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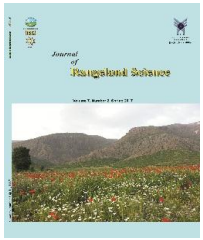

Research and Full Length Article:

Cryopreservation of *Smirnovia iranica* (Sabeti) Seeds and Evaluation of Cryopreserved Seeds under Laboratory, Greenhouse and Natural Habitat Conditions

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Abstract. *Smirnovia iranica* (Sabeti) synonym: *Smirnovia turkestana* (Bunge) is a deciduous perennial bushy species of the Fabaceae (Papilionaceae) family. The species grow on sand dunes of desert areas, having a deep vertical root and very long horizontal or lateral roots. They have an important role in natural vegetation of desert and sand dune stabilization. Limited growing areas, grazing due to good palatability, and foliage quality of the plant have put this species under threat. Seed preservation under cryogenic conditions at -196ºC is an important approach to conserve seeds for a long period. In this study, seeds of the *S. iranica* were collected from natural habitats of the plants and three pre-cryopreservation treatments including PVS2, Desiccation, and 30%Glycerol were applied before transferring the seeds into liquid nitrogen (LN) at -196ºC for 1 week, 1 month, and 1 year (in 2015). Subsequently, the seeds were removed from the liquid nitrogen, imposed to heat shock and evaluated under laboratory, greenhouse and natural habitat conditions. The cryopreserved seeds of various cryopreservation periods germinated normally under laboratory and greenhouse conditions. In laboratory conditions, there were no significant differences between periods of seed storage in cryogenic conditions for seed germination trait. The highest seed germination percent (84%) was observed in the desiccation pre-cryopreservation treatment. In pre-cryopreservation treatments as well as cryogenic storage periods under greenhouse conditions, seed germination and seedling establishment were significantly different. In natural habitat, the cryopreserved seeds germinate and grow to normal seedlings and plants. The results showed that *S. iranica* seeds can be successfully stored in cryogenic conditions for a long period.

Key words: *Smirnovia iranica*, *Smirnovia Turkestana*, Seed Cryopreservation, Seed Germination, PVS2, Desiccation

Introduction

Smirnovia iranica is an important desert species of Iran, growing on sand dunes of desert areas. In Iran, this species grows in sand dunes near Aran and Bidgol, and Choupanan in Isfahan province, few sand dune areas in south of Damghan and Shahroud in Semnan province, and some sand dunes of Sabzevar in Razavi Khorasan province. Besides Iran, the species are observed in Afghanistan and Karakum desert of Turkmenistan (Rechinger, 1984) as well as Kyzylkum desert of Uzbekistan (Kapustina, 2001).

This species is a perennial bushy plant of the Fabaceae (Papilionaceae) family. It has a deep vertical root and the mature plant produces very long lateral root system which grows out horizontally up to 25-30 m on top of sandy soils. Very long horizontal or lateral root system has an import role in sand dune stabilization. The whole plant system has an essential role in soil protection, sand dune stabilization, and desert vegetation. Limited growing areas, over grazing due to good palatability and foliage quality of the plant in some regions have put this species under threat.

This perennial legume species is a native plant and well adapted to harsh environments such as low precipitation that occurs from late autumn through late winter or early spring and high temperature from mid-spring to mid-autumn. Resistance of this species against such environmental conditions in desert ecosystems and the ability to establish on sand dunes reveal the effects of species on the sustainability of fragile and vulnerable desert ecosystems. Studying the ecological factors such as rainfall, soil, temperature, light, wind, flora and fauna, grazing, influence of man on vegetation as well as studies on plant characteristics such as botany, establishment, flowering, seed and seed dispersal, seed germination and establishment, vegetative propagation, and other abiotic and biotic factors help us in better understanding the plant and its environment. These sorts of knowledge are basic to recognize the ecological needs, conservation and re-establishment of the species if the species encounters the threat of extinction.

Collection and preservation of seeds of the desert plants especially endangered and under threat species are regarded as an important approach to preserve the germplasm as well as genetic biodiversity of desert species. In orthodox seeds, seed conservation in genebanks is possible only for medium terms. However, in cryopreservation conditions at -196ºC in liquid nitrogen (LN), seeds or plant organs can be stored for a long time. In this regard, Walters *et al.* (2004) calculated half-life of the seed germination of lettuce accessions with 6.5% water using Avrami (1941) thermodynamic equations. In this experiment, the exact mean half-life of the seed germination was calculated as 3375 years. In cryogenic conditions, metabolic activities of cell dramatically decrease and therefore, seed longevity significantly increases (Walters *et al.*, 2004; Caswell and Kartha, 2009).

Seeds of wide variety of plant species with orthodox seeds such as forest species (Naderi Shahab *et al.*, 2009) and range species (Naderi Shahab *et al.*, 2013) have been successfully preserved at -196ºC. In plants having recalcitrant seeds, vegetative organs such as buds, meristems, seed embryonic axes, calli, and axillary buds can be treated with cryoprotectant substances and conserved in cryogenic at -196ºC for the prolonged time periods (Al Zoubi and Normah, 2012; Wesley-Smith *et al.*, 2004; Chmielarz *et al.*, 2005; Blakesley and Kiernan, 2001; Tyagi and Hymowitz, 2003; Flachsland *et al.*, 2006; Makeen *et al.*, 2005; Stanwood, 1985; Hay and Muir, 2000). Cryoprotectant substances including glycerol, dimethyl sulfoxide (DMSO), sucrose, ethylene glycol, and dextran are used to protect cells of seeds,

tissues, or other biological organs from freezing damages caused by ice crystal formation in cryogenic conditions. Meanwhile, concentration of these chemical substances should be adjusted since high concentration of these substances can lead to toxic effects on cells, especially in some specific species. Some cryoprotectant solutions such as Plant Vitrification Solution2 (PVS2) consisting of the glycerol, ethylene glycol, dimethyl-sulfoxide (DMSO), and sucrose are widely used as cryoprotectant solutions.

