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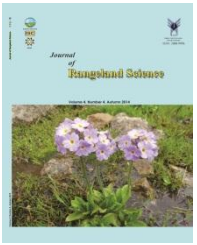

Research and Full Length Article:

Seed Germination of *Lilium ledebourii* (Baker) Boiss. after Cryopreservation

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Abstract. Seeds or plant organs are usually used as the materials for the long-term cryopreservation. The aim of this study is to investigate the possibility of seed cryopreservation of *Lilium ledebourii* (Baker) Boiss. as an endemic and endangered species because of genetic erosion. To evaluate seed potentials for the cryopreservation, four treatments including vitrification, 30% glycerol, desiccation and encapsulation-dehydration were applied on seeds before immerging in the liquid nitrogen (-196˚C) for a week. Then, seeds were removed from liquid nitrogen and exposed to heat shock (42˚C water bath), washed with the distilled water and eventually, sown in petri-dishes containing tissue papers. Some criteria including seed germination percent, root and shoot length values, root/shoot length ratio and seed vigor index were recorded and statistically analyzed after four weeks. Data of germination was converted to an arc-sine transformation prior to the analysis of variance. Results showed that germination percentages were 97.50, 97.43, 94.86 and 69.47% for 30%-glycerol, vitrification, desiccation, and encapsulation-dehydration treatments, respectively. They were not significantly different from control seeds (89.33%). On the other hand, other germination attributes of seeds almost showed no significant differences in comparison with control treatment in most treatments. In addition, 30%-glycerol, vitrification and desiccation experienced the highest amounts of germination attributes whereas they showed no significant differences with the control treatment in most qualities. In contrast, the encapsulated seeds showed the lowest amounts in germination indices though they had no significant differences with control treatment (except germination rate). Most of germination attributes of encapsulated seeds were significantly lower than the other cryogenic treatments. Both 30% glycerol and desiccation treatments showed some advantages over vitrification method. However, desiccation was the best treatment because it does not need any chemical substances. It was concluded that the cryopreservation technique is an important approach for the long-term preservation of the seeds of this endangered species.

Key words: Seed cryopreservation, Vitrification, Encapsulation-dehydration, Desiccation, 30%-glycerol

Introduction

Genus of *Lilium* is an important ornamental species which is grown in both natural habitats and fields such as pots, outdoors or cut flowers. *Lilium ledebourii* (Baker) Boiss. is a rare, endemic species in Iran which is distributed from southern Caspian Sea forests of Mazandaran and Gilan provinces in north Iran (Jalili and Jamzad, 1999) to south western Caspian Sea forests in Ardabil province. According to Red Data Book of Iran, *Lilium ledebourii* is vulnerable and unfortunately, faces high risk of extinction in natural habitats in future (Jalili and Jamzad, 1999).

For the germplasm conservation, there are two major *ex situ* and *in situ* approaches (Mandal *et al.,* 2003). There is a risk of material to be lost due to the environmental hazards in *in situ* method (Razdan, 2003). Germplasm conservation using seeds by a classical method is an efficient approach for short-term and mid-term periods (Pritchard and Nadarajan, 2008). Cryopreservation method is a suitable candidate method for *ex situ* germplasm conservation for long-term storage. The advantages of this procedure can be specified as greater security for collection, small space requirements, little input or cost of maintenance and the reduction in number of duplicates needed for the active collection (Reed *et al.,* 1998) and a method with low risk of genetic modification (Matsumoto *et al.,* 1998).

Vitrification technique has been successfully applied to the axillary shoot tips (Matsumoto and Sakai, 2003), apical meristems (Matsumoto *et al.,* 1998), mononodal micro-cuttings with an axillary bud (Charoensub *et al.,* 2003), embryogenic cells (Nishizawa *et al.,* 1993), callus (Shahrzad *et al.,* 2009) and seeds of many species (Naderi Shahab *et al.,* 2009; Hatami *et al.,* 2010; Kaviani *et al.,* 2008). However, vitrification is not always the most proper method. Hirata *et al.* (2002) have shown that vitrification was not suitable for root explants whereas they obtained more than 70% survival rate when they cryopreserved the *Vinca minor* roots in the liquid nitrogen by encapsulation-dehydration.

