Jour. Korean For. Soc. Vol. 95, No. 5, pp. 585~590 (2006)

JOURNAL OF KOREAN FOREST SOCIETY

Cryopreservation of *in Vitro* Grown Axillary Shoot-tip Meristems of *Lycium chinense* by Vitrification

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Abstract : *In vitro*-grown axillary shoot-tip meristems of *Lycium chinense* Mill. from cold-acclimated plant were successfully cryopreserved using a vitrification technique. After loading for 15 minutes with a mixture of 2.0 M glycerol and 0.4 M sucrose (20°C), small segments (1-2 mm, 3-4 mm, and 5-6 mm) were cut from axillary buds and exposed to the cryoprotectant solution containing 30% glycerol, 15% ethylen glycol, 15% dimethyl sulfoxide (DMSO), and 0.4 M sucrose at 0°C for 30-120 minutes prior to direct plunge into liquid nitrogen (LN). After rapid thawing (40°C), the segments were washed with MS medium containing 1.2 M sucrose for 0-35 minutes, and then transferred onto recovery-growth medium. The highest survival rate (about 90%) was obtained with cold-hardening treatment, and cryopreserved explants were successfully recovered to plantlets. No abnormal morphological changes were observed with the recovered plants after cryopreservation.

Key words **:** *axillary shoot-tip meristems, cryopreservation,* Lycium chinense, *PVS2, vitrification*

Introduction

Lycium chinense Mill. (Chinese matrimony vine), which belongs to the family Solanaceae, is a hard wood species, and one of the common and important medicinal crop growing throughout Korea. It occupies the warm and subtropical environmental area, and has been distributed in such as Japan, Southeastern Asia and European countries (Hou, 1984). In China, the fruits are infused with water or spirit liquor and cooked with broth of poultry or domestic animal meat, as a good visual acuity improver according to the traditional Chinese medicine (Qian *et al.*, 2004). The root barks of *L. chinense* has been shown to be clinically effective for hypertension, and also reported to exhibit hypotensive, hypoglyconic, antipyretic and anti-stress ulcer activity in experimental animals (Funayama *et al.*, 1980).

Genetic resources of this species are mainly conserved vegetatively in field gene banks such as grafting and rooting of cuttings, and stored in the large size refrigerators as seeds collected from seed orchards. These methods, however, are not always reliable and also not the best way of deploying improved genetic materials (Ford *et al.*, 2000). The amount of viable seeds obtained from seed orchards are not always consistent. Grafting is not economically viable on a large scale and rooting of cuttings from mature trees is not easy. Moreover, genotypes of interest can be lost due to contaminations or environmental stresses. To overcome these problems, cryopreservation was applied and several attempts have been made to optimize the techniques for a long term storage of plant germplasm safely (Panis *et al.*, 1996; Hirai and Sakai, 1999; Martinez *et al.*, 1999). Cryopreservation has been considered as an ideal tool for the long-term storage of germplasm. Theoretically, the plant materials can thus be stored without any changes for an indefinite period of time (Engelmann, 1997).

Cryopreservation protocols were developed for approximately 40 tropical plant species (Engelmann, 1991). Eight of them are only cryopreserved through meristems. More recently, the vitrification was proved as a successful method for cryopreservation of suger beet (Lambardi *et al.*, 2000; Vandenbussche *et al.*, 2000), *Acer mono* (Park *et al.*, 2005) and *Melia azedarach* (Yang *et al.*, 2006). But, there is no report on the cryopreservation using the axillary shoot-tip meristems of *L. chinense*.

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Table 1. Sequence of random OPC primers (Operon Tech. Inc., USA).

