

Cryopreservation of *Lilium ledebourii* (Baker) Bioss. By Encapsulation-vitrification and *In vivo* Media for Planting of Germplasms

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Abstract: Germplasms (seeds, embryonic axes, lateral buds and bulblets) of *Lilium ledebourii* (Baker) Bioss. were cryopreserved using a vitrification method combined with an encapsulation technique. The germplasms were encapsulated in sodium alginate before pretreatment on PVS2 solution. Encapsulated germplasms were vitrified in plant vitrification solution 2 (PVS2). Germplasms were then rapidly plunged into liquid nitrogen (LN). After cryopreservation and rapid warming in a water bath at 38°C for 2 min, capsules were removed from around the germplasms prior to transfer on the plastic pots containing perlite and vermiculate. About 10% of cryopreserved seeds and embryonic axes pretreated with PVS2 solution, sucrose (0.75 M) and encapsulation were able to sprouting, while there was no survival after LN storage for seeds and embryonic axes pretreated with PVS2 solution and sucrose. None of lateral buds and bulblets pretreated with sucrose and encapsulation-vitrification was survival after cryopreservation. The obtained results in the research showed cultivating the cryopreserved germplasms in perlite and vermiculate in comparison to *in vitro* culture medium is not suitable.

Key words: *Lilium ledebourii* (Baker) Bioss. • Sucrose • Germplasm conservation • Liquid nitrogen
• Encapsulation-vitrification • Cryopreservation

INTRODUCTION

Cryopreservation is an important tool for long-term storage of biological materials. It offers a safe and cost-effective option for long-term conservation of genetic resources in many plant species. At the temperature of LN (-196°C), all the metabolic activities of cells are at a standstill. Thus, they can be preserved in such a state for a long period [1]. Many new cryopreservation techniques such as simple freezing, vitrification, encapsulation-dehydration and encapsulation-vitrification have been reported for successful use for many cells, tissues and organs of plant species [2]. However, for successful cryopreservation, many factors are involved such as starting materials, pretreatment conditions, cryoprotocols and post-thaw treatment [3]. Therefore, in order to accomplish successful cryopreservation for each species and cultivar, a separate study must be carried out. Vitrification (glass formation) is a simple, fast and effective method for cryopreservation. It eliminates the

need for controlled slow freezing and permits cells and organs to be cryopreserved by direct transfer into LN [4]. Vitrification method is another cryopreservation procedure and has extended the applicability of cryopreservation to a broad range of plant materials from various species, including embryonic cultures [4], somatic embryos [5], apical tips [6] and hairy roots [7]. The vitrification method uses highly concentrated plant vitrification solution 2 (PVS2), by which cells are osmotically dehydrated at a non-freezing temperature [4]. The dehydrated cells are completely vitrified after immersion in LN and preserved safely at the temperature of LN (-196°C). Cryopreservation with encapsulation, in which cells and organs are embedded *in vitro* in alginate gel has been successfully achieved with many plant materials in combination with vitrification methods [8, 9]. The aim of the investigation reported here were: 1- the effect of encapsulation-vitrification method on survival of lily (*Lilium ledebourii* (Baker) Bioss.) germplasms after exposure and storage to LN. 2- The effect of different

procedure, reported for the first time, based on the removal of capsule from around the lily germplasms after cryopreservation and then planting in perlite and vermiculate instead of *in vitro* culture medium after exposure to LN.