Seed storage and survival rate in cryopreservation by the desiccation method is depended on the species. Roberts (1973) classified seeds into "Orthodox" or "Recalcitrant" based on their physiological responses to moisture content and their seed storage behavior. Orthodox seeds can be stored using standard seed storage conditions (Rao *et al.,* 2006), dried to moisture content ≤5% and stored at temperatures of −18 to −20˚C without any damages for long-term periods. Recalcitrant seeds, on the other hand, are damaged with desiccation below a critical moisture content point of 12–30% (Roberts, 1973) and they are sensitive to chilling temperatures <15˚C (Benson, 2008). The basic idea for the dry-freezing method was originated from the fact that non-germinated dry seeds are able to tolerate super low temperatures. In contrast, water-imbibed seeds showed susceptibility to cryogenic injuries (Razdan, 2003). Naderi Shahab *et al.* (2009) successfully cryopreserved *Biota orientalis* seeds using vitrification, 30% glycerol and desiccation treatments. They reported an average recovery and seed germination percent ranged from 93.69 to 95.04%. Furthermore, Hatami *et al.* (2010) reported relatively similar results (60.04 to 70.56%) using vitrification, 30% glycerol and desiccation methods for *Eucalyptus microtheca.*

The present study describes and compares vitrification, 30% glycerol, desiccation, and encapsulation-dehydration pre-treatments for storing *Lilium ledebourii* seeds in the liquid nitrogen (LN) at -196˚C. What's more, optimizing a simple, available and inexpensive protocol is required.

Materials and methods

Plant materials

Lilium ledebourii (Baker) Boiss. seeds were collected from natural habitats in late August 2009 from Damash of Emarlu district between Lahijan and Rudbar, Gilan province (36° 45' 0" N, 49°48'0" E) in north of Iran. The seeds were extracted from capsules. Then, a uniform seed bulk was used for the experiment (Fig. 1). In order to overcome seed dormancy, the collected seeds, after that, were stored at 2-4˚C for six weeks. Eventually, seeds were pretreated and subsequently immersed in the liquid nitrogen.

Cryogenic procedures

a) Vitrification pre-treatment

The cryotubes containing seeds were treated with a mixture of 2 M glycerol plus 0.4 M sucrose (LS[1] solution, Nishizawa *et al.*, 1993) for 20 min at 22˚C. The cryotubes containing the seeds were then filled with a cold (+4˚C) PVS2[2] solution consisting of 30% (w/v) glycerol, 15% (w/v) Ethylene Glycol (EG), 15% (w/v) Dimethyl sulfoxide (DMSO) and 0.4 M sucrose (Sakai *et al.*, 1990) and were stored at the same temperature for 20 min. Afterwards, they were rapidly plunged into the liquid nitrogen (-196˚C).

b) Encapsulation-Dehydration pre-treatment

At first, seeds were suspended in liquid MS media (Murashige and Skoog, 1962) containing Alginate (3%, w/v) and 0.75 M sucrose for 5-10 min at 22˚C. Calcium alginate beads containing seeds were dropped into CaCl2 solution (0.1 M) for 20 min. Subsequently, the encapsulated seeds were desiccated under the air in a laminar air flow cabinet for 3 hours (Fig. 1). Finally, the desiccated alginate beads were transferred into 1.8 ml cryotubes and immediately submerged in the liquid nitrogen.

