Korean J. Plant Res. 19(3) : 385~391 (2006)

Cryopreservation of *in vitro*-cultured Axillary Shoot Tips of Japanese Bead Tree (*Melia azedarach*) using Vitrification Technique

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Abstract - *In vitro*-grown axillary buds of *Melia azedarach* were successfully cryopreserved by vitrification. On the MS medium supplemented with BA 1 mg/L, multiple shoots were developed within 4~5 weeks. Plantlets of *Melia azedarach* were cold-hardened at 10℃ for a 16-hr photo-period for 6 weeks. Excised axillary shoot-tips from hardened plantlets were precultured on a solidified Murashige & Skoog agar medium (MS) supplemented with 0.7 M sucrose for 1 day at 25℃. Axillary shoot-tip meristems were dehydrated using a highly concentrated vitrification solution (PVS2) for 60 min at 0℃ prior to a direct plunge into liquid nitrogen (LN). The PVS2 vitrification solution consisted of 30% glycerol (w/v), 15% ethylene glycol (w/v), 15% DMSO (w/v) in MS medium containing 0.4M sucrose. After short-term warming in a water bath at 40℃, the meristems were transferred into 2 ml of MS medium containing 1.2 M sucrose for 15 min and then planted on solidified MS culture medium. Successfully vitrified and warmed meristems resumed growth within 2 weeks and directly developed shoots without intermediary callus formation. The survival rate of cold-hardened plantlets for 3 and 4 weeks was 90%. We did not find any difference in PCR-band patterns between control and cryopreserved plants. This method appears to be a promising technique for cryopreserving axillary shoot-tips from *in vitro*-grown plantlets of Medicinal plants.

Key words - Cryopreservation, Masspropagation, *Melia azedarach*, Vitrification technique

Introduction

Around 1.4 million living species have been identified in the natural ecosystems on the Earth (Wilson, 1988). Mayr (1969) estimated there are 10 million living species on the Earth and Erwin (1983) estimated more than 30 million. Of these, about 250 thousand are flowering plants. According to an IUCN report, about half a million to one million species of living organisms will be exterminated within the next 20 years. Reid and Miller (1989) reported that 19,078 living species are endangered or declining, and a further 384 already extinct. There is no doubt that the current extinction crisis threatening the Earth's biological diversity is worsening.

Germplasm preservation, essential for the maintenance of biodiversity and the avoidance of genetic erosion, is a costly activity for plant propagation enterprises. With respect to woody species, germplasm preservation using traditional systems (i.e. clonal orchards) will face serious problems in the near future because of the large spaces required for production and the high cost of maintenance. An alternative is offered by cryopreservation in LN (-196℃). Traditional cryopreservation techniques utilize expensive equipment to carry out gradual chilling and freezing of the explant and to avoid intracellular ice formation, which may result in lethal damage to cells (Lambardi *et al.*, 2000).

Recently, new approaches for plant tissue cryopreservation by direct plunging in LN have been explored (Benson, 1994) and, of these, the vitrification procedure has been proven to be the most promising. In this system, when tissues are plunged into LN, the highly concentrated aqueous solution solidifies into a non-crystal, glassy-state structure. Consequently, ice crystal nucleation is impeded, and vitrified cells can escape the danger of intracellular freezing (Fahy *et al.*, 1984). To date, the vitrification procedure has been applied mainly to the preservation of herbaceous species (Grout, 1995), although a still very limited number of woody species - i.e. navel orange (Sakai *et al.*, 1990), apple and pear (Niino *et al.*, 1992), mulberry (Niino, 1995), *prunus* spp. (Brison *et al.*, 1995; Niino *et al.*, 1997) - have been tested for cryopreservation following this approach. Cryopreservation of plant meristems or cells, somatic and zygotic embryos, is an important technique for long-term preservation without genetic alteration.

Melia azedarach is a tree of great important because it has therapeutic as well as insecticidal properties (Ascher *et al.*, 1995; Schmidt *et al.*, 1997). The tree has been used for producing termite resistant wood (Yaga, 1978), fodder and green manure, as well as oil from seeds (Ascher *et al.*, 1995). It originates from north-western India, and is now found in many subtropical countries. The tree bears seeds

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only during the summer and its natural regeneration is limited in a natural stand.

This study reports the cryopreservation procedure for *Melia azedarach* using the vitrification method.