MATERIALS AND METHODS

Seeds and nodes containing lateral buds of lily (*Lilium ledebourii* (Baker) Bioss.) obtained from Damash area of Guilan province in the north of Iran, were used in the present study. Nodes about 1 mm long were dissected from *Lilium* stems in vegetative phase. Embryonic axes were cut from mature seeds collected in generative phase. Bulblets were produced from seeds planted in plastic pots containing peat and vermiculate. Seeds, embryonic axes, lateral buds and bulblets of *L. ledebourii* (Baker) Bioss. were washed under running tap water for 30 min. Then, they were disinfected in ethanol 70% (v/v) for 1 min followed by sodium hypochlorite 0.5% (v/v) for 10 min and rinsed three times with sterile water. Seeds, embryonic axes, lateral buds and bulblets were suspended in MS [10] liquid medium containing 0.75 M sucrose for 1 h with slow agitation, then individually suspended in liquid MS medium supplemented with 3% (w/v) Na-alginate and 0.75 M sucrose for 1 h with slow agitation. Then, they were individually dispensed with a sterile forceps into liquid MS medium containing 100 mM CaCl₂ and 0.75 M sucrose for 1 h with slow agitation (Figure 1). In the case of controls with non-encapsulated, these were suspended in MS liquid medium and 0.75 M sucrose for 1 h. Seeds, embryonic axes, lateral buds and bulblets were put in plant vitrification solution 2 (PVS2) which was developed by Sakai *et al.* in 1990. The PVS2 consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide prepared in MS liquid medium. Vitrification was carried out at 25±2°C for 16 h, before plunging the cryotubes into LN where they were kept for at least 60 min. For cryopreservation, dehydrated seeds, embryonic axes, lateral buds and bulblets were transferred to sterile 2 ml polypropylene cryovials (5 per cryovials) and immersed directly into LN and held for at least 1 h. For control, a series of non-pretreated and non-encapsulated seeds, embryonic axes, lateral buds and bulblets were plunged in LN for 1 h immediately after disinfection. Cryovials containing frozen seeds, embryonic axes, lateral buds and bulblets were re-warmed rapidly in a water-bath at 37-38°C for 2 min. Then, capsules were removed from around the germplasms. Seeds, embryonic axes, lateral buds and bulblets lacking capsule were planted in plastic



Fig. 1: (Up): Encapsulated seed (bead) of *Lilium ledebourii* (Baker) Bioss. under Binocular (10x). (Down): Encapsulated bulblets of *Lilium ledebourii* (Baker) Bioss. under Binocular (10x). Bead (capsule) is an artificial seed which is made by integration of Na-alginate and CaCl₂

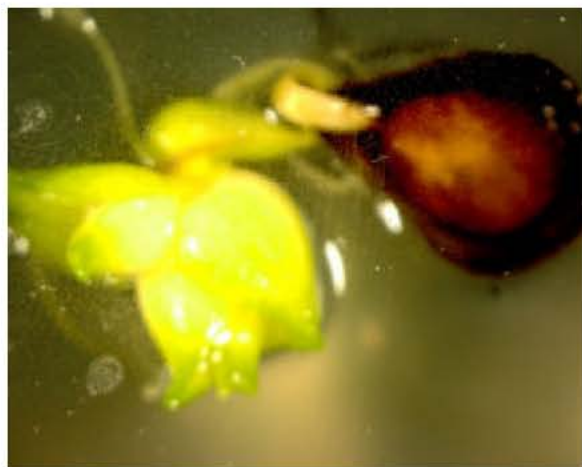
pots filled with perlite and vermiculate. All treated and untreated (control) cultures were incubated in a growth chamber set for 16-h photoperiod, a light flux of 30 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and day/night temperatures of 25/20°C. After growth under standard conditions the percentage of viability were recorded. In every experiment, approximately 12 seeds, embryonic axes, lateral buds and bulblets were treated for each of three replicates. Data were subjected to One Way ANOVA (analysis of variance).

RESULTS AND DISCUSSION

The osmotic dehydration process with PVS2 is crucial for successful cryopreservation of plant cells. Many reports have shown that osmoprotection is effective in enhancing the capacity of cells to tolerate

Table 1: Effect of pretreatments of sucrose, glycerol, encapsulation and vitrification on the viability of seeds, embryonic axes, lateral buds and bulblets of *Lilium ledebourii* (Baker) Bioss. after exposure to LN

Treatment	Viability (%)		
	Control	Pretreated, non-encapsulated	Pretreated, encapsulated-vitrified
Seeds	0	0	10
Bulblets	0	0	0
Embryonic axes	0	0	10
Lateral buds	0	0	0

Fig. 2: Sprouted seed of *Lilium ledebourii* (Baker) Bioss. after exposure to LN

severe dehydration with PVS2 [8, 11]. Current study showed that non-pretreated, non-encapsulated seeds, embryonic axes, lateral buds and bulblets of *Lilium ledebourii* (Baker) Bioss. control, did not survive after exposure to LN (Table 1). The same results were reported in many plants [12, 13]. Contrary to this result, embryonic axes of *Camellia sinensis* withstood after freezing in LN without any pretreatment [14]. The studies of Benelli *et al.* [15] on *Pupulus* spp. germplasm demonstrated that all seeds of *P. tremuloides* maintained 100% germinability after direct immersion in LN without any pretreatment.