PVS2 and other plant vitrification solutions may have positive effects on seed survival of cryogenically stored seeds. Treating the seeds of plant species with PVS2 before being stored in cryogenic at -196ºC has shown positive effects on seed survival and germination after removal from LN (Gale *et al.,* 2008; Rall, 1987; Thammasiri, 2000). However, some reports have pointed out the toxic effects of PVS2 on cryogenically stored plant organs (Kuleshova *et al.,* 1999). This may be due to the penetration of substances (for example ethylene glycol and DMSO) into plant cells, especially susceptible cells.

Treating seeds or plant organs with glycerol has also shown positive effects on survival rate of the cryogenically stored seed or plant organs (Jeyendran *et al.,* 1985). Glycerol is commonly used as cryoprotectant solely or as a component of multicomponent cryoprotectant solutions. Another extremely important factor in seed cryopreservation is moisture content of the seed. Seeds with high moisture content such as recalcitrant seeds are not able to tolerate cryogenic at -196ºC conditions. In orthodox seeds, lowering the seed moisture content to some extent before being transferred to LN at -196ºC showed positive effects on seed survival and germination after removing from LN. However, the reduction of seed water content or seed desiccation to below optimum level can have negative effects on seed survival and germination, especially under cryogenic conditions.

Regarding the effect of cryopreservation on ploidy level, genomic structure and gene expression of cryopreserved cells, a question may arise as whether this approach is safe or has adverse effects on plant genome. Most of the reports show that cryopreservation conditions did not have detrimental effects on gene expression, genomic structure, and ploidy level of cryopreserved cells (Harding and Benson, 2001; Sánchez *et al.,* 2008; Zhai *et al.,* 2003). Even in transgenic plants, the expression of transgene remains stable after cryopreservation (Hao *et al.,* 2002). Therefore, these reports show that most of the plant species developed from cryopreserved seeds, cells or organs were normal and did not show genetic abnormalities. Cryopreservation is an important and promising approach for long-term conservation of plant seeds, cells, and organs especially those vulnerable, threatened or endangered species.

The aim of this study was to evaluate the possibility of long-term seed cryo-preservation of the threatened species of *Smirnovia iranica* and assess the seedlings and plants derived from the cryopreserved seeds under laboratory, greenhouse and natural habitat conditions

Materials and Methods

Seed samples:

Seed pods were collected from Rigboland sand dunes near Aran and Bidgol town, close to Kashan city in Isfahan province, Iran. Depending on climatic conditions, pods usually ripen between 4th and 14th June. If harvesting is delayed, pods detach from the plants and undergo long-distance dispersal by wind or are quickly collected by rodents or other desert animals and insects. The number of seeds per pod is 1 to 15 (Fig. 1). The collected pods were threshed and cleaned. The

undamaged, healthy and normal seeds were scarified with emery paper (P100). This study was conducted in 2015.

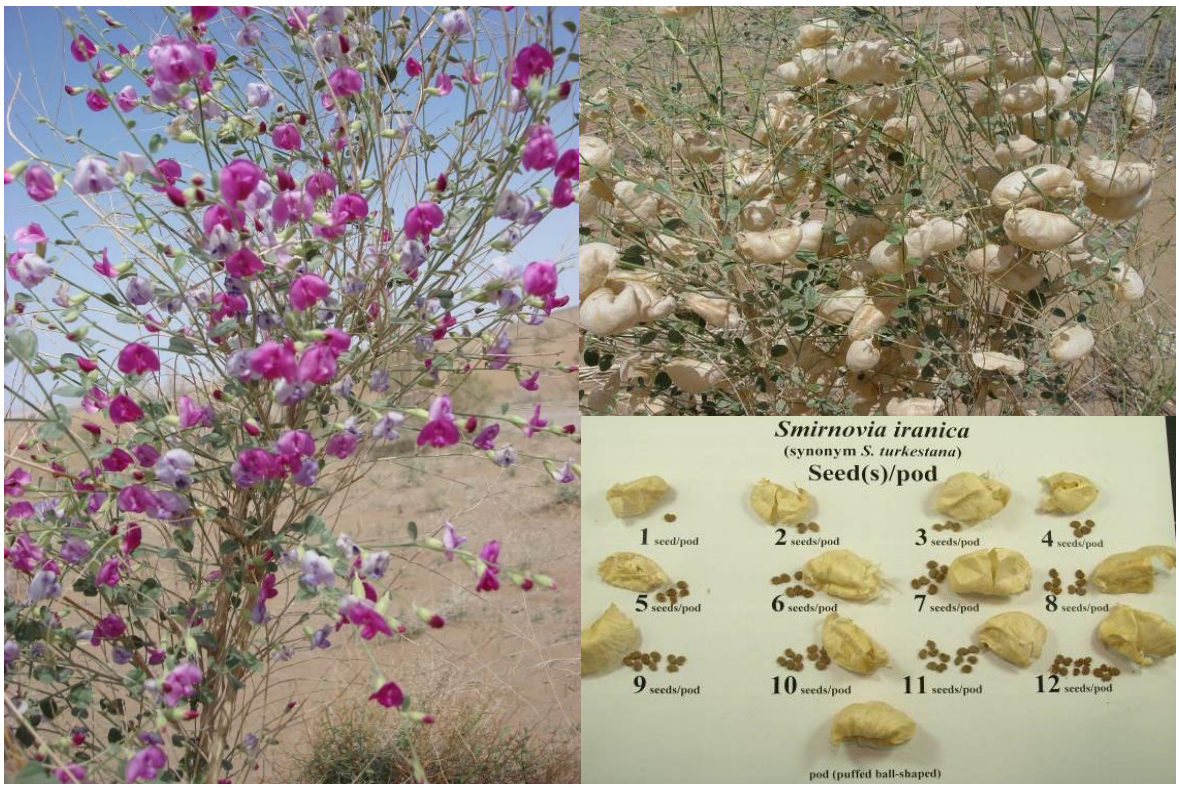

Fig. 1. *Smirnovia iranica* at full bloom stage (left), pods at last stage of ripening (top right), and variation in number of seeds per pod (bottom right)

