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Investigation on possibility of cryopreservation of *Satureja rechingeri* seeds

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Abstract

Satureja rechingeri, belonging to the Lamiaceae family, is one of the native range species of Iran. Considering the loss of *Satureja rechingeri* germplasm, due to the limited distribution, biotic and abiotic stresses, overgrazing of livestock, and generally the risks threatening the habitat of this species, fundamental actions need to be performed towards the preservation of *Satureja rechingeri* genetic diversity. Cryopreservation has the ability to preserve the seeds for a long time with much lower costs than other methods and without loss of seed viability. In the current study, treatments of vitrification, glycerol (30%), and the reduction of seed moisture before entering the liquid nitrogen were used. After one week, the treated seeds were removed from liquid nitrogen and were melted in sterile distilled water bath with a temperature of 42 °C. Then, the seeds were washed by sucrose solution (1.2 M) using shaker in three stages and were cultured with control seeds on sterile wet filter papers and transferred to a growth chamber. According to the obtained results of seed cryopreservation of *Satureja rechingeri*, no significant difference was found for all germination and seed growth characteristics except germination percentage and rate. Our results clearly indicate the effectiveness of this method for the seed preservation of *Satureja rechingeri* as a unique and endangered species for a very long time.

Abbreviations

DMSO: Dimethyle sulfoxide, PVS2: Plant Vitrification Solution 2.

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Introduction

Many studies have been conducted on seed cryopreservation of different species. Ozden-Tokatliet *et al.* (2007) reported a viability of 47% for the seeds of *P. ientiscus* under cryopreservation conditions.

Hay *et al.* (2000) studied the cryopreservation of *Najas flexiti* in liquid nitrogen. The cryopreservation of *Doritis pulcherrima* seeds in liquid nitrogen using vitrification has been reported successfully (Thammasir, 2000).

According to the findings of previous studies, the seeds of different species have been successfully cryopreserved.

Satureja rechingeri is a native range species of Iran, having a special place among medicinal species in terms of producing valuable essential oil.

Since the life of this valuable species has been threatened by biotic and abiotic stresses and excessive exploitation of natural areas always takes place, the preservation of genetic resources of *Satureja rechingeri* is of utmost importance.

Excessive population growth in recent decades and the growing pressures on natural resources, have led to the endangerment of many species of valuable natural areas. Excessive exploitation, effects of biotic and abiotic stresses, changes in atmospheric (climate) conditions, severe soil erosion, degradation by human activities, pests and diseases etc, exacerbate germplasm degradation, particularly genetic resources of natural resources arena Given the importance of the preservation of plant genetic resources, continued efforts are conducted globally to prevent the degradation of plants. This is particularly important in the case of native and endangered species.

Preservation of valuable plant species is done in several ways of which seed preservation in gene banks and at a temperature below zero degrees Celsius is of utmost importance (Saloma, 2002).

The preservation time under this condition is short and after a period of 10-20 years, the recultivation of the preserved seeds is needed with technical problems and costs (Hawksworth and Bull, 2007).Botanical Gardens and protected areas are the other preservation activities with heavy and enormous cost of plant species preservation (Li and Pritchard, 2009).Cryopreservation is a new technology that due to its unique importance and performance in genetic resources preservation is rapidly progressing. In this method, plant germplasm is preserved at very low temperature (-196 °C) using liquid nitrogen because very low temperature reduces plant cell activities through which biological activity could be preserved for a longer period.

Cryopreservation makes possible long-term preservation of seeds and vegetative organs, leading to reduced exorbitant costs (Hu *et al.*, 2013).In cryopreservation, samples remain immune against the risks of pests and diseases and minimal energy and space are needed for species preservation. In addition, it is possible to repeat the samples with minimum cost and facilities in any environment (Surencisk*iet et al.*, 2012). Seeds can be preserved for a long-term period without losing viability (Ganzalez Benito *et al.*, 1998; Touchell and Dixon, 1993). Vitrification is one the protection methods against ultracold temperatures in which the formation of ice crystals is prevented without significant decrease in intracellular water (Panis and Lambardi, 2005; Jitsopakul, 2012). In the cooling process, glycerol is used as a preservative against freezing. It reduces the formation of ice and lowers its freezing point. It seems that glycerol in the extracellular solution is entered the cells and prevents the decrease in cell volume due to the water exit.

Maintaining cell volume causes maintaining salt concentrations (Norton, 1999). dehydration leads to the reduction of cellular water content through which destructive effects of ice crystals formation is reduced and seed viability percentage is increased (Liu *et al.*, 2003; Bhat *et al.*, 2005).

