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Cryopreservation of Somatic Embryos of Soapberry (*Sapindus mukorossi* Gaertn.) by Vitrification

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Abstract - Somatic embryos do not survive at exposure to liquid nitrogen temperatures without cryoprotective treatments. A simplified technique which simultaneously induces and cryoprotects embryogenic calli using plant vitrification solution 2 (PVS2) followed by dehydration was developed for the cryopreservation of Soapberry genetic resources. Vitrification is a way of removing the moisture in vegetation through PVS2. The PVS2 vitrification solution consisted of 30% glycerol (w/v), 15% ethylene glycol (w/v), 15% Dimethylsulfoxide (w/v) in B5 medium containing 0.4M sucrose. Two tests were done. The one was to eliminate moisture at 0℃ and the other at 25℃. In both cases the best results came out at a vitrification time of 10~20 minutes. It was also found that the survival rate was higher at 0℃ than at 25℃. In particular, the survival rate reached more than 80%. Water-damaged embryos turned brown and stoped growth, but energetic embryos took on a milky hue and show a very vigorous growth rate. Successful cryopreservation of somatic embryos of soapberry can be used to establish *in vitro* genebanks for long-term conservation of Soapberry genetic resources to complement field genebanks and other *in vitro* methods already being used.

Key words - Cryopreservation, Somatic embryo, *Sapindus mukorossi*, Vitrification

Introduction

Soapberry (*Sapindus mukorossi* Gaertn.) is a tree that at one time the Taoists used to plant in China. They believed that its fruit could expel ghosts. Its black seeds were used in Buddhist rosaries because they are very hard and smooth. *Sapindus* means 'Indian Soap' in Latin from sapo for soap and *indus* for Indian (Langdon, 1996; Ugent, 2000). It is called the "soap fruit tree" in English. The surfactant called saponin is included in the fruit peel and in the inner layer of the skin on the branch. It is used as washing soap in India and as caustic soda in Korea. It is also used for washing hair and is very helpful in promoting healthy hair (Takagi, *et al*., 1980; Sengupta and Basu, 1982).

The demand for a 'laboratory-scale storage method' for valuable research material and specific genotypes of soapberry entered a new period following the appearance of suitable conservation methods. Cryopreservation protocols of *in vitro*-shoot tips of *melia azedarach* were an answer to this requirement (Scocchi, *et al*., 2004). The possibility of using cryopreservation as an alternative or a duplicate storage method for the traditional *ex situ* clone collections used for the storage of endangered elite trees, and tree expressing rates, valuable or interesting characteristics, including genetically modified lines, has also been rewarding.

No costly equipment is needed in vitrification based cryopreservation methods. The highly concentrated solutions of cryoprotective agents prevent the intra and extra cellular crystallization of ice in the cells and tissues as they pass rapidly through the temperatures where ice crystal growth occurs (Martinez, *et al*., 2003; Lambardi, *et al*., 2005). The vitrification of soapberry's cell has, so far, not been very successful. However, some improvements have been achieved in the cold hardening and regeneration of somatic embryo of soapberry cryopreserved by the loading treatment preceding plant vitrification solution 2 (PVS2) like dehydration in the vitrification protocol applied for other deciduous trees (Lambardi, *et al*., 2000; Jokipii, *et al*., 2004). These methods could be applicable to the vitrification of somatic embryos of soapberry. On the other hand, a number of studies on the precultivation and dehydration of tree material have shown that the testing of species/meristem genotype specific concentrations and exposure times (Kuranuki and Sakai, 1995; Shatnawi, *et al*., 1999; Lambardi, *et al*., 2000) is essential for the development of suitable vitrification protocols. The recent good survival results obtained for *melia azedarach* create a promising basis for the further development of a vitrification protocol for soapberry.

To our knowledge, no successful cryopreservation method using

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vitrification has been published for soapberry. The aim of the present study was to develop a vitrification protocol that can easily be applied for the cryopreservation of the Sapindus species.

Materials and Methods

Plant materials

Seeds of the soapberry were obtained from the campus of Kyungpook National University, Daegu, Korea. This tree was a shrub which grew to a height of 34m. The embryos were immersed in 70% ethyl alcohol for 3minutes and were disinfected by dipping in a solution mixed with Sodium Hypochloite (NaOCl) and 0.1% Tween-20 (Polyoxyethylene Sorbitan Monolautate: Sigma, St. Louis Mo) for 20-30minutes. Then they were rinsed 3-4 times with sterile water. The sterilized embryos were planted on a growth medium.

MS (Murashige and Skoog) basal medium was used as cultivation medium, in which 3% sucrose, 100mg/L myoinostol, 100mg/L vitamin, and 0.4% Gelrite were included.