Seed germination test

1- Approximately 3g seeds were placed in a 30ml tube and washed 3 times with sH_2O.
2- Tubes were drained off and 20% bleach was added to the tubes and incubated at +22°C for 15 min.
3- Bleach was discarded and seeds were washed 3 times with sH_2O under aseptic conditions in laminar air flow.
4- Seeds were transferred between sterile moist papers in Petri dishes.
5- The Petri dishes were incubated in a +22ºC germinator under continuous light at an intensity of $10W/m^2$.

Pre-cryopreservation treatments

1. Plant Vitrification Solution 2 (PVS2) contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO (dimethyl sulfoxide), and 0.4 M sucrose (Sakai *et al.*, 1991; Naderi Shahab *et al.*, 2009, 2013). Approximately 50g of fresh seeds were transferred into screw-cap tubes, filled with PVS2 and submerged in the liquid nitrogen (LN) container.
2. Desiccation: Fresh weigh (FW) of the seeds were determined by weighing 10g of freshly collected seeds. The seeds were oven dried at +75°C for 72h, weighed and recorded as seed dry weight (DW). The percentage of the total seed moisture content was obtained using the $Moisture\% = (FW - DW)/DW \times 100$ formula. Total seed moisture content of the collected seeds was 4.46%. Approximately 50g of fresh seeds were weighed and placed in air tight desiccators containing 500g silica gel for 7 days at +4°C. The moisture content of the seeds dropped from 4.46% to 3.33%. Based on the total moisture content, the reduction of seed moisture content was

approximately 25.25%. The seeds were removed from desiccator and immediately transferred into screw-cap tubes and submerged in a liquid nitrogen (LN) container.

3. 30% Glycerol: Approximately 50g of fresh seeds were transferred into screw-cap tubes, filled with 30% glycerol and submerged in a liquid nitrogen (LN) container (Naderi Shahab *et al.*, 2009; 2013).
4. Control seeds: Four samples of fresh seeds were stored at +4°C for 1 week, 1 month, and 1 year and were used as Control for different experiments.

Post-cryopreservation method

The seeds were removed from liquid nitrogen (LN) after 1 week, 1 month, and 1 year and were subjected to post-cryopreservation treatments as described (Naderi Shahab *et al.*, 2009; 2013).

Laboratory experiments

Cryopreserved and Control seeds were sterilized and transferred between sterile moist papers in Petri dishes. The Petri dishes were transferred into a +22°C germinator (as described above). Next, the germinated seeds were counted and recorded every 3 days (from beginning of germination) until no more seeds germinated. The following attributes were measured: seed germination percent, germination speed, shoot length, root length, root/shoot length ratio (R/S), and seed vigor index (VI) (Naderi Shahab *et al.*, 2009; 2013).

Experiments layout

A factorial experiment consisting two factors: 1) three pre-cryopreservation treatments including PVS2, 30% Glycerol, Desiccation, and Control seeds which formed 4 levels of factor A and 2) three LN storage periods of seeds including 1 week, 1 month, and 1 year made up three levels of factor B using a Completely Randomized Design with three replications. The experimental units were single Petri dishes. The above mentioned attributes were measured and data analysis was carried out using SAS software. The differences between the treatments means were tested using Duncan's Multiple Range Test.

Greenhouse Experiments

Fifteen cm diameter plastic pots were filled with sandy soil brought in from sand dunes of Rigboland. Seeds of various treatments were sown and irrigated with tap water in greenhouse at 22±3°C. The pots were irrigated and maintained at field capacity. The number of emerged seedlings was recorded and subjected to data analysis. The experimental design was a factorial design consisting of two factors: 1) three liquid nitrogen incubation times including 1 week, 1 month, and 1 year were three levels of factor A and 2) three pre-cryopreservation treatments including PVS2, 30% Glycerol, Desiccation and Control seeds were four levels of factor B using a Completely Randomized Design with three replications. Data analysis was carried out using SAS software and the differences between treatment means were tested using Duncan's Multiple Range Test. In order to study root development, seeds were sown in a glass box with dimensions of 120×40×60 cm (L×W×H), filled with the plant habitat's soil. All of the laboratory and greenhouse experiments were carried out at Research Institute of Forests and Rangelands, Tehran, Iran.

Seed sowing depth

Seed sowing experiment was carried out under greenhouse conditions. Round plastic washing up bowel with 60 cm diameter and 15 cm depths was filled with the plant habitat's soil. Seeds were sown in depths of 2.5, 5.0, 7.5 and 10.0 cm and irrigated at 7-day intervals.

Salt tolerance of seeds

In this experiment, *S. iranica* as a legume species was used for salt tolerance

evaluation and *Medicago sativa* (alfalfa), a relatively salt sensitive crop (Grattan *et al.,* 2004) was used as a control legume species. Seeds of the *S. iranica* and *M. sativa* species were exposed to NaCl concentrations of 0.03M, 0.06M, 0.09M, 0.12M, 0.15M, 0.18M, 0.21M, 0.24M, 0.27M, 0.30M, 0.33M, 0.36M, 0.39M, 0.6M, 0.9M and 1.2M plus control (dH_2O) with three replications in laboratory conditions. The germination of seeds was measured and subjected to data analysis using a Completely Randomized Design with three replications.

