Research Article

Seed Cryopreservation of Some Medicinal Legumes

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Seed survival after storage in liquid nitrogen ($-196°C$) was examined in 12 wild medicinal legume species occurred Far East of Russia. Dry seeds of all species survived cryostorage without loss of viability. Initial germinability varied from 3 to 85%. The stimulatory effect of cryogenic temperature on germination, with or without subsequent chemical scarification, was observed in all species studied with deep physical dormancy or heterogeneous levels of hardseededness. Frozen seeds demonstrated higher germination percentages (the percentage of germinated seeds) and germination rates (time for first seed to germinate (T_0) and time required (in days) to reach 50% of the final germination percentage (T_{50})) than the control ones. The anomalous seedlings were not observed after storage of seeds in liquid nitrogen. This study shows that cryostorage may be successfully applied for conservation of native species without detrimental effects on germination and growth.

1. Introduction

Fabaceae family is the one of the richest families of Russian Far-Eastern flora, and it includes a lot of valuable medicinal plants. The set of Far-Eastern legume species arouse particular interest due to pharmaceutical properties. *Astragalus membranaceus* (Fisch.) Bge., a herbaceous perennial, inhabits the western to northern part of China, Korea, and Japan. This herb is one of the important medicinal plants used as an adaptogenic in China, Korea, and Japan [1]; compounds from *A. membranaceus* roots may be utilized as immunostimulants, tonics, diuretics, antidiabetics, and sedatives [2]. *Hedysarum austrokurilense* (N.S. Pavlova) N.S. Pavlova and *H. sachalinense* B. Fedtsch. are perennial herb species endemic to the Sakhalin Island and South Kuril Islands [3]. The chemical components found in the plants of both related species include flavonoids, xanthones-mangiferin and isomangiferin, and polysaccharides many of them are of interest for traditional and modern medicines [4]. A lot of representatives of the genus *Oxytropis* have attracted attention thanks to the wide use in Tibetan and Mongolian medicine and also in folk medicines of Siberia and central Asia as the effective drugs [5]. The plants of some *Oxytropis* species are the richest in an alkaloid content [6], and alkaloid from *O. ochrocephala* has antitumor activity [7]; one can suppose that other *Oxytropis* species (*O. chankaensis* Jurtz., *O. kamtschatica* Hult., *O. ochotensis* Bunge, *O. revoluta* Ledeb., and *O. retusa* Matsum.) have also valuable bioactive substances. *Sophora flavescens* Soland., a perennial shrub, has been used in the herbal medicine for centuries in China, Japan, and Korea; the dried roots of *S. flavescens* have well-known antibacterial, antiviral, antiprotozoal, anti-inflammatory, antitumor, and antipyretic effects ([8] and references herein, [9]). A perennial herb *Trifolium lupinaster* L. contains flavonoids and vitamins and has been used in Tibetan medicine against hepatitis [10]. *Vicia amurensis* Oett. is a perennial plant occurred in northern China, Japan, Korea, and Manchuria, contained flavonol glycosides [11], and used against tick-borne encephalitis and respiratory infections [12]. It was shown that alcohol extract of *Vicia subrotunda* seeds has antioxidant properties in experiments [13].

Nowadays, these legume species are attracting increasing attention of the modern pharmaceutical industry; however, natural resources of medicinal plants are limited, so it is reasonable to bring medicinal legumes under cultivation by seed conservation and reproduction. Cryopreservation (storage in liquid nitrogen (LN) at $-196°C$) is the only currently available long-term storage technique that ensures

Table 1: Seed collection site and ecological preference for 12 wild legume species studied.