Immature leaves 3-6mm in length from 5 to 14 days old *in vitro* shoot cultures were cut into 1cm^2 pieces and placed in a B5 induction medium supplemented with 2,4-D (dichlorophenoxyacetic acid), NAA (naphthalineacetic acid) as auxin, BA (6-Benzyladenine), as cytokinin each 0.1-5mg/L of B5 basal medium, 3% sucrose, 100mg/L myoinostol, 100mg/L vitamin, and 0.4% Gelrite. The pH was adjusted to 5.8 and it was autoclaved at a temperature of 121℃ and an atmospheric pressure of 1.5kg/cm^2 for 15min. The embryos were subcultured at 4 weeks intervals under cultivation conditions of 25±2℃ in the dark for 21 days.

Cryopreservation procedure (vitrification methods)

Vitrification method # 1

Somatic embryos were loaded for 20min 0℃ at a loading buffer which had B5 medium with 2M glycerol and 0.4% sucrose in 2ml cryo-tubes, followed by immersion in the PVS2 (Sakai, *et al*., 1990) consisting of 30% glycerol (w/v), 15% ethylene glycol (w/v), 15% DMSO (w/v) in B5 medium containing 0.4 M sucrose (pH 5.8) for 0 to 120 min on ice. Somatic embryos were preserved at -196 ℃ for a day by plunging directly into liquid nitrogen (LN).

Vitrification method # 2

Somatic embryos were loaded for 20 min 25℃ at a loading buffer which had B5 medium with 2M glycerol and 0.4% sucrose in 2ml cryo-tubes, followed by immersion in the PVS2 in B5 medium containing 0.4 M sucrose (pH 5.8) for 0 to 120 min at room temperature. Somatic embryos were preserved at -196℃ for a day by plunging directly into LN.

Thawing and recovery

Somatic embryos were rapidly warmed in a water bath at 40℃. PVS2 was removed and embryos were plunged into liquid B5 medium containing 1.2M sucrose for 15min. Somatic embryos were planted on regeneration medium.

Acclimatization

Rooted plantlets were washed in water to remove agar from the roots and transferred to pots containing perlite and vermiculite [1:1 (by volume)] and placed in the culture room. Glass covers were used to ensure high humidity around the plants at the initial stage of growth and were gradually opened day by day during the acclimatization period. However, the percentage of plants established *ex vitro* was not evaluated.

Statistical analysis

The data shown represent the mean ±S.E. of three independent experiments. An analysis of significant difference was carried out using Duncan's Multiple Range Test (DMRT) using SPSS 10.0 (SPSS Inc. USA).

Results and Discussion

The induction of embryogenic callus pieces and somatic embryos in the solid medium culture

Most plant cells had their own totipotency. For the differentiation of plants, many researchers have used high-density plant hormones. In the current experiment, we have succeeded in inducing differentiated callus pieces by utilizing auxin 2,4-D, NAA, and cytokinin BA. Every callus was not a regenerated piece. However, the individually induced callus pieces with auxin and cytokinin could be regenerated (Table 1). Therefore, we carried out an additional experiment combining cytokinin (BA) with the auxin (2, 4-D). The induction of somatic embryos were the best on 0.1mg/L 2,4-D, 0.01mg/L BA (Table 2).

In the case of soapberry pieces leaves, as shown in Fig. 2 a, b, two types of callus pieces were induced. One type of callus piece induced from leaves of soapberry showed pale, crispy, and crumbly callus pieces and the others were a yellowish, shiny and solid callus. Both of them were cultivated. The second type had the capacity for re-differentiating embryogenic callus pieces.

Cryopreservation

Cryopreservation is one of the *in vitro* genetic conservation ways since it is cheaper and can be stored in a very limited space. Water is crystallized in the process of freezing. Thus, when the temperature drops below 0℃, the moisture in vegetations gets condensed and crystallized. Then, the frozen sharp crystals may destroy cell membranes or walls. The most important factors, one needs to be most cautious in using cryopreservation is how to safely eliminate moisture in vegetation.

Vitrification is a way of removing the moisture in vegetation through PVS2. Two methods of cryopreservation were tested. One was to eliminate moisture at 0℃ and the other at 25℃. With both methods, the best results came out at the vitrification time of 10~20 minutes. It was also found that the survival rate was higher at 0℃ than at 25℃. In 10 minutes vitrification treatment, the survival rate reached more than 80% (Fig. 1). Water-damaged cells turned brown and stoped growth, but energetic embryos took on a milky hue and

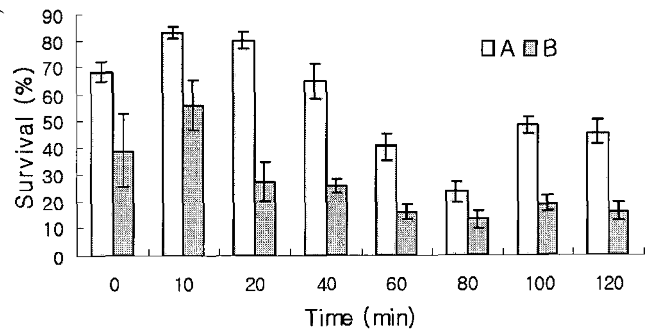

Fig. 1. Survival of somatic embryo exposed to PVS2 for different lengths of time. A: PVS2 at 0℃; B: PVS2 at 25℃.
a) Means within columns followed by the same letter are not significantly different at P=0.05 according to the Duncan Multiple Range test.