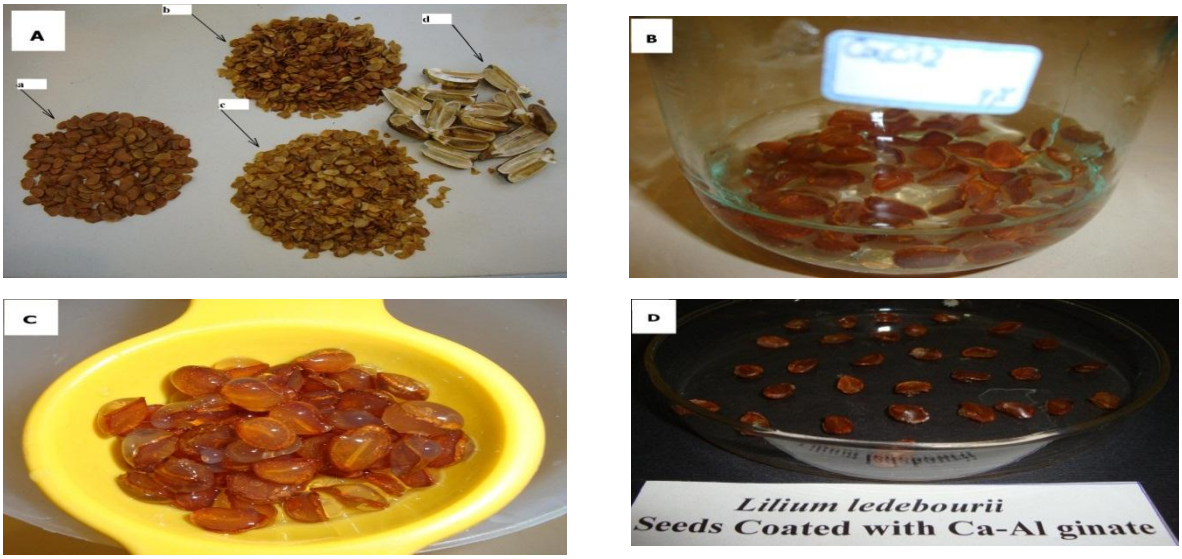

Fig. 1. A (a-d) Separating abnormal and normal seeds from their capsules. (a & b) normal and healthy seeds used in experiments. c abnormal and unhealthy seeds removed from experiments. d capsules that seeds separated from them. (B-D) the stages of encapsulation-dehydration method. **B** the calcium alginate beads containing seeds encapsulated when the seeds with a little medium was dropped into CaCl2 solution, 20 min. **C** removing beads from CaCl2 solution. **D** the dehydrated beads under the air current of a laminar air flow cabinet for 3 hours

1: Linsmaier and Skoog medium

2: Plant Vitrification Solution

c) 30% Glycerol pre-treatment

Cryotubes containing seeds filled with 30% glycerol solution and incubated at 22 ˚C for 20 minutes. After that, cryotubes were submerged rapidly in liquid nitrogen.

d) Desiccation pre-treatment

Fresh seeds weight was recorded. Then, seeds were dried in an oven at 90˚C for 48 hours to obtain dry matter weight. Seed moisture content dropped from 11.90% to 4.86% (40.87% based on total seed moisture content) when fresh seeds were placed in an air-tight desiccators containing 300g silica gel for 21 h at 22˚C. Eventually, the desiccated seeds were put into cryotubes and then, rapidly plunged in liquid nitrogen. On the other hand, the control seeds were included whereby the whole steps of pre-treatments and placing in LN were omitted and the seeds were transferred directly in petri dishes to assess germination attributes.

Heat shock process (post-treatment)

After one week, the cryopreserved seeds were removed from LN and rapidly, warmed in a water bath at 42˚C for 2 min. They were then placed between wet papers in 10-cm petri dishes. The petri dishes were transferred into the growth chamber at 22˚C with a 16 h photoperiod for 32 days.

Evaluation seed germination

Number of the germinated seeds was recorded once a week and then, the germinated seeds were transferred in an ordinary tray onto wet paper and covered with the transparent plastic (Fig. 2). After 32 days, root and shoot length values were recorded. Seed germination percent, germination rate, root/shoot length ratio and seed vigor index (VI) were also obtained. Vigor index was calculated via the following formula (Abdul-Baki and Anderson, 1973) (Equation 1).

VI = Germination percent × (shoot length ^{mm} + root length ^{mm}) (Equation 1)

Statistical analysis

A completely randomized design with four replications was used. Experimental units were petri dishes containing 20 seeds. The treatments included vitrification, desiccation, 30% glycerol and encapsulation–dehydration. The ANOVA was applied and Duncan's multiple range tests were used for the means comparison. The raw data of germination percentages were transformed using Arc-sine method prior to ANOVA.

Results and Discussion

The mean values of the cryopreserved seeds showed no significant differences with control seeds for germination percent, root length and root/shoot length ratio (Table 1). The highest seed germination percentages were observed with three cryopreservation treatments including 30% glycerol, vitrification, and desiccation with the average values of 97.50%, 97.43% and 94.86%, respectively with no significant differences with control treatment (89.33%). In encapsulation-dehydration treatment, germination percent of the cryopreserved seeds with the average value of 69.47% was not significantly lower than control seeds while it showed a considerable decrease in comparison with the other cryopreservation treatments.

Table 1. Means comparison of *Lilium ledebourii* seed attributes under different cryogenic treatments and control

Treatments	Germination (%)	Germination Rate (%)	Root Length (mm)	Shoot Length (mm)	Root/Shoot Length Ratio	Vigor Index (VI)
Control	89.33 ^{ab}	5.22 ^{a}	14.24 ^{a}	19.58 ^{bc}	0.71 ^{a}	30.34 ^{bc}
30% glycerol	97.50 ^{a}	5.47 ^{a}	22.03 ^{a}	23.95 ^{a}	0.92 ^{a}	44.81 ^{a}
Vitrification	97.43 ^{a}	5.18 ^{a}	17.70 ^{a}	21.26 ^{abc}	0.82 ^{a}	38.83 ^{ab}
Desiccation	94.86 ^{a}	5.19 ^{a}	21.83 ^{a}	22.87 ^{ab}	0.95 ^{a}	42.46 ^{ab}
Encapsulation-Dehydration	69.47 ^{b}	3.60 ^{b}	17.58 ^{a}	17.57 ^{c}	1.00 ^{a}	24.23 ^{c}