Materials and Methods

Plant materials

Fruits of *Melia azedarach* L. (Meliaceae) were obtained from several trees at Jinju National University, Gyeongnam, Korea. Seeds were first washed in running tap water for 1 day and disinfected by immersion in 70% (w/w) ethanol alcohol for 1 min, in sodium hypochlorite (25~30 min) and 0.1% Tween-20 (polyoxyethylenesorbitan monolaurate; Sigma) with agitation for 10 min, followed by four to five rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from the megagametophytes and placed horizontally on a solidified induction medium in 150-mm Petri dishes.

Media were prepared using the MS salt composition, sucrose (3%) and agar (0.8%). Growth regulators were incorporated into the media and pH was adjusted to 5.8 before autoclaving. The media were then sterilized in an autoclave at 121℃ for 15 min. The cultures were incubated under a 16:8-hour photo-cycle at 25℃, 50~60% relative humidity (RH). Plant growth regulators were added prior to autoclaving as optional additives, according to the experimental objectives.

Direct somatic embryogenesis and micropropagation

For direct somatic embryogenesis of *Melia azedarach*, forty petiole segments (0.5 cm) were cultured on MS (Murashige and Skoog) medium + BA (6-Benzyladenine) 0.1, 0.5, 1, 5 mg/L.

An optimal micropropagation procedure was achieved using nodal segments with two axillary buds as the explant. In each treatment, five nodal segments with two axillary buds were cultured on MS medium with BA (0.1, 0.5, 1, 5 mg/L).

Cryopreservation procedure

Melia azedarach plantlets were cold-hardened on hormone-free MS medium gelled with 0.75% agar and supplemented with 3% sucrose for 1~6 weeks at 10℃ under a 16:8-h photo-cycle. Excised axillary shoot-tips from cold-hardened plantlets were precultured on a solidified Murashige & Skoog agar medium (MS) supplemented with 0.7 M sucrose for 1 day at 25℃. Cold-hardened axillary buds were loaded for 20 min at 25℃ with a cryoprotectant (2 M glycerol and 0.4 M sucrose; from Matsumoto *et al.*, 1994) in 2 ml cryotubes, followed by immersion in the PVS2 vitrification solution (Sakai *et al.*, 1990) consisting of 30% glycerol (w/v), 15% ethylene glycol (w/v), 15% DMSO (w/v) in MS medium containing 0.4 M sucrose (pH 5.8). Sixty buds were cooled to -196℃ by plunging them directly into LN weekly. The axillary buds were then rapidly warmed in a water bath at 40℃.

Thawing and plating

The axillary buds were thawed by removing the vials from the LN and then plunging them for 3 min in a water bath adjusted to 40℃. After rapid warming, PVS2 was drained and replaced with liquid MS medium containing 1.2 M sucrose and the buds were immersed in this for 15 min before being planted. The axillary buds were then transferred onto 0.75% agar MS medium and cultured on MS medium.

After 3 weeks on MS medium, shoot tips (3 cm) were isolated and transferred to MS + IBA (Indole-3-butyric acid) 0.1, 0.5, 1, 2, 4 mg/L. The number of rooted shoots and the quality of roots was recorded after 2 weeks.

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.

DNA extraction and RAPD analysis

Genotypic variations between non-cryopreserved and cryopreserved axillary shoot tips were compared by PCR protocol. DNA was extracted by 2×CTAB protocols. A about 50 mg of leaves of both cryopreserved and non-cryopreserved plants respectively were ground in a mortar with liquid nitrogen for 5-10 min. The ground leaf samples were then transferred to microcentrifuge tubes (1.5 ml), 400 to 600 μl of hot (65℃) 2×CTAB was added and the samples were incubated for 30 min at 65℃ in a water bath. An equal volume of Chloroform : Isoamyl alcohol (24:1) was then added to each sample and mixed thoroughly by shaking the tubes sharply. The samples were then centrifuged for 5 to 10 min at 10,000 rpm two times, and the top layers of the samples were placed in new 1.5 ml E-tubes with Isoprophenol (0.6v) or 2 volume of 95 or 100% Et-OH (ethyl alcohol). The tubes were swirled until fiber appeared, and the samples washed with 70% Et-OH after decanting the supernatant DNA and dissolving the pellets in ddH_2O with RNase.

