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Cryopreservation of Lily [*Lilium ledebourii* (Baker) Bioss.] Germplasm by Encapsulation-Dehydration

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Abstract: A cryopreservation process using encapsulation-dehydration was set up for seeds of lily [*Lilium ledebourii* (Baker) Bioss.]. Seeds were subjected to a rapid freezing protocol in liquid nitrogen following encapsulation in MS medium supplemented with 3% Na-alginate, 100 mM $CaCl_2$ and 0.6 M sucrose and then desiccation for 1 h under the laminar air flow. Survival after freezing was nil for control seeds, 22% for seeds treated with sucrose and dehydration and up to 50% for seeds treated with sucrose, dehydration and encapsulation.

Key words: Cryopreservation, encapsulation, dehydration, germplasm conservation, lily, sucrose

INTRODUCTION

Cryopreservation of germplasm at liquid nitrogen (-196°C) is a perfect method for the long-term conservation of plant genetic resources.

Lilium ledebourii (Baker) Bioss., the rarest lily, is an endiginous species to Iran which is under surveillance carefully.

In vitro conservation of the plant germplasm is essential for plant breeding programs, also, provides a source of compounds to the pharmaceutical, food and crop protection industries. In the last decade, some reliable cryogenic procedures, new cryopreservation technique, such as vitrification, encapsulation and desiccation methods have been developed and the number of cryopreserved species has enormously been increased (Grout, 1995; Sakai, 1997).

For the long-term conservation of plant germplasm, cryopreservation, freeze- preservation at ultra-low temperature (liquid nitrogen, -196°C), is actually the valuable technique. At a cryogenic temperature, the rate of chemical and biophysical reactions is so slow that the biological growth and development of the ultra-frozen organ/tissue are hampered and genetic alterations do not take place during the very long period of storage.

Cryopreservation of biological tissues can be successful only if intra-cellular ice crystal formation is avoided. Crystal formation, can be prevented through vitrification (Sakai, 2000). Two requirements must be met for a cell to vitrify; rapid freezing and a concentrated cellular solution (Panis and Lambardi, 2005). In the method of cryopreservation, encapsulation of tissues within alginate beads allows to withstand drastic treatments such as desiccation and freezing (Bernard *et al*., 2002). Sugars play a very important role in the acquisition of resistance to desiccation and to freezing in LN (Suzuki *et al*., 2005).

Generally, tissues which have low water content, such as meristematic tissues, embryonic axes and seeds are more resistant to the stress of these techniques (Janeiro *et al*., 1996; Radhamani and Chandel, 1992).

In this study, we applied sucrose as a non-penetrating cryoprotective substance. Also, cryopreservation technique used was encapsulation-dehydration.

MATERIALS AND METHODS

Plant material: Seeds of *Lilium ledebourii* (Baker) Bioss. were collected (July, 2007) from Damash area of Guilan province in the North of Iran. Seeds were disinfected in ethanol 70% (v/v) for 1 min and sodium hypochlorite 0.5% (v/v) for 10 min.

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Encapsulation and osmoprotection procedure: Seeds were suspended in MS (Murashige and Skoog, 1962) medium supplemented with 3% (w/v) Na-alginate and 0.6 M sucrose for 1 h with slow agitation. Then, seeds were dropped into MS medium containing 100 mM $CaCl_2$ and 0.6 M sucrose for 1 h with slow agitation.

Dehydration procedure: Encapsulated and non-encapsulated seeds were transferred to empty open petri dishes and desiccated in the air current of a laminar flow chamber for 1 h.

Determination of the moisture content: To determine the moisture content, 20 seeds, encapsulated and non-encapsulated, were maintained in MS liquid medium with 0.6 M sucrose for 1 h with agitation. After that they were dessicated 1 h under laminar flow. The dehydrated seeds were weighted and dried in oven at 110°C for 20 h. Moisture content was expressed as a percentage of their initial fresh weight.

Cryopreservation and thawing procedure: For cryopreservation, control, osmoprotected and encapsulated seeds were placed in a 1.8 mL cryotube and directly plunged into LN and held for 24 h. Cryotubes were thawed in a water-bath at 37-38°C for 3 min.

Recovery and viability assessment: Following cryopreservation, samples were cultured on solid MS medium (Agar-agr 0.8%) with 3% sucrose. Cultures were incubated at 25°C under a 16 h photoperiod. After growth the percentage of seeds surviving were recorded.