Means of treatments followed by the same letters are not significantly different (P< 0.01) using Duncan's multiple range tests

The same results were also observed for seed germination rate. The 30% glycerol, vitrification, and desiccation treatments with the average values of 5.47, 5.18, and 5.19 respectively showed no statistical differences as compared to the control treatment (5.22). However, they were significantly higher than that of the encapsulation-dehydration treatment (with the average value of 3.60). Furthermore, there were no significant differences between 30% glycerol, vitrification, and desiccation treatments for all of the attributes (Table 1). Likewise, Naderi-Shahab *et al.* (2009) reported the same results for *Biota orientalis* seeds. They showed that three cryopreservation treatments including 30% glycerol, vitrification and desiccation had no significant differences in comparison with the control treatment for most of the seed attributes.

For all the germination attributes as germination percent, germination rate, root length, and root to shoot length ratio, there were no significant differences between the control and 30% glycerol treatment (Table 1). Glycerol penetrates the cells and on a colligative basis, reduces the amount of ice formation in cryogenic conditions (Reed, 2008). The 30% glycerol has been successfully used in seed storage under cryogenic conditions or at -196˚C for *Biota orientalis* and *Eucalyptus microtheca* seeds (Naderi Shahab *et al.*, 2009; Hatami *et al.*, 2010).

Vitrificated seeds after the cryopreservation treatment in LN showed no significant differences with control and normally germinated and grew after being removed from LN (Table 1). The positive effects of vitrification protocol utilizing PVS2 have also been reported by the other researchers (Matsumoto *et al.*, 1998; Matsumoto and Sakai, 2003; Charoensub *et al.*, 2003; Panis *et al.*, 2005; Halmagyi *et al.*, 2010; Chen *et al.*, 2011).

Desiccated and cryopreserved seeds germinated with high values and showed the normal growth. Although the mean values of all attributes in desiccation treatment were higher than those of the control, there were no significant differences among them. It can be concluded that it is possible to cryopreserve the desiccated *Lilium ledebourii* seeds under cryogenic conditions for a long period without any damages. As shown by the others, cryogenic storage as compared to conventional freezer storage drastically prolonged longevity of dried seeds (Reed, 2008). However, the dehydrated plant materials demonstrated a different behavior to cryogenic conditions according to tissue types (Razdan, 2003), seeds or organs of species. In addition, mature orthodox seeds can tolerate drying up to 5% or less moisture content (Malik *et al.*, 2010) without loss of viability (Razdan, 2003). In conclusion, seeds of the *L. ledebourii* are characterized as orthodox seeds and can be stored in LN for a long period.

Except negative impacts of the encapsulation-dehydration treatment on germination rate, none of the other treatments showed any negative effects on the other seed attributes. Seed germination rate was decreased in encapsulation-dehydration treatment after the removal from LN (Table 1). Similar results were also obtained by Kaviani *et al.* (2008) on *L. ledebourii* seeds. They reported that seeds coated with alginate beads and cryopreserved in LN showed only 50% germination after removing from LN. Overall, it seems that this treatment is not an appropriate approach for the storage of this species' seeds under cryopreservation conditions as compared to those of 30% glycerol, vitrification, and desiccation.

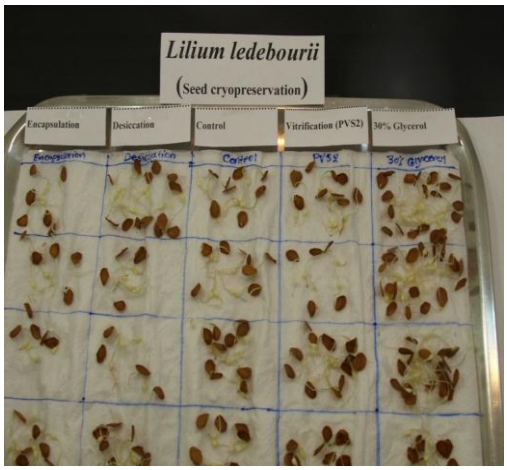

Fig. 2. Control and cryopreserved seeds of *Lilium ledebourii* treated with different cryogenic treatments (transferred from petri dishes)