An important purpose of this research was to achieve an efficient technique for the preservation of *S. rechingeri* seeds. The possibility of cryopreservation of *Satureja rechingeri* seeds has been investigated for the first time in the current research.

Materials and methods

The seeds of *Satureja rechingeri* were collected from Nasirian region in Ilam province. Collected seeds were winnowed. All steps were performed under sterile laminar flow hood to remove surface contamination.

The following pretreatments were applied before placement of seed samples in liquid nitrogen (temperature -196°C). Vitrification Pretreatment: PVS2 solution and loading solution were applies as pretreatment.

PVS2 solution contains 0.3 M sucrose, 30% w/v glycerol, 15 % (w/v) DMSO, 15% w/v ethylene glycol (Sakai and Engelman, 2007).

Seeds were loaded with 2M glycerol solution in 0.6M sucrose for 20 min., then loaded seeds were transferred into cryovials. The cryovials were sealed with para-film and transferred to liquid nitrogen (temperature -196°C).

Glycerol pretreatment (30%): Seeds were transferred into cryovials containing 30% glycerol. The cryovials were sealed with para-film and transferred to liquid nitrogen (temperature -196°C).

Dehydration pretreatment: The seeds were placed in desiccator for three days, dehydrated by silica gel and transferred to liquid nitrogen.

Control treatment: After the sterilization, seeds were placed in Petri dishes as control treatments.

All treatments except control were immersed in liquid nitrogen with temperature of -196 °C for a week.

Afterward, cryovials were removed from liquid nitrogen and were immersed in sterile distilled water bath with a temperature of 42 °C. The seeds were washed in 1.2 M sucrose solution for 2 min and were sown on Petri dishes containing moistened filter paper (previously autoclaved).

Petri dishes were sealed with para-fim and transferred to a growth chamber. Five replicates were applied for each treatment containing 20 seeds.

Germination percentage and seedling production were calculated after 21 days.

After calculating germination percentage and mean length of radicle and plumule, under laboratory conditions, seed vigor index was calculated as follows (Abdul-baki and Anderson, 1973):
Vigor index= mean length of seedling * germination percentage.

Germination percentage and germination rate were calculated as follows(Ruan *et al.*, 2002):
GP= Ni / N × 100, where Ni is the number of germinated seeds on the ith day and N is total number of seeds.

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

The experiment was performed in a completely randomized design and data were analyzed by SAS software.

Results

Our results showed that none of the seeds, dehydrated by silicagel and transferred to liquid nitrogen (-196 °C), did not germinate.

The results of ANOVA

According to the results of the analysis of variance (Table 1), no significant difference was found among control treatment, 30% glycerol and vitrification (PVS2) in traits such as seedling length, plumule length, radicle length, seed vigor index, seedling fresh

weight, number of leaflets, and radicle/plumule length. This indicates that mentioned traits were not affected significantly by cryotreatments and, as control samples, the seeds were able to germinate and produce seedlings after removal of liquid nitrogen.

Germination rate and germination percentage showed significant difference between the control seeds and cryopreserved seeds at 1% level of probability (Table 1).

Table 1. ANOVA of *S. rechingeri* seed traits under cryopreservation condition for one week in liquid nitrogen (-196 °C).

	Mean Square								
S.O.V	Germination percentage	Germination rate	Seed vigor index	Seedling length (mm)	Radicle length (mm)	Plumue length (mm)	Seedlig fresh weight (mg)	Numbr of leaflet	Rdicle / plumuleratio
Treatment	11/449**	2/223**	15/976^{ns}	0/148^{ns}	0/087^{ns}	0/027^{ns}	0/914^{ns}	0/027^{ns}	0/25^{ns}
Error	0/988	0/293	4/712	0/074	0/074	0/0183	0/246	0/175	0/212
(cv%)	25/53	34/72	55/37	29/72	35/67	24/89	17/74	25/77	30/42

**،ns: Significant at 1% level، not significant.

Mean comparisons of treatments

Maximum and minimum seed vigor index were recorded for PVS2 (2.94) and 30% glycerol (1.655) under cryopreservation for one week (Table 2).

Table 2. Mean comparisons of treatments under cryopreservation for one week in liquid nitrogen (-196 °C)

Treatment	Germination percentage	Germinatio n rate	Seed vigor index	Seedling length (mm)	Radicle length (mm)	Plumue length (mm)	Seedlig fresh weight (mg)	Numbr of leaflet	Rdicle / plumule ratio
control	5/88^{a}	2/372^{a}	6/033^{a}	0/975	0/845	0/48	2/77^{ab}	1/637	1/785
Glycerol	3/107^{b}	0/902^{b}	1/655^{b}	0/58	0/5	0/45	1/61^{b}	1/41	1/22
(PVS_2)	2/93^{b}	1/436^{b}	2/94^{ab}	1/035	0/812	0/63	3/12^{a}	1/67	1/32

Different letters indicate a significant difference among treatments.