Statistical analysis: In every experiment approximately 12 seeds were treated for each of three replicates. Data were subjected to ANOVA (analysis of variance) and significant differences between treatments were determined by Duncan's Multiple Range Test (DNMRT) using the MSTATC software package.

RESULTS AND DISCUSSION

Non-pretreated and non-encapsulated seeds, control, did not survive after exposure to LN (Table 1). In other words, none of control seeds showed any sign of germination on MS medium. The same results were reported in many plants (Bernard *et al*., 2002; Reed *et al*., 2006). Contrary to present results, embryonic axes of *Camellia sinensis* withstood after freezing in LN without any pretreatment (Janeiro *et al*., 1996).

The percentage of germination of seeds pretreated with sucrose and dehydration but non-encapsulated was 25% (Table 1). In contrary, non-encapsulated embryonic axes of *Melia azedarach* L. even those pretreated with sucrose and dehydration for 1 h, did not survive after exposure to LN. Blakesley *et al*. (1995) showed 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%. But almost all encapsulated germplasm in combination with high sucrose and dehydration survived. The study of Suzuki *et al*. (2005, 2006) on *Gentiana scabra* germplasm have revealed that preculturing with sucrose and desiccation induce high dehydration tolerance, the method has been found to be effective for encapsulation-desiccation based cryopreservation.

Table 1: Effect of pretreatments, sucrose and dehydration and encapsulation on the viability of lily after exposure to LN

Treatments	Viability (%)
Control	$0.00^{C\dagger}$
Pretreated, non-encapsulation	25.00^{B}
Pretreated, encapsulation	50.67^{A}

†Values followed by the different letter(s) are significantly different at $p<0.01$, using DNMRT

Table 2: Statistical analysis the effect of pretreatments, sucrose and dehydration and encapsulation on the viability of lily exposed to LN

Source of variance	Sum of squares	df	Mean square
Replication	27.556	2	13.778
Factor A	3872.889	2	1936.444**
Error	19.111	4	4.778

CV: 9.02%, **Significantly different at $p<0.01$

Sucrose in high concentration must be maintained in contact with the seeds during the whole time of the procedure and this is permitted by their encapsulation in alginate beads. Preculture with a high concentration of sucrose greatly increase the intracellular concentration which will act as the principal agent of tolerance to desiccation (Suzuki *et al*., 2006).

In the present study, seeds of *Lilium ledebourii* (Baker) Bioss., encapsulated within alginate along with sucrose and dehydration may offer a better resistance and the rate of viability after freezing reaches 50% (Table 1). Duncan's multiple range test showed that the differences between the survival rates of control, pretreated but non-encapsulated and pretreated-encapsulated seeds were statistically significant (Table 1). Similar results were obtained with many plants such as *Melia azedarach* L. (Bernard *et al*., 2002), *Cynodon* spp. (Reed *et al*., 2006) and *Camellia japonica* L. (Janeiro *et al*., 1996). Chang and Reed (2000) reported successful cryopreservation of both temperate and subtropical grasses.

The rate of viability after freezing in encapsulation seeds was significantly better ($p<0.01$) than controls and non-encapsulated seeds (Table 2).

Current study demonstrated that the moisture content of *Lilium ledebourii* (Baker) Bioss., seeds before exposure to LN was 15-20%. The study on

cryopreservation of *Melia azedarach* L. seeds was revealed that the optimum moisture content was 16-18% (Bernard *et al*., 2002). It is appears the optimum moisture content for germplasms of the more plants before exposure to LN is normally about 20% (Reed *et al*., 2006; Chang and Reed, 2000; Blakesley *et al*., 1995; Dumet *et al*., 2000). Reduction of water content to a critical level seems to be a necessary step for successful cryopreservation (Dumet *et al*., 1993).

The encapsulation-dehydration method has been successfully applied to a wide range of materials (Hirai *et al*., 1998; Matsumoto *et al*., 1995). Dehydration tolerance is required for successful cryopreservation by encapsulation-dehydration because the must have no freezable water (Benson, 1999). Cryopreservation is now a viable long-term storage technique for plants germplasm.

CONCLUSION

Cryopreservation is now a viable long-term storage technique for use with many species. Encapsulation plays an important role in the success of cryopreservation. For successful cryopreservation, it is necessary to induce high levels of dehydration tolerance for preservation. It has been shown that encapsulation and dehydration increased viability of cryopreserved lily seeds.

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