions and seed companies in tropical and sub-tropical conditions can be stored in bulk for short and medium term in cold arid region for practical application at a lower cost without the limitation of space.

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