Experiments conducted in natural habitat

The field tests were only observational and cryopreserved seeds (one month incubated in liquid nitrogen) and Control were sown in natural habitat of the species in Rigboland sand dunes under natural conditions using pit-seeding method. Approximately 10 seeds were located in each pit and covered with 4 cm soil. The experiment was carried out for three years from December 2007 up to spring 2010. Seed sowing dates in 3 successive years were as follows: 25th December 2007, 27th February 2009, 15th March 2009, and 7th December 2009.

Results

Laboratory experiments

Results of the analysis of variance (ANOVA) showed significant differences among different levels of pre-cryopreservation treatments for all of the traits, except seed germination percent and R/S ratio. There were no significant differences between incubation periods and interaction between incubation period and treatments for all of the traits (Table 1).

In Table 2, the total mean percentage of seed germination, root length, shoot length, seedling length, vigor index, and root to shoot ratios of *S. iranica* after 1 week, 1 month, and 1 year incubation in cryogenic (-196°C) conditions are presented. In all of the attributes, no significant differences were observed between cryogenic incubation periods. The results indicated that the incubation of the seeds in cryogenic conditions for a short or long time did not show any differences. The effect of pre-cryopreservation treatments and Control on total mean germination and other attributes is presented in Table 3. Except seed germination percent and R/S ratio, the other attributes showed different responses to the pre-cryopreservation treatments. For example, Desiccation showed the highest values as compared to those of the 30% Glycerol and PVS2. This reveals that 30% Glycerol and PVS2 pre-cryopreservation treatments have adverse effects on the preservation of *S. iranica* seeds under cryogenic conditions.

Table 1. Analysis of variance and mean of squares of *Smirnovia iranica* seed attributes under laboratory conditions

Source of variation	DF	MS					
		Germination %	Root Length (mm)	Shoot Length (mm)	Seedling Length (mm)	Vigor Index	R/S
Incubation Period	2	23.44 ^{ns}	2.32 ^{ns}	2.49 ^{ns}	9.62 ^{ns}	3.93 ^{ns}	0.020 ^{ns}
Treatment	3	13.81 ^{ns}	128.14**	61.99**	362.87**	285.32**	0.471 ^{ns}
Period x Treatment	6	2.93 ^{ns}	12.23 ^{ns}	6.35 ^{ns}	31.42 ^{ns}	24.92 ^{ns}	0.298 ^{ns}
Error	24	29.94	6.73	5.19	15.93	12.51	0.461
CV%		6.71	11.17	14.05	10.12	10.98	14.84

ns and**: non-significant and significant at P=0.01 level.

Table 2. Mean comparison of *Smirnovia iranica* seed attributes at different cryogenic incubation periods under laboratory conditions

Incubation Period	Germination %	Root length (mm)	Shoot length (mm)	Seedling length (mm)	Vigor Index	R/S
1 Week	82.33 a	23.37 a	16.31 a	39.68 a	32.78 a	1.45a
1 Month	82.50 a	22.73 a	15.71 a	38.43 a	31.64 a	1.46a
1 Year	80.00 a	23.57 a	16.60 a	40.17 a	32.21 a	1.43a

Means with the same letter are not significantly different (P=0.01)

Table 3. Mean comparison of *Smirnovia iranica* seed attributes at different pre-cryopreservation treatments under laboratory conditions

Pre-cryopreservation Treatment	Germination %	Root length (mm)	Shoot length (mm)	Seedling length (mm)	Vigor Index	R/S
Control	82.22 a	26.64 a	17.75 ab	44.39 a	36.46 a	1.55 a
30% Glycerol	81.00 a	21.89 b	15.72 b	37.61 b	30.45 b	1.40 a
Desiccation	83.00 a	25.86 a	18.63 a	44.49 a	36.92 a	1.39 a
PVS2	80.22 a	18.50 c	12.72 c	31.22 c	25.02 c	1.45 a

Means with the same letter are not significantly different (P=0.01).

The interaction between incubation periods and pre-cryopreservation treatments revealed differences amongst the pre-cryopreservation treatments and incubation periods for most of the attributes except germination percent and R/S ratio (Table 4). Incubation periods and pre-cryopreservation treatments did not show adverse effects on seed germination percent and R/S ratio as compared to that of the Control seeds. However, pre-cryopreservation treatments showed different effects on other attributes. While PVS2 and 30% Glycerol (respectively) had the lowest cryoprotective effects on root length, shoot length, seedling length, and vigor index, Desiccation showed the best cryoprotective effects on these attributes. The lowest figures for PVS2 probably arise through toxicity of PVS2's chemical components such as DMSO, ethylene glycol, and glycerol on seeds of the species. Yet 30% Glycerol regarded as another pre-cryopreservation treatment showed medium inhibitory effects on the above mentioned attributes. The effects of time period of cryogenic storage on different seed attributes showed that Germination% and R/S did not change over cryogenic storage time. While some of the other attributes in 30% Glycerol and Desiccation revealed changing patterns over cryogenic storage time. In PVS2, none of the attributes showed any significant changes over cryogenic storage time (the possible reason for this will be discussed later in Discussion). In contrast to the pre-cryopreservation treatments, root length, shoot length, seedling length, and vigor index significantly increased in Control seeds over cryogenic storage time. These increases probably arise through positive effects of seed storage duration under cool room conditions (+4°C).