Species	Collection site	Distribution and ecological preference
Astragalus membranaceus	Primorye, Muraviev-Amurskii Peninsula	Widespread (Russia-Siberia, Far East; Mongolia, China, Korea)/Forest meadows, dry meadows, steppe
Hedysarum austro-kurilense	Sakhalin, Makarov region, Magutan mud volcano	Endemic of North Sakhalin, South Kurils/Rocky hillsides, coastal terraces
H. sachalinense	Sakhalin, Makarov region, Tikhaia River, rocky hillsides	Endemic of Sakhalin/Coastal cliffs, rocky hillsides
Oxytropis chankaensis	Primorye, Khanka Lake, Sosnovyi Island	Endemic of Primorye, west coast of Khanka Lake/Sandy lakeshores
O. kamtschatica	Kamchatka Peninsula, Central Kamchatka, Kljuchevskaia Sopka volcano	Endemic of Far East of Russia (South Chukotka, Koryak Coast, Kamchatka Peninsula)/Alpine tundra, rock debris, old lava flows, shingle beach
O. ochotensis	Kamchatka Peninsula, Central Kamchatka, Kljuchevskaia Sopka volcano	Regional (Russia-East Siberia, Far East (West Chukotka, Kolyma, coast of the Okhotsk Sea, Kamchatka Peninsula))/Alpine tundra, cliffs, rocky hillsides
O. revoluta-1	Kamchatka Peninsula, Central Kamchatka, Tolbachik volcano	Regional (Russia-Far East (Koryak Coast, Kamchatka Peninsula, North Kuril Islands); Aleutians)/Alpine tundra, stone, rock debris, old lava flows, tephra, slag fields
O. revoluta-2	Kamchatka Peninsula, Central Kamchatka, Kljuchevskaia Sopka volcano	As before
O. retusa	Kuril Islands, Paramushir Island, Shelihov Bay, coastal rocky hillside	Endemic of Kuril Islands/Coastal cliffs, rocky hillsides
Sophora flavescens	Primorye, Hasan region, Gamov Peninsula	Widespread (Russia-East Siberia, Far East; Mongolia, China, Japan)/Forest meadows, dry meadows, river banks, xerophytic scrub
Trifolium lupinaster	Primorye, Ternei region, Svetlaya Bay, rock outcrops	Widespread (Russia-Siberia, Far East; Mongolia, China, Japan)/Open forest, meadows, shores
Vicia amurensis	Primorye, Muraviev-Amurskii Peninsula, sea shores	Widespread (Russia-East Siberia, Far East; China, Japan/Hillsides, roadsides, old railway, xerophytic scrub, meadows

safe conservation of genetic resources of valuable plant species [14–16]. Orthodox seeds of wild-growing legumes (moisture content from 5 to 12%) are one of the most convenient systems available for prolonged storage of genetic information. Cryogenic storage has been successfully carried out in many orthodox seed species [15–20]; however in some species authors pointed out problems after cryopreservation, such as cotyledon breakage [15, 16, 21], abnormal germination [1], or seed death by internal freezing injury [22]. In these cases, the role of seed characteristics, in particular seed size has been studied. The conventional regime of seed storage in seed banks at 5°C is not reliable, because low above-zero temperatures retard the loss of viability but fail to ensure its long-term preservation. The aim of the present study was to evaluate the effect of cryopreservation on the twelve Far-Eastern wild-growing legume species with different seed characteristics (size and mass of seeds) for their *ex situ* conservation. The germination of wild legumes is often complicated by physical dormancy which may require scarification for germination, and thus combinations of dormancy-breaking procedures and exposure to LN have been tested to examine the interaction of cryopreservation and

scarification. The results obtained suggest that seeds of species studied have the ability to tolerate cryostorage.