PCR reactions were carried out in a 25 μl reaction mix containing approximately 2 ng template DNA, 2.5 picomoles of each dATP, dCTP, dGTP and dTTP (Promega, Madison, Wis.), 5 picomoles of a single 10-base primer, 0.5units of DynaZymeTM polymerase (Finnzymes Oy,). The buffer (10mM Tris-HCL, pH 8.8 at 25℃, 1.5 mM $Mgcl_2$, 50 mM KCL, 0.1% Triton X-100) used was the one provided by the manufacturer of the enzyme. The PCR reactions were run in a Progene thermal cycler (Techne Ltd., UK) programmed for and initial denaturation step of 94℃ followed by 35 cycles as follows; 30 sec at 94℃, 30 sec at 36℃ and 1.5 min at 74℃. A final elongation step of 5 min at 74℃ was included. The PCR products were separated on 1.5% agarose gel stained with ethidium bromide. The gels were visualized with a UV transilluminator and photographed using Polaroid 667 film. A DNA ladder (Lambda/Hind Ⅲ) was used as a size marker. The number of bands were varied according to the Random primer (OPC-01, OPC-02, OPC-03, OPC-08, OPC-09, OPC-10, OPC-12, OPC-14, OPC-19; Table 1).

Table 1. Random primer sequences used in this study.

Code	Sequence
OPC-01	5′ TTC GAG CCA G 3′
OPC-02	5′ GTG AGG CGT C 3′
OPC-03	5′ GGG GGT CTT T 3′
OPC-08	5′ TGG ACC GGT G 3′
OPC-09	5′ CTC ACC GGT C 3′
OPC-10	5′ TGT CTG GGT G 3′
OPC-12	5′ TGT CAT CCC C 3′
OPC-14	5′ TGC GTG CTT G 3′
OPC-19	5′ GTT GCC AGC C 3′

Results

Direct somatic embryogenesis and micropropagation

Direct somatic embryogenesis of *Melia azedarach* was achieved from microcultured petiole segments on MS medium with 0.5 mg/L BA. After 4 weeks of culture on regeneration medium, approximately 95% of petiole segments were regenerated with shoots. These shoots had a soft structure and were pale-yellow in colour. Each shoot was transferred to the MS basal medium and grown to whole plantlet (Fig. 1).

Optimal micropropagation procedure was achieved using nodal segments with two axillary buds as explants on MS medium with 1 mg/L BA (Fig. 2). In each treatment, five nodal with axillary buds were cultured. These shoots were subcultured every three weeks on a

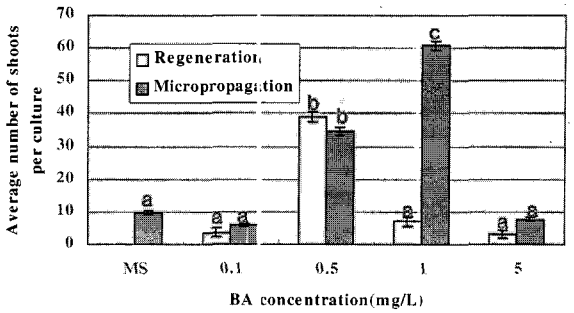

Fig. 1. The effect of BA concentration in the induction medium on the direct somatic embryogenesis and micropropagation. Different letters indicate Duncan's multiple range tests (Significant at $p<0.05$).

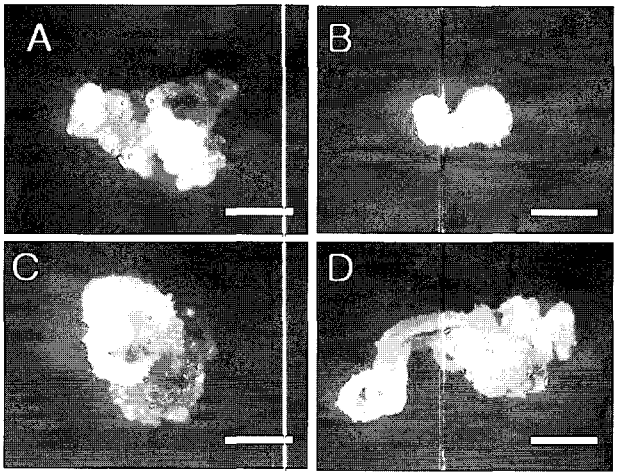

Fig. 2. Plants via direct somatic embryogenesis from petiole segments of *Melia azedarach* (Bar mean 0.5 cm). (A) White callus from rachis, (B, C) Shoot formation from white callus, (D) Shoots of green color.