Table 4. Means comparison of *Smirnovia iranica* seed attributes as affected by different pre-cryopreservation treatments at different cryogenic incubation periods under laboratory conditions

Incubation Period	Treatment	Germination%	Root length (mm)	Shoot length (mm)	Seedling length (mm)	Vigor Index (VI)	R/S
1 Week	Control	82.67 a	24.81 ab	16.21 dc	41.02 b	33.93 bc	1.59 a
	30%Glycerol	81.33 a	21.18 bc	16.48 abc	37.66 bc	30.76 cd	1.29 a
	Desiccation	84.00 a	29.09 a	19.78 a	48.88 a	41.06 a	1.49 a
	PVS2	81.33 a	18.40 c	12.77 d	31.18 c	25.38 d	1.43 a
1 Month	Control	82.33 a	26.11 ab	16.57 abc	42.68 ab	35.06abc	1.62 a
	30%Glycerol	83.33 a	22.02 bc	15.75 dc	37.77 bc	31.34 cd	1.40 a
	Desiccation	83.33 a	24.48 ab	17.92 abc	42.39 ab	35.28abc	1.37 a
	PVS2	81.00 a	18.29 c	12.59 d	30.88 c	24.87d	1.45 a
1 Year	Control	81.67 a	29.00 a	20.47 a	49.47 a	40.39 ab	1.44 a
	30%Glycerol	78.33 a	22.47 bc	14.93 d	37.40 bc	29.24 cd	1.51 a
	Desiccation	81.67 a	24.00 b	18.20 ab	42.20 ab	34.43 bc	1.31 a
	PVS2	78.33 a	18.80 c	12.80 d	31.60 c	24.80 d	1.47 a

Means with the same letter are not significantly different (P=0.01)

Greenhouse experiments

Analysis of variance of the seedling establishment percent under greenhouse conditions is presented in Table 5. Cryopreserved seeds treated with various pre-cryopreservation treatments, as well as Control seeds germinate and grow into normal seedlings in greenhouse conditions. Although there were no significant differences between incubation periods (Table 5 and last column of Table 6), the treatments showed significant differences for seedling establishment percent (Table 5 and 2nd column of Table 6). The cryopreserved seeds showed different patterns in response to cryogenic storage periods. As shown in Table 7, there were significant differences between pre-cryopreservation treatments with respect to seedling establishment.

Although Desiccation showed the highest seedling establishment over cryogenic storage time, PVS2 and 30% Glycerol showed the lowest seedling establishment. These variations in seedling establishment may arise through adverse effects of chemical component(s) on the PVS2 solution or inhibitory effects of glycerol as mentioned above. However, the Control showed the highest seedling establishment with statistically similar patterns over incubation periods. The highest establishment percent belongs to Control (84%) in 1 Year treatment followed by Desiccation (63.33%). As shown in Fig. 2, *S. iranica* seeds vigorously grow into seedlings either in pots or in glass box under greenhouse conditions. Regarding the vertical and lateral root development in seedlings grown in pots or glass box, it should be noted that only vertical roots developed in this stage and lateral or horizontal roots were not observed.

Table 5. Analysis of variance and mean of squares of seedling establishment of *Smirnovia iranica* under greenhouse conditions

Source of variation	DF	SS	MS	F Value	Pr > F
Incubation Period	2	102.38	51.19	1.11	0.35
Treatment	3	5668.53	1889.51	41.03	0.00
Period × Treatment	6	78.05	13.03	0.28	0.94
Error	24	1105.33	46.05		
CV%	11.37				

Table 6. Means comparison of *S. iranica* seed attributes at different pre-cryopreservation treatments and incubation periods under greenhouse conditions

Treatment	Establishment%	Incubation Period	Establishment%
30%Glycerol	46.11 c	1Week	60.00 a
Control	79.11 a	1Month	57.41 a
Desiccation	61.66 b	1Year	61.50 a
PVS2	51.66 c		

Means with the same letter are not significantly different (P=0.01)

Table 7. Seedling establishment of *Smirnovia iranica* as affected by different pre-cryopreservation treatments at different cryogenic incubation periods under greenhouse conditions

Incubation Period	Treatment	Establishment%
1 Week	Control	78.33 a
	30%Glycerol	46.00 d
	Desiccation	61.67 b
	PVS2	54.00 bcd
1 Month	Control	75.00 a
	30%Glycerol	45.67 d
	Desiccation	60.00 bc
	PVS2	49.00 d
1 Year	Control	84.00 a
	30%Glycerol	46.67 d
	Desiccation	63.33 b
	PVS2	52.00 bcd

Means with the same letter are not significantly different (P=0.01)

Experiments conducted in natural habitat

Field experiments were carried out in natural habitat at Rigbolnd sand dunes utilizing an observational method, as described before. At the beginning of the experiment in December 2007, shortly after sowing the seeds, all of the seeds were dug up and collected by the rodents (Fig. 3, Top left). To control the rodents' damage, the seeded area was covered by a thin steel mesh (Fig. 3, Top right). From the Control and cryogenic seeds sown in 4 different dates from 2007 to 2009 (see Materials and Methods), only the seeds sown on 7th December 2009 were germinated and continued to grow. The germinated seeds were developed to normal seedlings in spring 2010 (Fig. 3, Middle). In early spring, the seedlings were thinned to 1 plant per pit (Fig. 3, Bottom left). Only a few seedlings were grown into young normal plants (Fig. 3, Bottom right). Although the number of the established plants was low (approx. 5%), the results looked promising.