2. Material and Methods

2.1. Plant Material. The study was performed from 2002 to 2008. Mature seeds of twelve wild legume species were collected from wild plants growing in natural habitats from different regions of Far East of Russia (Table 1). Collected seeds stored in tightly closed bags in a laboratory at ambient temperature for 2–4 months until assays were carried out. Nomenclature, range, and distribution for species are according to the monograph on vascular plants of the Russian Far East [3]. For each species or population, seed mass was determined by weighting three samples of 100 seeds each; seed size, by measuring 25 seeds from each sample (Table 2). A batch of 50 seeds from each species was subjected to moisture determination by oven drying at 105°C for 24 h, when the seeds had reached constant dry mass. Moisture content (MC) was obtained from 3 independent determinations and expressed as mean percentage of fresh weight (Table 2). For 3 species (*Hedysarum austrokurilense*, *H. sachalinense*,

TABLE 2: Rate of germination and final germination percentage (G) of Far-Eastern legumes after scarification, cryopreservation (LN), or cryopreservation and scarification (scarification after LN). MC: seed moisture content. L: seed length. SM: seed mass, g per 100 seeds.

Species	Control			Scarification			LN			Scarification after LN			$G \pm SE$, %*	MC, %	L, mm	SM, g
	T_0, days	T_{50}, days	$G \pm SE$, %	T_0, days	T_{50}, days	$G \pm SE$, %	T_0, days	T_{50}, days	$G \pm SE$, %	T_0, days	T_{50}, days	$G \pm SE$, %				
Astragalus membranaceus	2	11	3.3 ± 1.2	1	2	99.3 ± 1.2	2	11	14.0 ± 3.5	2	2	75.3 ± 6.1	89.3 ± 4.2	6.90 ± 0.18	2.8 ± 0.03	0.4 ± 0.01
Sophora flavescens	7	10	3.0 ± 1.0	3	7	86.8 ± 7.9	4	13	48.9 ± 1.9	4	4	29.9 ± 4.3	78.8 ± 3.3	8.60 ± 0.04	4.86 ± 0.06	4.0 ± 0.18
Trifolium lupinaster	1	7	8.0 ± 2.0	1	2	94.0 ± 5.3	1	3	84.0 ± 2.0	1	2	12.7 ± 1.2	96.7 ± 2.3	7.98 ± 0.75	2.0 ± 0.04	0.33. ± 0.01
Oxytropis chankaensis	2	6	6.0 ± 2.0	2	2	80.7 ± 8.1	2	6	91.3 ± 4.6	2	2	4.7 ± 1.2	96.0 ± 3.5	7.05 ± 0.34	2.2 ± 0.03	0.24 ± 0.003
O. kamtschatica	2	11	68.0 ± 1.2	2	3	93.6 ± 4.2	2	9	89.3 ± 4.8	—	—	—	—	6.64 ± 0.77	2.26 ± 0.02	0.25 ± 0.005
O. ochotensis	2	12	36.0 ± 1.2	2	2	87.3 ± 5.2	2	9	62.0 ± 3.1	—	—	—	—	7.10 ± 0.18	1.98 ± 0.03	0.2 ± 0.002
O. retusa	6	10	9.0 ± 1.0	2	3	67.0 ± 2.0	4	7	41.3 ± 6.4	—	—	—	—	5.74 ± 0.71	2.68 ± 0.04	0.41 ± 0.003
O. revoluta-1	2	33	42.0 ± 2.0	2	2	79.3 ± 1.9	2	6	84.7 ± 7.1	—	—	—	—	6.68 ± 0.25	1.79 ± 0.03	0.13 ± 0.001
O. revoluta-2	2	25	49.3 ± 4.8	2	2	79.3 ± 3.0	2	7	80.7 ± 2.7	—	—	—	—	6.45 ± 0.40	1.81 ± 0.03	0.13 ± 0.002
Hedysarum austrokurilense	1	8	70.7 ± 1.3	1	2	87.7 ± 4.6	1	7	85.0 ± 1.5	—	—	—	—	—	2.84 ± 0.05	0.47 ± 0.011
H. sachalinense	1	2	85.3 ± 1.8	—	—	—	1	3	73.0 ± 7.5	—	—	—	—	—	4.46 ± 0.10	0.93 ± 0.056
Vicia amurensis	6	11	30.0 ± 4.2	2	3	100	5	11	33.3 ± 2.7	—	—	—	—	—	3.35 ± 0.04	2.47 ± 0.06
V. subrotunda	8	26	5.3 ± 0.7	2	3	74.3 ± 1.8	5	16	53.3 ± 7.1	—	—	—	—	6.97 ± 0.23	3.79 ± 0.07	2.57 ± 0.123

* Summarized germination percentage after cryopreservation (LN) and scarification (scarification after LN).

