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Cryopreservation the seeds of the medicinal plant *Satureja bachtiarica* Bunge

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Abstract

Many species of the genus *Satureja* are reported to have aromatic and medicinal properties. Seed storage techniques at very low temperature (-196 °C) using liquid nitrogen is one way of maintaining germplasm with much less cost and without loss of viability on storage. In order to preserve *Satureja bachtiarica* seeds under -196 °C condition, three protocols, Plant vitrification solution (PVS2), Desiccation and Glycerol were studied. The treated seeds were transferred into Liquid Nitrogen (LN) and stored for 7, 30 and 90 days respectively. In this study the effect of cryopreservation on germination and growth indices (germination percentage, germination rate) of plant species (*S. bachtiarica*) in storage conditions of cryopreservation (-196°C) were evaluated for 7, 30 and 90 days. This study showed that liquid nitrogen did not affect growth indices (germination percentage, germination rate) from cryopreserved seeds when compared with control seeds. So the seed of this endangered species can be preserved for a long period. According to the results there were no significant differences among 7, 30 and 90 days liquid nitrogen storage treatments in trait germination percentage and rate. This study revealed that cryopreservation did not have significant effects on seed germination percentage and germination rate of this species. Therefore cryopreservation is a suitable procedure for the long-term preservation of *S. bachtiarica*.

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Introduction

The genus *Satureja* belongs to the family Lamiaceae and over 30 species of this genus are distributed in the eastern parts of the Mediterranean area (Cantino *et al.*,1992). Many species of the genus *Satureja* are reported to have aromatic and medicinal properties. The aerial parts of these species such as leaves, flowers, and stems are used in traditional medicine, to treat various disease for example cramps, nausea and infectious diseases (Eminagaoglu *et al.*, 2007). It has been reported that *Satureja,* having the essential oils, play an important role in the protection of the plants by acting as anti-bacterial, anti-viral, and anti-fungal agents. Some oils have also been shown to elicit anti-oxidative, anti-genotoxic (Ahmad *et al.*, 2012; Alpsoy *et al.*, 2011; Goncalves *et al.*, 2012).

S. bachtiarica, having a special place among medicinal species in terms of producing valuable essential oil is an endemic plant of Iran. Reports on the composition of essential oils from the aerial flowering parts of *S. bachtiarica* from Yazd-Iran showed the list of major components as Linalool (4.6%), p-cymene (15.2%) carvacrol (66.5%) (Sefidkon and Jamzad, 2005).

Ethanolic extract of *S. bachtiarica* have antimicrobial activity on microorganisms. *S. bachtiarica* extract showed the more effective impact on the growth of *B. subtilis* PTCC 1720 and *L. innocua* ATTC 33090 than *E. aerogenes* ATTC 13048 ($p<0.05$). According to result, ethanolic extract of *S. bachtiarica* had maximum effect on Gram positive bacteria *B. subtilis* PTCC 1720 and *L. innocua* ATTC 33090 (Heidari-Sureshjani *et al.*, 2014). *S. bachtiarica* methanolic extract can improved Beta amyloid induced memory impairment and can be a candidate for treatment of Alzheimer disease (Soodi *et al.*, 2012). Methanolic extract of seven plants from *salvia* and *satureja* species were evaluated for their protective effects against beta-amyloid induced neurotoxicity. *Satureja bachtiarica*, *Salvia officinalis* and *Salvia macrosiphon* methanolic extracts exhibited high protective effects against Aβ induced toxicity Protective effects of *Satureja bachtiarica* and *Salvia officinalis* were dose-dependent (Balali *et al.*, 2012).

Conservation of genetic resources of *S. bachtiarica* is important. Because the life of this valuable species has been threatened by biotic and abiotic stresses. Through the recent remarkable progress in cryobiological studies of plant materials, cryopreservation has been recognized as a practical and efficient tool for long-term storage of plant germplasm with minimum space and maintenance requirements. Cryopreservation has an advantage of long-term storage without subjecting frequent subculturing which is known to induce somaclonal variation (Jain, 2011).

Efficient cryopreservation protocols have been designed for numerous crop and medicinal plant seeds like *Thymus lotocephalus* (Coelho *et al.*, 2012) Citrus (Graiver *et al.*, 2011). The germination ability of seeds of the 11 endemic species (*Ermania parryoides*, *Hedysarum austrokurilense*, *H. sachalinense*, *Myosotis sachalinensis*, *Oxytropis chankaensis*, *O. kamtschatica*, *O. retusa*, *Saxifraga purpurascens*, *Stellaria eschscholziana*, *Vaccinium vulcanorum*, *Vicia subrotunda*) from the Russian Far East did not change after the effect of ultra-low temperatures (–196°C)(Voronkova and Kholina, 2010). Germination percentage and rate of cryopreserved seeds of *Satureja rechingeri* decreased after the effect of ultra-low temperatures (–196°C)(Shahbazi *et al.*, 2014).