Fig. 2. *Smirnovia iranica* seeds germinated and developed to seedlings into pots (left) and glass box (right) under greenhouse conditions

Fig. 3. Seed sowing method (pitting), and covering seeded area with a thin steel mesh (top), seed germination and seedling growth (middle), thinning the bunched seedlings (bottom left) and establishment of young *Smirnovia iranica* plants in natural habitat (bottom right)

The salinity effects on seed germination under laboratory conditions

Soil layers of Rigboland sand dunes (0-120 cm depth) were tested and analyzed. The analysis and test results of the layers showed that the soil was almost homogeneous (data were not shown) with the following characteristics:

Soil texture was 88% sand, 1.6% silt and 10.4% clay. Soil pH was 8.41 and EC of the saturated soil extract was 0.371 dS/m. The results showed that the soil was sandy with low salinity and moderate alkalinity. Hence, soil of the plant habitat was not saline although the habitat climate is hot and dry from mid spring to mid-autumn and it receives almost no rainfall for eight to nine months.

To evaluate the salt tolerant behavior of *S. iranica* seeds under laboratory conditions, salinity experiment was conducted using *S. iranica* and *M. sativa* (control) seeds. The seed germination percent and germination speed of both species reduced significantly by increasing Molarity of NaCl (Table 8). However, seed germination percent and germination speed in *S. iranica* dropped sharply by increasing salt concentration and at 0.36 M NaCl, seed germination stopped completely (Table 8) whereas *M. sativa* seeds continued to germinate at 0.36 M NaCl. Table 8 indicates that salt tolerance of *M. sativa* seeds as a forage crop is significantly higher than that of the *S. iranica* seeds as a desert species (Fig. 4).

Table 8. Seed germination and germination speed of *Smirnovia iranica and Medicago sativa* seeds at various NaCl concentrations

NaCl Concentration	***S. iranica***		***Medicago sativa***	
	Germination (%)	Germination Speed	Germination (%)	Germination Speed
Control (dH_2O)	89.67 a	29.94 g	98.00 a	47.35 a
0.03M	88.33 a	24.94 b	96.33 a	46.47 ab
0.06M	79.33 b	20.50 c	96.00 a	46.26 ab
0.09M	80.67 b	17.57 d	95.33 a	46.18 ab
0.12M	77.67 b	15.14 e	94.67 ab	45.17 b
0.15M	75.33b c	13.37 f	94.33 ab	43.65 c
0.18M	69.00 c	9.89 g	91.67 bc	41.92 d
0.21M	57.00 d	7.41 h	88.33 cd	36.27 e
0.24M	43.00 e	6.61 h	86.33 d	32.25 f
0.27M	29.33 f	4.39 i	76.67 e	21.42 g
0.30M	12.33 g	1.73 j	67.00 f	13.13 h
0.33M	5.33 g	0.48 j	15.33 g	2.70 i
0.36M	00.00	00.00	10.33 h	1.21 j

Means with the same letter are not significantly different (P=0.01)

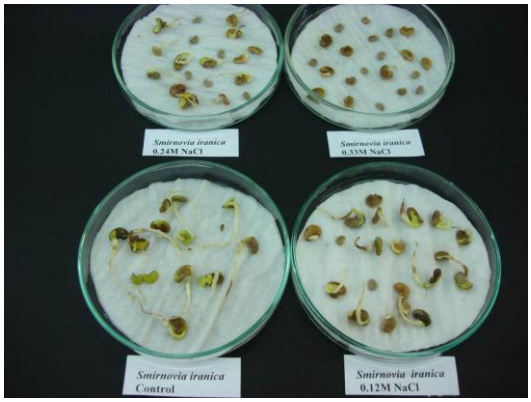

Fig. 4. Effects of salt concentration on seed germination of *Smirnovia iranica*

Seed sowing depth effect on seedling emergence under greenhouse conditions

Although the main objective of this experiment was to show the depth effects on seed germination and seedling growth, the effects of seed scarification on seed germination and seedling growth was also evaluated. The best seed sowing depth for seed germination was 2.5 cm in scarified seeds (Fig. 5). Although seed germination and seedling growth in Control (non-scarified) seeds were observed, the number of seedling was significantly lower than those of the scarified seeds. Seeds of the 5.0 and 7.5 cm sowing depth germinated and developed to seedlings in a longer time (20 and 25 days, respectively). However, in 10 cm sowing depth, germination was longer and the number of the germinated seeds and seedlings was significantly low.

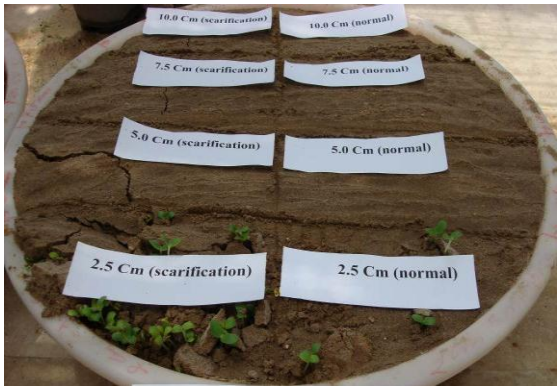

Fig. 5. Effect of seed sowing depth on *Smirnovia iranica* seed germination (13 days after seed sowing)

Discussion

Smirnovia iranica seed is an orthodox seed with low moisture content (4.46%) without dormancy. For quick and steady germination, the seeds require scarification; otherwise, seed germination percent reduces significantly and seed germination takes a longer time. In good conditions, maximum seedling emergence of 84% was observed under greenhouse conditions (see last column of Table 7). In laboratory conditions, seed germination occurred after 5-6 days and in greenhouse conditions, seed germination and seedling emergence were observed 13 days after seed sowing date (Fig. 5).