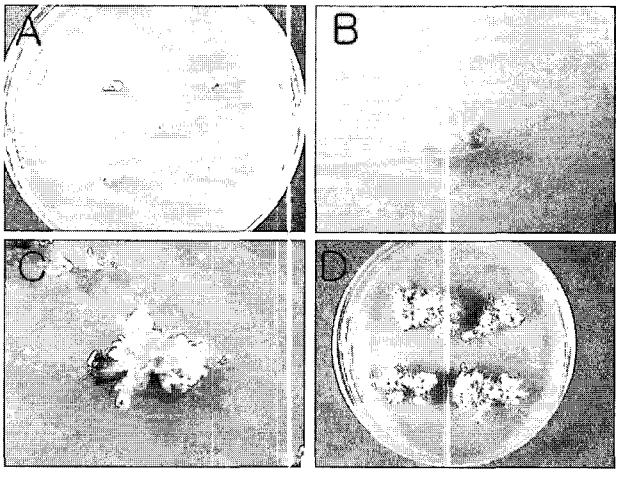

Fig. 3. Proliferating nodal cultures of *Melia azedarach* on MS with BA. The culture was maintained on 1 mg/L BA and subsequently transferred for tree subcultures. (A) 3 days after planting, (B) 7 days after planting, (C) 15 days after planting, and (D) 30 days after planting.

differentiation medium. A high concentration of BA produced a callus that was white or cream-colored. In addition, a low concentration of hormone did not encourage shoot tip growth but induced callus formation. In each treatment, about 40-50 shoots were obtained from planted axillary buds. Formation of axillary shoots was observed on media with cytokinin (BA), where the explant developed shoots rapidly at all concentrations. Shoot proliferation was influenced by cytokinin type and concentration. Thus, a medium containing 1 mg/L BA produced an average of 6 times more shoots than hormone-free media (Fig. 1, 3).

Cryopreservation

In the preliminary experiments, excised axillary shoot-tip meristem from segments without cold-hardening were precultured on a solidified Murashige & Skoog agar medium (MS) supplemented with 0.7 M sucrose for 1 day at 25℃ and loaded for 20 min at 25℃ with a cryoprotectant (2 M glycerol and 0.4 M sucrose) in 2 ml cryotubes. The cryoprotectant was then replaced with the PVS2 vitrification solution. This preculture resulted in a considerable decrease in the rate of shoot formation of vitrified meristem cooled to -196℃ (Fig. 4).

Therefore we investigated the effects of cold-hardening on the shoot formation of vitrified meristem. Shoot tips from *in vitro*-grown meristems on MS basal medium, using cold-hardened stock plants of *Melia azedarach* at 10℃ were successfully cryopreserved at -196℃ by one-step vitrification. After cold-hardening at 10℃ for 1~6 weeks on hormone-free MS medium containing 3% sucrose, shoot tips were loaded with cryoprotectant then treated with the PVS2 vitrification solution for 0~120 min and plunged directly into LN (liquid nitrogen).

The best survival rate as shown by shoot formation increased gradually with an increasing period of cold-hardening, and reached a max-

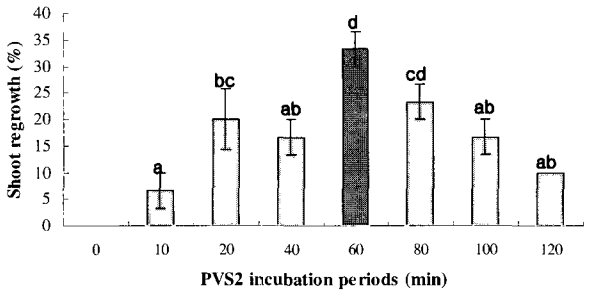

Fig. 4. Effect of PVS2 solution without cold-hardening on the rate of shoot formation of axillary buds from meristems of *Melia azedarach* cooled to -196℃. Different letters indicate Duncan's multiple range tests (Significant at $p<0.05$).