Conclusion

In this study, high levels of post-cryopreservation recovery (Table 1 & Fig. 2) were obtained in *L. ledebourii* seeds by 30% glycerol, vitrification, and desiccation treatments. Each of the three approaches can be performed easily. Both treatments of 30% glycerol and desiccation showed some advantages over vitrification method. On the other hand, 30% glycerol treatment is applied easier than vitrification mainly due to the difficulty of vitrification medium preparing in comparison with 30% Glycerol. Likewise, the 30% glycerol is superior to vitrification method. However, desiccation treatment does not need any chemical substances; hence, it is economically and environmentally superior to vitrification and 30% glycerol treatments. The high recovery of seeds and normal growth of the cryopreserved *L. ledebourii* seeds showed that seeds of this endangered and endemic plant can be collected and successfully preserved under cryogenic conditions for a long period. In the case of extinction danger, the seeds can be removed from cryogenic conditions and recovered easily. It is worth mentioning that in our knowledge, this is the first study which obtained the highest germination percent for control treatment and cryopreserved seeds of *L. ledebourii*.

Acknowledgements

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جوانه‌زنی بذرهای سوسن چلچراغ (***Lilium ledebourii* (Baker) Boiss.**) پس از نگهداری در شرایط فراسرد

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چکیده. بذرها و یا اندام‌های گیاهی، به‌طور معمول، به‌عنوان مواد گیاهی، جهت ذخیره‌سازی بلندمدت در شرایط فراسرد، استفاده می‌شوند. سوسن چلچراغ، بومی ایران و از گونه‌های در معرض خطر، به‌دلیل فرسایش ژنتیکی، می‌باشد. به‌منظور ارزیابی پتانسیل ذخیره‌سازی بذرها در شرایط فراسرد، پیش از قرارگیری در ازت مایع به‌مدت یک هفته، چهار تیمار ویتریفیکاسیون، گلیسرول ۳۰٪، آبگیری و آبگیری-کپسوله‌کردن بر روی بذرها صورت گرفت. سپس، بذرها از ازت مایع خارج و پس قرارگیری در حمام آب گرم$42^{\circ}C$ با آب مقطر شسته و در پتری‌دیش‌ها کاشته شدند. پس از ۴ هفته، برخی صفت‌ها شامل درصد و سرعت جوانه‌زنی، طول ساقه و ریشه، نسبت طول ریشه به ساقه و شاخص قدرت جوانه‌زنی ثبت و به‌طور آماری آنالیز گردیدند. داده‌های درصد جوانه‌زنی، پیش از تجزیه واریانس، به معکوس سینوس تبدیل شدند. درصد جوانه‌زنی بذور در تیمارهای گلیسرول ۳۰٪، ویتریفیکاسیون، آبگیری و آبگیری-کپسوله‌کردن به ترتیب با ۹۷/۵۰، ۹۷/۴۳، ۹۴/۸۶ و ۶۹/۴۷ بود که تفاوت معنی‌داری با شاهد (۸۹/۳۳) نداشتند. همچنین سایر صفت‌های جوانه‌زنی در بیشتر تیمارها اختلاف معنی‌داری با شاهد نداشتند. با وجود آنکه تیمارهای گلیسرول ۳۰٪، ویتریفیکاسیون و آبگیری در شاخص‌های جوانه‌زنی برتر بودند ولی اختلاف آنها با شاهد معنی‌دار نبود. در مقابل، بذرهای کپسوله شده کمترین مقدار در صفات جوانه‌زنی را داشتند، اگرچه به استثنای سرعت جوانه‌زنی تفاوت معنی‌داری نیز با شاهد نداشتند. صفت‌های جوانه‌زنی بذرها در تیمار آبگیری-کپسوله‌کردن در مقایسه با سایر تیمارهای فراسرد، به‌طور چشمگیری پائین‌تر بود. هر دو تیمار گلیسرول ۳۰٪ و آبگیری در مقایسه با تیمار ویتریفیکاسیون برتری داشتند. با این وجود، تیمار آبگیری به دلیل عدم استفاده از هر گونه ماده شیمیایی، بهترین تیمار شناخته شد. در نتیجه نگهداری بذرهای گونه سوسن چلچراغ در شرایط فراسرد می‌تواند از مهم‌ترین روش‌های حفاظت بلندمدت این گونه باشد.

کلمات کلیدی: حفاظت فراسرد، ویتریفیکاسیون، آبگیری-کپسوله، آبگیری، گلیسرول ۳۰٪