Laboratory

The results of the laboratory experiments showed that *S. iranica* seeds treated with different pre-cryopreservation treatments (30%Glycerol, Desiccation and PVS2), tolerate the cryogenic conditions (-196°C) excellently in different cryopreservation periods including 1 Week, 1 Month, and 1 Year (Tables 2 and 3). The interaction effects of pre-cryopreservation treatments and cryopreservation periods on various attributes (Table 4) were different. Although seed germination percent and R/S attributes did not show significant differences in different pre-cryopreservation treatments and cryopreservation periods, other attributes showed different reactions to those pre-cryopreservation treatments and cryopreservation periods. Seed germination and R/S were statistically similar to that of the control seeds. The results revealed that seed germination percent and R/S remain constant over cryopreservation periods and even pre-cryopreservation treatments had similar effects on these attributes. For most of the treatments, seedling emergence percent was more than 80% and the highest percent (84%) belonged to Desiccation in 1 Week incubation period

(Table 4). However, the other attributes showed different responses to Pre-cryopreservation treatments and periods. In this regard, Desiccation showed the highest mean values for root length, shoot length, seedling length, and vigor index in various cryopreservation periods followed by 30% Glycerol and PVS2 (Table 4). Based on the results, it can be concluded that under laboratory conditions, *S. iranica* seeds tolerate the cryogenic (-196°C) conditions excellently and the cryopreserved seeds germinate normally and vigorously.

Another important point was the effects of pre-cryopreservation treatments on seed cryopreservation. Desiccation showed the best results as compared to those of 30% Glycerol and PVS2. Hence, regarding the negative effects of 30% Glycerol and PVS2 on attributes such as root length, shoot length, seedling length, and seed vigor for cryopreservation of *S. iranica* seeds, Desiccation was an excellent pre-cryopreservation treatment. This may be due to the penetration of chemical components of PVS2 solution such as ethylene glycol, DMSO, glycerol, and sucrose into seeds and causing negative effects as pointed in some reports (Kuleshova *et al.,* 1999). Although positive effects of the PVS2 solution are reported in most cryopreservation studies (Gale *et al.*, 2008; Rall, 1987; Thammasiri, 2000), the PVS2 solution showed adverse effects on *S. iranica* seed germination and other seed attributes. Whereas glycerol was the main component of cryoprotectant solutions, 30% Glycerol also showed negative effects on the above mentioned attributes. This could be due to negative effects of the glycerol on above mentioned attributes.

Greenhouse

In greenhouse conditions, the cryopreserved seeds treated with different pre-cryopreservation treatments germinated and developed to normal seedlings. Seedling establishment of the cryopreserved seeds was lower than the control seeds (Table 7). This reveals that cryopreserved seeds in soil environment suffered from higher mortality than the control seeds which may be due to negative effects of ultra-low temperature on seed vigor under soil conditions (severe than the laboratory conditions, Table 4). In this regard, it can be concluded that in greenhouse conditions, the damaged seeds and seeds with low vigority were not able to tolerate the soil environment and suffered mortality. This mortality was not relevant to cryopreservation period or duration, since the number of seedlings established in Desiccation statistically did not change over the incubation periods of 1 Week, 1 Month, and 1 Year. Although in 30% Glycerol and PVS2, the number of established seedlings was significantly lower than that of the Desiccation, almost similar patterns were observed.

In contrast to the results of laboratory experiments, the number of established seedlings in 30% Glycerol was lower than PVS2 pre-cryopreservation treatment. This reveals the different responses of cryopreserved seeds to different germination conditions and media. In most cases, vigority of seeds, organs or cells reduced just after entering into cryogenic conditions and remained almost constant over the incubation period (Ozden-Tokatli *et al.*, 2007; Walters *et al.*, 2004; Naderi Shahab *et al.,* 2013; Wood *et al.*, 2003).

Natural habitat

From 3 successive years of seed sowing experiments in natural habitat of the species in Rigboland sand dunes, only the seeds sown on 7th December 2009 germinated successfully and the seedlings were established in early spring. In 2009, rainfall occurred from late autumn to early spring. Control and cryopreserved seeds germinated well although the germination rate and consistency were

poor (Fig. 3, middle). Because the number of seedlings in some of the pits was high, the number of seedlings was thinned to one per pit. However, the results revealed that in natural habitats, percentage of seeds developed into the established mature plants was significantly low. Furthermore, in natural habitats, seed germination, seedling establishment, and mature plant development were occasional and occurred in some years with particular climatic conditions. In natural habitats, plant propagation is mainly occurring through vegetative propagation. Therefore, plant propagation through seeds is rare. Since plant propagation through seed increases genetic variation and plant diversity, the replantation of natural habitats via seed is an important issue.

Survey of the plant natural habitats revealed that the species exclusively grew on sand dunes. The species had vertical and horizontal roots. The horizontal or lateral roots (approximately 25m in length) are within the top 15-30 cm of soil. It seems that the horizontal roots in some extent quickly absorb top soil water, oxygen, and soil minerals and they are responsible for vegetative propagation in a natural habitat. The species altitudinal range in Rigboland is around 980-1085 m and in Choupanan, it is around 920-950 m above sea level. Generally, on sand dunes of Iran, *S. iranica* is grown between 900 and 1200 m in elevation. Annual mean precipitation in Rigboland is approximately 90 mm and usually occurs from early January up to late March and therefore, the dry season is approximately 9 months. The temperature from mid-spring up to mid-autumn is hot and dry. However, Kapustina (2001) pointed out that the species altitudinal range in Kyzylkum desert of Uzbekistan was 100-300 m above sea level with annual rainfall of 150 mm. These figures are quite different from the altitudinal range of the species in Iran. The reported annual rainfall of 150 mm by Kapustina (2001) is much more than annual rainfall of the species habitat in Iran.