However, no information is available on cryopreservation of *S. bachtiarica* germplasm. In comparison, desiccation is a simple cryopreservation procedure which does not required the use of expensive cooling equipments.

Material and methods

Plant material

S. bachtiarica seeds were collected from Yazd Province located in 12 km southwest of Mehriz (latitude: N 31° 31' 1", longitude: E 54° 18' 56", Altitude: 2357 m). Before transferring seed samples in liquid nitrogen (temperature -196°C), three

protocols were tested for cryopreservation:

Vitrification with PVS2

The complete vitrification procedure involves (a) loading of seeds with a mixture containing 2 M glycerol and 0.4 M sucrose for 20 min (b) dehydration of seeds by exposing them to a PVS2 (Sakai *et al*. 1990) for 60 min at 4 °C. The PVS2 contains (w/v) 30% glycerol, 15% ethylene glycol (EG) and 15% dimethyl sulfoxide (DMSO) in medium containing 0.4 M sucrose (pH 5.8) (c) transferring the cryotubes in liquid nitrogen.

Glycerol

Seeds were transferred into cryotubes containing glycerol. They were incubated for 60 min at 4°C and subsequently transferred into liquid nitrogen.

Desiccation

For determination of water contents, seeds were weighed and dried in an oven at 103 °C for 17 h (ISTA 1996). Water content was calculated on a fresh weight basis (FW). In order to reduction moisture, seeds were placed in an airtight desiccator containing silica \ gel for 7 days at 4°C (Table 1) and immediately submerged in liquid nitrogen. Non-cryopreserved seeds were stored at +4°C temperature for 7, 30 and 90 days as a control.

All treatments except control were immersed in liquid nitrogen with temperature of -196 °C for 7, 30 and 90 days. Afterward, the cryotubes were rapidly thawed in a warm water bath at 40 °C for 100 sec (Engelmann, 1990). The seeds were then transferred to MS medium supplemented with 1.2 M sucrose for 20 min at 25°C.

Due to reduction ice crystal, after thawing, seeds were sterilized by 1.5% hypochlorite (V/V) for 5 min. Seeds were washed three times with sterile distilled water and placed on sterile filter paper in an covered Petri dish and kept at 25 °C under a 16 h photoperiod. Three replicates were applied for each treatment containing 30 seeds. These experiments were performed twice. Germination percentage and rate were calculated after 30 days (Ruan *et al*., 2002).

Germination Percent=Ni/N × 100, where Ni is the number of germinated seeds on the ith day and N is total number of seeds.

Germination rate = ΣNi/Ti, where Ni is the number of germinated seeds in each count and Ti is the number of days after sowing.

Statistical analysis

Unless stated otherwise, A factorial experiment, consisting of two factors (treatment and liquid nitrogen exposure period), using a completely randomized design, two replications, was established. The data were analyzed using MSTATC (Michigan State University, East Lansing, MI, USA).

Results and discussion

This study revealed that pre-cryopreservation treatments such as desiccation, vitrification with PVS2, and glycerol did not have significant effects on seed deterioration under liquid nitrogen storage (Fig1,2). Based on this result, there were no significant differences among 7, 30 and 90 days liquid nitrogen storage treatments on germination percentage and germination rate. It was concluded that the increase in liquid nitrogen storage duration from 7 days to 90 days did not significantly decrease the seed germination percentage and germination rate. This study revealed that cryopreservation did not have negative effects on seed germination percentage and germination rate (Table 2).

We may conclude that using frozen seed in germination experiments is safe for establishing germination percentage and germination rate. Cryopreserved seed did not have any difference in germination compared to control seed (Fig. 3).

There were no significant differences among liquid nitrogen exposure period on germination percentage and germination rate. Popov *et al* showed the cell strains of carrot, *D. deltoidea* and *P. ginseng* successfully resumed their growth and biosynthetic

capacities after 6, 9, 12 and 14 years of storage in liquid nitrogen. The longest storage time exceeded 25 years.

This study can contribute to the development of an efficient method for long-term conservation of seeds from this *Satureja* species. In conclusion, and according to the results obtained in this work, *S. bachtiarica* can be desiccated to water contents of 3.88% and cryopreserved. The germination percentage of cryopreserved seed was 76.39% in Desiccation-treated seed of *Satureja. bachtiarica*, while Shahbazi *et al* (2014) showed that none of *Satureja rechingeri* seed, dehydrated by silica gel and transferred to liquid nitrogen (-196 °C), did not germinate.

Table 1. Seed characteristics of *S. bachtiarica*.

Species	Geographical location	Moisture content (After desiccation)	Moisture content	1000-grain weight (gr)
S.bachtiarica	12 km southwest Mehriz in Yazd	of 3.88	5.66	0.2

Beardmoreand Whittle (2005) reported that *Acer saccharinum* seed can be successfully desiccated and cryopreserved.