Discussion

In this study, it was observed that the cryopreserved seeds of *S. rechingeri,* as well as control samples, were able to germinate and produce seedlings after removal of liquid nitrogen.

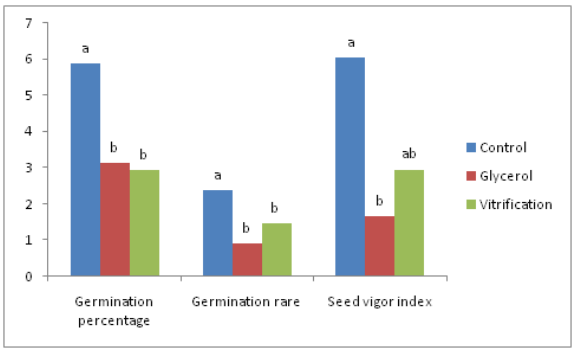

Fig. 1. Effects of different treatments on germination percentage, germination rate and seed vigor index of *S. rechingeri* after cryopreservation.

Desiccation is a process during which intracellular water content decreases to the extent that it does not harm the viability and seed vigor and consequently destructive effects of the formation of ice crystals in seeds and plant organs are reduced. As a result, after cryopreservation the viability of seeds improves or increases (Bhat *et al*., 2005; liu *et al*., 2003; Dussert *et al*., 1998).

The prevention of intracellular ice crystals formation is considered important for success in cryopreservation experiments. Therefore, before transferring into liquid nitrogen, tissues and cells should be desiccated sufficiently (Iskikawa *et al*., 1997).

In this process, desiccation was obtained by placing the seeds in a desicator for three days. The effects of desiccation on seed viability of several species after crypreservation have been reported as positive or negative.

The seeds of a species have different genetic potentials for germination and generally, the use of cryoprotectant guarantees the viability of cells and tissues.

However, the exact mechanism of their function has not been known yet (Schmale *et al.*, 2006).

Cryoprotectant by reducing the amount of ice and balancing the increasing concentration of the solutions maintain cell viability (Panis and Lambordi, 2005).

Immature seeds of orchids would not survive under cryopreservation treatments without the use of cryoprotectant (Surenciski *et al.*, 2012), which is consistent with our results.

Kolahdoozan *et al.* (2009) reported a higher germination percentage for the seeds of *Medicago rigidula*, cryopreserved in liquid nitrogen.

In 30% 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.

Volk *et al.* (2006) showed that glycerol treatment and sucrose reduced the freezing temperature and then vitrification solution further lowered the temperature. Morphological and physiological variations were not observed in the seeds grown under cryopreservation conditions (Kolahdoozan *et al.*, 2009). Considerable germination percentage and radicle and plumule growth rate will result in higher establishment and production. Germination percentage showed significant difference between control and liquid nitrogen treatments and control seeds had a higher germination percentage and rate as compared to the cryopreserved seeds.

Gonzalez_Benito *et al.* (1998) reported that seed cryopreservation of 13 plant species (liquid nitrogen and -196 °C) did not reduce seed germination percentage.

A germination percentage of 63% was reported for the cryopreserved seeds of *Najas flexitis*, continuing to grow to maturity stage (Hay *et al.*, 2000).Seed germination rate showed no significant difference between cryopreservation treatments. Seed vigor index indicates the germination ability (Gelmord *et al.*, 1978).

Under cryopreservation conditions, seed quality and seedling fresh weight are well maintained (Aryakia *et al.*, 2012) which corresponds with our findings.

According to Zanotti *et al.* (2012), no significant difference was found between the leaf fresh weight and number of leaf in *Caesalpinia echinata*.

Our results showed that both vitrification and 30% glycerol treatments could be used for seed cryopreservation of *S. rechingeri* in liquid nitrogen. In addition, cryopreserved seeds were not damaged in terms of seed viability, confirmed by laboratory assessments. Plants (seedlings) obtained from cryopreserved seeds were similar to the control samples and no significant difference was observed in leaf condition and shoot length as compared to control.

In general, according to the obtained results, cryopreservation of *Satureja rechingeri* seeds by above mentioned treatments is recommended, leading to the preservation of genetic resources and the prevention of extinction of this valuable species.

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