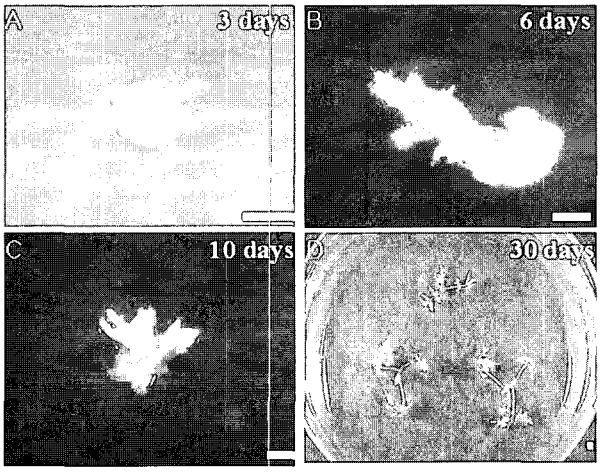

Fig. 5. Shoot development from successfully cryopreserved meristems (Bar means 2mm).
(A) Recovered axillary bud meristem, (B) Dead and live organs, (C) Multiple shoots from the buds, and (D) Developed plant.

Table 2. Effect of cold-hardening on the shoot formation of shoot tips cooled to -196℃ following dehydration with PVS2 solution

Cold-hardening \ PVS2	Survival rate (%) (Mean ± S.D.)						
	10min	20min	40min	60min	80min	100min	120min
1 week	33±11.54	33±15.27	40±20	46±15.27	50±10	43±5.77	40±5.77
2 weeks	43±11.54	50±0	63±5.77	73±15.27	80±10	66±5.77	56±5.77
3 weeks	63±5.77	66±5.77	73±5.77	90±10	86±5.77	73±5.77	60±10
4 weeks	50±5.77	56±5.77	80±20	90±10	83±5.77	70±10	60±0
5 weeks	65±5.77	63±5.77	75±10	76±0	85±2.24	43±11.54	50±5.77
6 weeks	55±10	40±5.77	80±10	73±5.77	75±2.24	50±0	63±5.77

Approximately 10 axillary shoot tips were tested for each of four replicates; Cold hardening: 10℃ under 16:8-hr photo cycle; PVS2 treatment: 10~120 min at 0℃.

imum (90%) at 3 to 4 weeks. It also increased with increasing time of exposure to PVS2 and reached a maximum at 60 min (Table 2). Successfully vitrified and cooled axillary shoot tips were planted on gelled MS medium and started to develop and resume growth within 2 weeks (Fig. 5).

Optimum rooting procedures

In our study, we compared the growth rates of roots on the excised terminal bud sections with those of the same size from *in vitro* plants. Some roots were formed on the base of the excised shoot in the 2nd week and significantly more roots generated with continuous exposure to either 0.5 mg/L or 1 mg/L of auxin. Using these concentrations of auxin (IBA), three to five adventitious roots formed on about 100% of the shoots (Table 3).

Table 3. Effect of IBA on the root and shoot formation of the *Melia azedarach*

Medium	Length of root (cm)	Length of shoot(cm)	Callus induction(○,×)
MS	0	0	○
MS+IBA 0.1ppm	0	0	○
MS+IBA 0.5ppm	0.5	2	×
MS+IBA 1ppm	5	6	×
MS+IBA 2ppm	2	3	×
MS+IBA 4ppm	0	0.5	○

RAPD analysis

In this paper, we confirmed the phenotypic or genotypic changes of both the cryopreserved and, and non-cryopreserved plants using DNA extraction and RAPD analysis. Agarose gel electrophoresis revealed a band of approximately 500 bp~2000 bp (Fig. 6).

Acclimatization

Acclimatization was affected directly by rooting conditions. Survival was best when plantlets were transferred to pots after a short period of root emergence on rooting medium. Multiplication medium was also important for successful acclimatization, since shoots transferred to rooting media from media with BA, where the shoots had better leaf expansion and better quality in general, resulted in better acclimatization. For acclimatization, shoots were rooted (0.5~1 mg/L). After 4 weeks, rooted plantlets were washed in water to remove agar from the roots, transferred to pots containing perlite and vermiculite (1:1) 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 (Fig. 7).