In this study, the percentage of germination in pretreated, non-encapsulated seeds, embryonic axes, lateral buds and bulblets was nil (Table 1). Previously studies showed that the percentage of germination in seeds of *L. ledebourii* (Baker) Bioss. pretreated with sucrose and air dehydration was 75% [16]. Blakesley *et al.* [17] showed that the pretreatment of embryonic tissues of *Ipomoea batatas* with high levels of sucrose alone resulted in up to 28.6% survival. After dehydration the maximum survival demonstrated was 9.1%. The study of Suzuki *et al.* [18, 19] on *Gentiana scabra* germplasm have

revealed that pre-culturing with sucrose and desiccation induce high dehydration tolerance, the method has been found to be effective for cryopreservation. The studies of Sanchez-Romero and Panis [20] on olive embryonic cultures revealed that after controlled-rate cooling only 10% viability was observed. The highest viability percentage and re-growth rates were obtained with the ultra-fast method with vitrification on aluminium foil strips. Embryonic cultures responded better to this method when previously dehydrated during 60 min. At these conditions, 100% of cultures showed viability. Studies on recalcitrant seeds species showed that 60 min vitrification treatment was optimum for shoot tips with survival above 70% after freezing [21].

Current study showed that the percentage of germination in seeds and embryonic axes of *L. ledebourii* (Baker) Bioss. pre-treated with sucrose, vitrification and encapsulation was 10% (Table 1 and Figure 2).

The studies of Benelli *et al.* [15] on *Pupulus* spp. germplasm demonstrated that no survival was achieved with the encapsulation-vitrification technique. Benson *et al.* [22] also reported that vitrification and encapsulation-dehydration exceeded the conventional freezing method in terms of shoot formation and recovery growth in *Ribes*. The studies of Janeiro *et al.* [14] on cryopreservation of embryonic axes and somatic embryos of *Camellia japonica* L. showed that none of the protective pretreatments applied to these experiments (desiccation, chemical protectors, hardening by culture at low temperatures and encapsulation in alginate beads) allowed cryopreservation of the somatic embryos [14]. In contrary, Nukariet *et al.* [23] proposed that in cryopreservation of somatic embryos, encapsulation is especially useful.

There were no statistically significant differences between the survival rates of control and pretreated seeds, embryonic axes, lateral buds and bulblets of *L. ledebourii* (Baker) Bioss. in our studies. Many researchers have used a combination of encapsulation, high sucrose

pretreatment and dehydration [12, 13, 17]. Pre-culturing before vitrification might be an essential step for successful cryopreservation by vitrification. Increasing the sucrose concentration in the pre-culture medium improved the survival of vitrified tobacco shoot tips [24]. According to the literature, desiccation and direct immersion in LN is probably the simplest and most successful method of cryopreservation [25, 26]. The studies of Lambardi *et al.* [27] showed that the embryogenic tissues of both olive and horsechestnut proved to be a highly suitable material for cryopreservation. After a 90 min incubation in the vitrification solution, more than 81% of the cryopreserved samples of horsechestnut and 38% of olive survived. The studies of Thammasiri and Soankul [28] on cryopreservation of *Vanda coerulea* Griff. ex Lindl. seeds by vitrification showed that about 67% of cryopreserved seeds treated with PVS2 for 70 min were able to develop into normal seedling *in vitro*, while without PVS2 treatment, there was no survival after LN storage. Vitrification could be an appropriate and practical method for cryopreservation of many accessions of orchid seeds [29, 30]. For the successful cryopreservation of plant germplasm by vitrification approach, it is essential to adapt the general technique to the specific requirements of each species.

The use of perlite and vermiculite as *in vivo* medium instead of *in vitro* medium after exposure of germplasms to LN which was done for the first time in this work was not a suitable method at all.

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