Vicia amurensis), MC was not determined due to small number of seeds. No desiccation procedures were carried out due to low level of seed moisture. Seeds of species tested contained 5.7–8.6% moisture; seed mass ranged from 0.13 to 4.0 g per 100 seeds (Table 2).

2.2. Cryopreservation. The seeds in tightly closed aluminium foil bags were immersed directly into liquid nitrogen ($-196°C$) and stored for 1 month, then warmed at room temperature ($21 \pm 3°C$) for few hours before planting in germination dishes. Unfrozen seeds remained at room temperature.

2.3. Germination Test. For all treatments, the germination tests were carried out at 22–25°C under a 16 h light/8 h night photoperiod. Three replicates of 50 seeds were placed on moist filter paper in Petri dishes and watered daily with tap water. The tests were monitored every day, and germinated seeds (radicle extension ≥2 mm) were counted and removed. Incubation was continued until it was obvious that no further germination could occur. The first test was a control germination without any treatments. The second one presents germination after scarification. The water-impermeable legume seeds before plating in Petri dishes were scarified with a concentrated sulphuric acid: *O. kamtschatica*, *O. ochotensis*, *O. revoluta*, *O. retusa*—20 min; *A. membranaceus*, *T. lupinaster*, *H. austrokurilense*—30 min; *S. flavescens*—45 min; *V. amurensis*, *O. chankaensis*—60 min; *V. subrotunda*—70 min. Then the seeds were washed in tap water and sown as described previously. The third attempt was germination after cryogenic storage. For most species, the major part of not-germinated after-cryopreservation seeds became soft and decayed. In four species—*A. membranaceus*, *O. chankaensis*, *S. flavescens*, *T. lupinaster*, the seeds, which failed to germinate during 2 months after cryopreservation, remained intact and undamaged. Then they were treated with a concentrated sulphuric acid, then washed in a tap water, and sown to evaluate a combined effect of cryopreservation and scarification. For each treatment, time of initial germination (T_0) was determined, and final germination percentage (mean values $\pm$ SE) and a number of days needed to reach 50% of the final germination percentage (T_{50}) were calculated. To analyse the significance of differences between mean values, analysis of variance (ANOVA) was applied, as was the Tukey test for pairwise comparisons. The Tukey test was performed after arc-sine transformation at a significance level of $P < 0.05$.

In two species—*H. austrokurilense*, *H. sachalinense*, the seedling emergence tests, like the germination tests, were conducted out at 22–25°C under 16 h light/8 h night photoperiod, until the seedlings reached ca. 2-3 cm in height. Seedling vigor was measured by a shoot and root length on 15 d after sowing. Twenty five plantlets were measured in each sample.

In *S. flavescens*, plantlets from the control and cryopreserved seeds were transplanted to 1-L pots with a soil to assess the quantitative traits (plant height, leaf size and number of the leaves, and root diameter) over three months (by measuring 30 plantlets from each sample). The data were analyzed using Student's *t*-test with significance at $P = 0.05$.

3. Results

In control, the time of initial germination varied from 1 to 8 days, with both *Hedysarum* and *T. lupinaster* seeds germinating most rapidly. The germination of studied species seeds varied considerably, ranging from 3 to 85%; this indicates the different levels of hardseededness. The seeds of six species (*A. membranaceus*, *O. chankaensis*, *O. retusa*, *S. flavescens*, *T. lupinaster*, *V. subrotunda*) showed very low germination percentages (less than 10%). The germination rate also showed evident differences among species, with slowest rates in *V. subrotunda* and two populations of *O. revoluta*. The depth of hardseededness is species-specific and does not depend on seed size.