Table 1. The effects of auxin in a induction medium on percentage embryogenesis and a mean number of somatic embryos from leaves of soapberry

Growth regulator (ppm)		No. of occurred callus (%)	No. of calli showing embryogenesis (%)±SE*	Total number of somatic embry
2,4-D	0.1	100.00	23.40±1.34[b]	-
	1	100.00	10.34±1.99[a]	-
	2	100.00	6.67±0.46[a]	-
	5	100.00	6.16±0.36[a]	-
NAA	0.1	100.00	-	-
	1	100.00	-	-
	2	100.00	-	-
	5	100.00	-	-
BA	0.1	30.00	-	-
	1	60.00	-	-
	2	93.75	-	-
	5	80.00	-	-

*Mean Standard error (SE).
[a, b]Means within columns followed by the same letter are not significantly different at P=0.05 according to the Duncan's Multiple Range test.

Table 2. The effects of 2,4-D and BA combination on percentage embryogenesis and the mean number of somatic embryos from the leaves of soapberry

Growth regulator (ppm) 2,4-D	BA	No. of calli showing embryogenesis (%)±SE*	Total number of somatic embryos±SE*	Mean no. of somatic embryos per responding explant ±SE*
0.1	0.01	33.33±6.41[b]	13.67±2.40	1.52±0.26
0.1	0.1	11.11±6.41[ab]	-	-
0.1	1	7.40±7.41[a]	-	-
0.1	2	-	-	-
0.1	5	-	-	-

*Mean Standard error (SE).
[a, b, ab]Means within columns followed by the same letter are not significantly different at P=0.05 according to the Duncan's Multiple Range test.

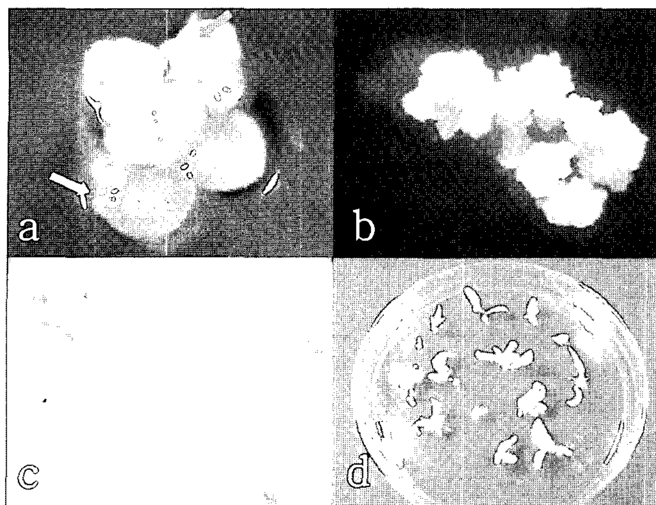

Fig. 2. Embryos after vitrification cryopreservation. (a) After 1 week (red arrow: active embryos, white arrow: dead embryos); (b)~(c): After 6 weeks; (d): After 8 weeks

showed a very vigorous growth rate (Fig. 2).

In conclusion, we provide experimental evidence supporting two mechanisms by which PVS2 aids cryoprotection of somatic embryos of soapberry at 0℃ and 25℃. Because solution components permeate into cells, PVS2 treatment can broaden allowable water contents in cryopreserved somatic embryos by lessening the damage from excessive cell shrinkage and limiting the risk of ice formation and growth. These effects were observed at both 0℃ and 25℃. In most of the studies in which both methods have been compared, vitrification is reported to be preferable, i.e., for herbaceous *Asparagus officinalis* (Nishizawa *et al.*, 1992) and for woody plants, i.e., *Citrus sinensis* (Sakai, 1991), *Malus domestica* (Wu, *et al.*, 1999), *Ribes* (Barbara, *et al.*, 2001), and *Populous tremuloides* (Jokipii, *et al.*, 2004).

The fact that not only different species but also different genotypes behave differently and require different cryopreservation protocols for successful recovery that was clearly seen in the present study. With soapberry, the average recovery of different genotypes varied from 86% to 25% in best vitrification methods (Vitrification #1) and from 53% to 12% using Vitrification #2. This result showed that the correct dehydration period and temperature with PVS2 is also important factors these should be tested in the vitrification protocols. According to Corredoira *et al.*, (2004), the exposure time to PVS2 is species-specific and it may be necessary to increase dehydration times and temperatures. In the present study the best treatment peried with PVS2 is 10min, which is a relatively average time for dehydration of somatic embryos at 0℃.

This study concluded that successful cryopreservation of somatic embryos of soapberry could be used to establish *in vitro* genebanks for long-term conservation of soapberry genetic resources, to complement field genebanks and other *in vitro* methods already being used.

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