Seed sowing depth and salinity effects on germination of Control seeds

Salt tolerance experiment revealed that the species was not a salt tolerant species because salinity of the habitat soil was low (0.371 dS/m) and salt tolerance of the species was significantly lower than that of the *M. sativa* as a relatively salt sensitive legume crop species (Grattan *et al.*, 2004). Taking into account the discussion on plant habitat characteristics (discussed before), it can be concluded that although this species is a drought and heat tolerant plant, it is sensitive to soil salinity.

The results of the sowing depth showed that the depth of 2.5 cm was the optimum depth for rapid seed germination and seedling emergence under greenhouse conditions (see Fig. 5). However, in natural habitats, shallow sowing depth caused rapid drying of the top soil and seeds encountered dryness shortly after rainfall. Deep sowing depth also causes a reduction in seed germination and delays the seeding emergence. Based on the results, it can be concluded that empirically, the optimum seed sowing depth in natural habitats of Iran is around 6 cm. A very important point regarding seed sowing in natural habit that should be kept in mind is seed scarification before sowing because seed scarification causes rapid seed germination and enhances germination percent.

Conclusion

S. iranica seeds are orthodox and are able to tolerate cryogenic (-196°C) conditions. Under cryogenic conditions, the cryopreserved seeds maintain their germination ability and vigor as same as control seeds. Lack of significant

differences among the cryogenic preservation durations indicated the possibility of long term *S. iranica* seed storage under cryogenic conditions. The cryopreserved seeds germinated and developed to normal seedlings under greenhouse conditions. Although the cryopreserved seeds under natural conditions in plant habitat germinated and grew to seedlings and young vigor plants, the establishment rate was fairly low. Significant differences among pre-cryopreservation treatments under laboratory, greenhouse, and natural habitat conditions revealed the differences related to the efficacy of the pre-cryopreservation treatments. In this regard, Desiccation showed the best results regarding protection of *S. iranica* seeds under cryogenic conditions. Hence, seeds of the *S. iranica* can be collected from various natural habitats of the species, pre-treated with Desiccation method and preserved under cryogenic conditions for a long period. If the species is threatened or endangered due to such factors as destruction of habitat, overgrazing, pests, diseases or other natural and human-made factors in near or far future, it will be possible to use the cryopreserved seeds to replant and rehabilitate the natural habitats using the plant establishment methods as described above.

To replant the natural habitats, the following considerations must be taken into account. The plant only grows on almost non-saline sand dunes at altitudes between 900 and 1200 m above sea level. Preferred seed sowing time is late autumn or early winter and the suitable seed sowing depth in natural habitats is around 6 cm. For rapid seed germination and enhancement of germination percent, seed scarification should be carried out before sowing. Annual precipitation of the species habitats is about 90 mm. Nevertheless, the seedling establishment and plant growth are occasional and depend highly on environmental factors and precipitation characteristics such as intensity, occurrence, and total amount.

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نگهداری بذر دُم‌گاوی (*Smirnovia iranica* (sabeti)) در شرایط فراسرد و ارزیابی بذور فراسردی در شرایط آزمایشگاه، گلخانه و عرصه رویشگاهی

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چکیده. دُم‌گاوی (*Smirnovia iranica* (Sabeti) synonym: *S. turkestana* (Bunge)) گیاهی است خزان کننده و چندساله از خانواده پروانه آسا (Fabaceae). این گونهٔ گیاهی بر روی تپه‌های شنی عرصه‌های بیابانی رشد می‌کند و دارای ریشه عمیق عمودی و ریشه طویل افقی است. این گونه گیاهی نقش مهمی در پوشش بیابانی و تثبیت شن‌های روان دارد. گستره محدود رویشگاهی، چرای گیاه به‌دلیل خوش خوراکی و کیفیت خوب آن باعث گردیده این گیاه در معرض تهدید قرار گیرد. ذخیره‌سازی بذر در شرایط فراسرد یا ۱۹۶°C- روش مهمی برای نگهداری طولانی مدت بذر می‌باشد. در این بررسی بذور دُم‌گاوی از عرصه‌های طبیعی گیاه جمع‌آوری و با سه پیش تیمار شامل PVS2، آب‌گیری (Desiccation) و گلیسرول ۳۰٪ تیمار و به مدت یک هفته، یک ماه، و یک سال وارد ازت مایع (۱۹۶°C-) (در سال ۱۳۹۵) گردیدند. پس از خروج بذور از نیتروژن مایع یا شرایط فراسرد در معرض شوک حرارتی قرار گرفته و تحت شرایط آزمایشگاه، گلخانه، و رویشگاه طبیعیِ گیاه کشت و مورد بررسی قرار گرفتند. بذوری که برای مدت‌های متفاوت در شرایط فراسرد ذخیره شده بودند تفاوت معنی‌داری در صفات مختلف جوانه‌زنی نشان ندادند. بیشترین درصد جوانه‌زنی بذر (۸۴٪) در تیمار آب‌گیری (Desiccation) مشاهده گردید. در شرایط گلخانه، پیش تیمارهای فراسردی و زمان‌های ذخیره‌سازی بذر در فراسرد رفتارهای متفاوت و معنی‌داری در رابطه با جوانه‌زنی و استقرار بذر نشان دادند. در شرایط عرصه رویشگاهی، بذور فراسردی جوانه زده و تا مرحله گیاه جوان رشد و مستقر گردیدند. نتایج این بررسی نشان داد که می‌توان بذر دُم‌گاوی را برای مدت بسیار طولانی در شرایط فراسرد ذخیره‌سازی نمود.

کلمات کلیدی: دُم‌گاوی، *Smirnovia iranica*، *Smirnovia turkestana*، جوانه‌زنی بذر، فراسرد، PVS2 Desiccation