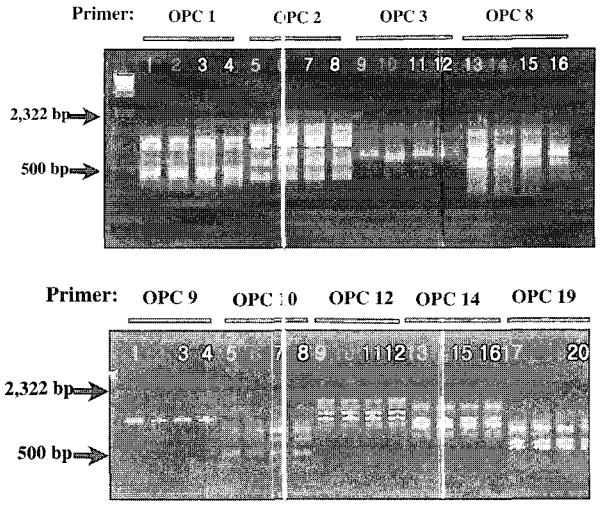

Fig. 6. RAPD analysis of control and cryopreserved plants. Lane 1,5,9,13,17: control, Others: plants immersed in LN.

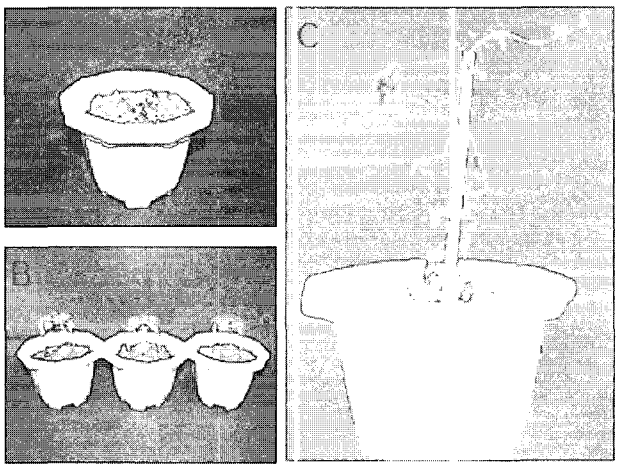

Fig. 7. An fully acclimatized plantlet in perlite and vermiculite (1:1). (A) Plant established in pots, (3) Plants into the glass cover, (C) Hardened plants in pots.

Discussion

Cryopreservation is an important tool for the long-term storage of germplasm using a minimum of space and maintenance. In the vitrification method, with or without encapsulation, meristems are sufficiently dehydrated osmotically by exposure to a highly concentrated vitrification solution (PVS2). Towill (1990) was the first to report the cryopreservation of mint by vitrification. For successful cryopreservation, it is necessary to avoid lethal intracellular freezing during rapid cooling. Thus, cells and meristems have to be suffi-

ciently dehydrated prior to a plunge into LN (Sakai, 1993). Cells and meristems are dehydrated using a highly concentrated vitrification solution. However, prolonged exposures to a concentrated vitrification solution can produce injury to the experimental material due to chemical toxicity or strong osmotic stress. On the other hand, survival of non-frozen shoot tips following PVS2 treatments at the above time/temperature combination, was always higher than 95% (Caccavale *et al.*, 1998). Earlier studies have emphasized the positive effect of cold-hardening on successful cryopreservation of fruit-tree organs (Reed, 1990; Niino *et al.*, 1997; Reed *et al.*, 1998). A short period of cold-hardening (less than 6 weeks) greatly improved the survival of apple shoot tips cryopreserved by conventional (Kuo and Lineberger, 1985; Stushnoff, 1987; Brison *et al.*, 1995) or vitrification-based procedures (Niino *et al.*, 1992, 1997). Therefore, we investigated the significance of both exposure time to PVS2 and cold-hardening. *In vitro*-grown axillary shoot-tip meristems of *Melia azedarach* successfully vitrified, and warmed meristems directly developed vigorous shoots directly within 2 weeks of planting. Axillary bud survival was significantly higher when the axillary shoot tips were exposed to PVS2 solution for 60 min at 0℃ after 3~4 weeks cold-hardening at 10℃. No morphological abnormalities and callus formation were observed during the development of plants from vitrified meristems.

In this study, we have described an efficient procedure for germplasm cryopreservation of *Melia azedarach* species through direct somatic embryogenesis and rapid clonal propagation after cryopreservation using vitrification methods.

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(Received 12 October 2005; Accepted 10 May 2006)