First report on desiccation and cryopreservation of seeds of *P. cincinnata*,*P. coriacea*, *P. foetida*, *P. giberti*, *P. micropetala*, *P. morifolia*, and *P. mucronata* was reported by Veiga-Barbosa *et al* (2013). *P. alata*, *P. cincinnata*, *P. coriacea, P. edulis*, *P. giberti*, and *P. morifolia* seeds can be desiccated to water contents of 2.1-3.6% and cryopreserved, while those of *P. foetida*, *P. micropetala*, *P. mucronata*, and *P. nitida* were damaged by desiccation but can be cryopreserved at 6.1-7.3% wc.

Table 2. Comparison of control and three protocols used for cryopreservation of seed. Factor A: Liquid nitrogen exposure period Factor B: treatment.

S.O.V	DF	Germination percent	Germination rate
Main effect (A)	2	15.524^{ns}	0.428^{ns}
Main effect (B)	3	201.624^{ns}	3.607^{ns}
AB interaction	6	26.275^{ns}	0.499^{ns}
Error	12	84.265	0.635
% CV		% 11.85	15.19%

ns: Not significant at 1% of probability level.

In our study, germination percentage between control and PVS2 -treated seeds of *S. bachtiarica* was the same. Hu *et al* (2013) showed *B. formosanaseeds* seeds can be preserved for long periods by using cryopreservation and vitrification. When seeds are treated with LS and PVS2 the germination percentage of *B. formosana* seeds during cryopreservation period was enhanced (Hu *et al*., 2013).

In glycerol treatment, glycerol, as a cryoprotectant, reduced the risks of ice crystals and prevents the formation of ice by lowering the freezing point (Turner *et al*., 2001). According to the previous studies, glycerol pretreatment have been used singly in a few plant species. Our results indicate the use of glycerol in seed cryopreservation (73.7% GP).

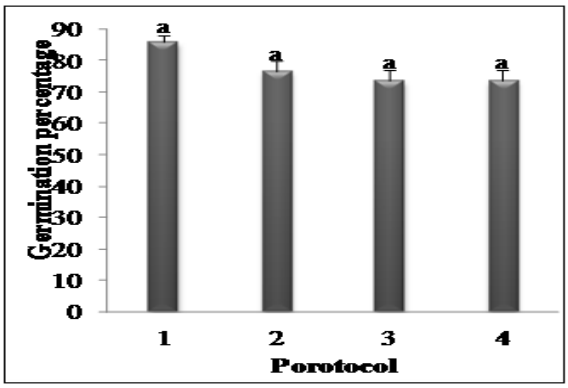

Fig. 1. Germination percentage (%) of cryopreserved seeds and control. Vertical bars: SE (n=6); 1: Control, 2: Desiccation, 3: Vitrification with PVS2, 4: Glycerol.

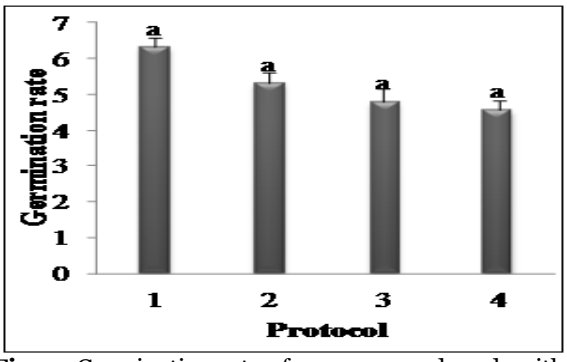

Fig. 2. Germination rate of cryopreserved seeds with control. Vertical bars: SE (n=6); 1: 1: Control, 2: Desiccation, 3: Vitrification with PVS2, 4: Glycerol.

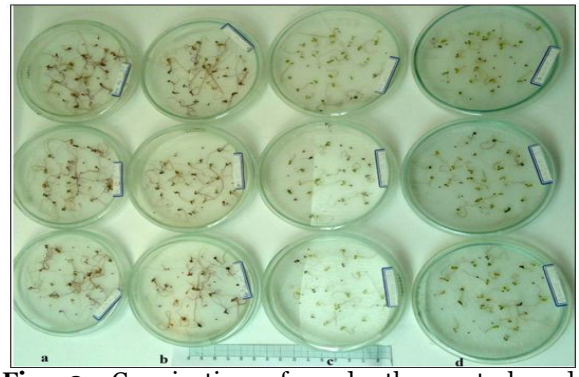

Fig. 3. Germination of seeds the control and cryopreserved seeds of *S. bachtiarica* one month after thawing. LN exposure period: 30 days. a: Vitrification with PVS2 b: Glycerol c: Desiccation d: Control.

The germination ability of seeds of *S. bachtiarica* did not change after the effect of ultra-low temperatures (−196°C).The germination of *S. bachtiarica* remained at the same level as in the control. In general, Based on this result, cryopreservation of *S. bachtiarica* seeds by above mentioned treatments is successful, but desiccation treatment (76.39% GP) is suggested for cryopreservation of *S. bachtiarica* seeds for reducing cost.

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