After scarification, germination was substantially higher and much faster as compared with control. It is indicative that seed dormancy was only of the physical type. For the most species T_0 and T_{50} generally occurred at the same time (2-3 days after incubation), regardless of seed size. Seeds of *A. membranaceus*, *O. chankaensis*, *S. flavescens*, and *T. lupinaster* after scarification germinated about 30–40 days, though most of them germinated in first 10–14 days (Table 2). For the rest of the species, there were no any marked differences among them with respect to the germination rate; all seeds germinated rapidly for 7–12 days. Seed size and weight did not affect germination of scarified seeds apparently.

No damage was observed in control and scarified seeds. Cryopreservation was successful, and seeds of all species began to germinate after exposure to a cryogenic temperature. All tested species, except *H. sachalinense* and *V. amurensis*, exhibited a significant positive response to the 30-day exposure to LN when compared with control. Seed germination of *H. sachalinense* after cryostorage was high (≥70%) and did not differ significantly from control ($P = 0.203$), while for *V. amurensis* one was rather low (Table 2) and also did not differ significantly from control ($P = 0.527$). Both populations of *O. revoluta* respond identically to the treatment though they differ in the initial levels of hardseededness. Cryostorage generally did not influence T_0, with an exception of seeds of *O. retusa*, *S. flavescens*, and both *Vicia* species, which germinated earlier than in control. For all species T_{50} decreased to 3–16 days.

The germination rate of cryopreserved seeds was generally higher than for the unfrozen seeds, but lower than for scarified ones. No general trends between seed size and the ability of seed to survive to LN storage were observed. Nevertheless, a germination of the large seeds with high and moderate levels of hardseededness (*S. flavescens*, *V. amurensis*, *V. subrotunda*) did not exceed 50% after LN exposure, while a germination of the small seeds with high levels of hardseededness (*O. chankaensis*, *T. lupinaster*) was above 80%. For all species with small seed size (*O. chankaensis*, *O. kamtschatica*, *O. ochotensis*, *O. revoluta*, *T. lupinaster*) germination after cryostorage was very high (60–90%). For these species and *H. austrokurilense* there was no significant differences

between germination after scarification and LN treatment. A germination of *A. membranaceus, O. chankaensis, S. flavescens,* and *T. lupinaster* after freezing was enhanced by a scarification of the nongerminated seeds (Table 2). *A. membranaceus* and *S. flavescens* seeds reached significantly higher germination percentage than before scarification (from 14 to 89% and from 49 to 79%, resp.).

Despite some variability in the seed germination among species studied, all seeds germinated after storage in LN developed into the normal seedlings with healthy shoot and root formation; no diseases were observed. The initial stages of seedling ontogenesis in the control and experimental samples of *Hedysarum* species went on synchronously. The seedlings coming from frozen and control seeds were morphologically and developmentally similar; that is, we could not detect differences in growth rate of shoot and root and in stem and root shape as well.

For *S. flavescens*, the formation of juvenile plants (an appearance of first two leaves) from the control seeds began earlier than from seeds stored in LN. There were no significant differences in the plant height, size and number of leaves, and root diameter between the plantlets derived from cryopreserved and control seeds during the entire period of culturing [23]. It is indicative of normal development of plantlets derived from the seeds treated by LN. Only slight increase of a root diameter was found in the 70-day-old seedlings from frozen seeds.

4. Discussion

The effect of an exposure to ultralow temperature (liquid nitrogen, LN) on germination of the seeds was investigated in 12 wild medicinal legume species occurring Far East of Russia. The results show that the seeds of all investigated species can survive cryostorage.

There was no trend between seed size and survival of seeds after LN treatment. We observed higher germination percentages after cryostorage in the species with small seeds as compared to species with large seeds, which could be conditioned by presence of physical dormancy (hardseededness) in the tested species. The only exception was *A. membranaceus* seeds (0.4 ± 0.01 g per 100 seeds) with the lowest germination after cryostorage, which evidently had a highest level of hardseededness, and scarification following LN treatment, allowed to reach high germination values (above 90%).

The seeds of the studied species have a hard seed coat with a layer of macrosclereids (palisade-like cells with strongly lignified walls), typical for many wild Fabaceae species [24, 25], and most part of them is characterized by high and moderate levels of hardseededness (Table 2). A physical dormancy often occurs as a means of adaptation to the different habitats with the severe climatic conditions, and such seeds are highly resistant to unfavorable environmental factors [26, 27]. It is low permeability of hard seed coat that may play some role in the cryotolerance of these seeds [19, 28]. However, dormancy poses one of the main problems for work with wild species [15, 29]. For the tested seeds of wild legumes, apparently, immersion in LN effects as a means of

overcoming seed dormancy like scarification. It was reported that seed coat cracks, formed in the seeds impermeable to water during freezing, favoured a water uptake, and consequently germination was stimulated [30].

The final germination capacity of cryostored seeds differed significantly from nonstored ones for most species studied except *H. sachalinense* and *V. amurensis*. The stimulatory effects of LN exposure were revealed for other wild legumes with hard seeds [1, 15, 21, 31, 32]. Besides, after LN exposure, the seeds required less time to germinate than controls. In agreement with those results, it could be permitted to correlate them to the beneficial effects of low-temperature exposure, namely, stimulating germination and increasing germination rate. In addition, previous scarification or other dormancy-breaking treatment before germination seems not to be necessary for the most of these seeds. However, for seeds with deep physical dormancy LN is not effective scarifying agent, as it was shown for seeds of some *Rubus* species [33] and for seeds of *A. membranaceus, S. flavescens,* and *V. amurensis* in the present study. At the same time, for *A. membranaceus* and *S. flavescens* LN treatment, when combined with chemical scarification, favoured the germination resulting in considerable increase in germination percentage (Table 2). The similar action of combined treatment by LN and sulfuric acid has been observed in some orthodox legume seeds of Brazilian tropical species [32]. Necessity of scarification (mechanical or chemical scarification, heat treatment) combined with cryogenic storage to enhance the final germination percentage was shown for other species with hard seed coats [28, 29, 34]. The intensity of seed hardness varies among species and within individual species (e.g., in populations of *O. revoluta*), possibly due to the differences in their seed coat structure, physical, and chemical properties [25]. These differences might be reflected in the seeds response to LN treatment. As it is shown in previous studies, it is not possible to predict the behaviour of the seeds of a species after cryopreservation by their size or by taxonomic relatedness [19, 35]. Therefore, it is important to continue screening different species for effective cryostorage and to investigate possible interactions with germination enhancement treatment.

In our experiments, cryopreserved seeds of *Hedysarum* species and *S. flavescens* developed into the normal seedlings and plants. Similar results were reported by Tikhonova [36] for 30 wild-growing plant species: plant growth and development was not adversely affected by cryopreservation. The normal growth of plants from the seeds treated by LN was shown for two Spanish endemic species [35] seven species of Orchidaceae [37, 38]. It appeared that LN exposure only or combined treatments did not induce seed physical damage or abnormal seedling development for all species studied.

Thus, the obtained results have demonstrated that the seeds of the Far-Eastern medicinal legume species can be deep-frozen at $-196°$C without loss of viability. Taking into account the tolerance to liquid nitrogen, cryogenic storage would be a suitable method for a long-term conservation of wild medicinal legumes. Additionally, the very small size of seeds of the most studied species results in an effective cryogenic procedure which is easy to operate.

Acknowledgments

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