Code	Sequence	Code	Sequence
OPC 01	5' TTCGAGCCAG 3'	OPC 11	5' AAAGCTGCGG 3'
OPC 02	5' GTGAGGCGTC 3'	OPC 12	5' TGTCATCCCC 3'
OPC 03	5' GGGGGTCTTT 3'	OPC 13	5' AAGCCTCGTC 3'
OPC 04	5' CCGCATCTAC 3'	OPC 14	5' TGCGTGCTTG 3'
OPC 05	5' GATGACCGCC 3'	OPC 15	5' GACGGATCAG 3'
OPC 06	5' GAACGGACTC 3'	OPC 16	5' CACACTCCAG 3'
OPC 07	5' GTCCCGACGA 3'	OPC 17	5' TTCCCCCCAG 3'
OPC 08	5' TGGACCGGTG 3'	OPC 18	5' TGAGTGGGTG 3'
OPC 09	5' CTCACCGTCC 3'	OPC 19	5' GTTGCCAGCC 3'
OPC 10	5' TGTCTGGGTG 3'	OPC 20	5' ACTTCGCCAC 3'

This study was aimed for developing a simple and reliable protocol for the cryopreservation of *in vitro* grown axillary shoot-tip meristems of *L. chinense* by vitrification.

Materials and Methods

1. Plant materials

Fruits of *L. chinense* were collected during the late summer from extant natural populations. The seeds were stored in a refrigerator (4°C) for 1 month to break down the dormancy. Seeds were washed with running tap water for one day and surface-disinfected by immersing in 70% ethanol for one minute. They were sterilized by dipping in 0.1% sodium hypochlorite (NaClO) and 0.1% Tween-20 (polyoxyethylenesorbitan; Sigma, USA) solution for 25 to 30 minutes with occasional agitation, followed by washing five times using sterile distilled water. Mature zygotic embryos were aseptically removed from the megagametophytes and placed horizontally on induction MS (Murashige and Skoog, 1962) medium (1.5× MS salts supplemented with 100 mg/l myo-inositol, MS vitamin, 4% sucrose and 0.15% gelatin). The cultures were maintained in a growth room at 25°C and 16h/8h photoperiod (96 μmol $m^{-1}s^{-2}$) for 2 weeks. pH of the media was adjusted to 5.8 before autoclave. The media were then sterilized in an autoclave at 1.5 lb pressure and 121°C for 20 minutes. After 2 weeks of culture, the explants were cold-hardened on hormone-free MS medium from 2 to 60 days at 10°C incubator. The axillary buds including stems were used as plant materials for the crypreservation experiments.

2. Pretreatment and vitrification procedures

The axillary buds with small part of stem were dissected in different sizes (1-2, 3-4, and 5-6 mm) and transferred into cryotubes containing loading solution consisted with 2.0 M glycerol and 0.4 M sucrose. Those were incubated at 5°C for 0 to 35 minutes. PVS2 vitrification solution consisting of 30% glycerol, 15% ethylene glycol, 15% DMSO and 0.4 M sucrose in liquid MS medium replaced the loading solution. The cryotubes were then incubated at 0°C for 30 to 120 minutes, and directly immersed into liquid nitrogen (LN) for one hour.

3. Thawing and recovery

Axillary shoot-tips were rapidly thawed by removing the vials from the LN and warmed in 40°C water for 1-2 minutes. After rapid thawing, PVS2 solution was drained from the cryotubes and held into liquid MS medium containing 1.2M sucrose in which the axillary shoot-tips were washed for 0-35 minutes before plating. Later, the explants were cultured on 3/2 MS semi-solid medium for one day before transferring them onto a new medium.

4. DNA extraction and RAPDs analysis

DNA was extracted from cryopreserved and non-cryopreserved materials using modified 2x CTAB protocols (Edwards *et al.*, 1991; Choi *et al.*, 1999).

20 random primers (Operon Tech. Inc., USA) were used to perform PCR amplification (Table 1). Reaction mixture was consisted with approximately 2 ng of template DNA, 2.5 picomoles of each dATP, dCTP, dGTP and dTTP (Promega, Madison, Wis., USA), 5 picomoles of a single 10-base primer, and 0.5 units of Dyna Zyme™ polymerase (Finnzymes Oy, Finland). It was performed using a Progene thermal cycler (Techne Ltd., UK) programmed for an initial denaturation step at 94°C, followed by 35 cycles as follows; 30 seconds at 94°C, 30 seconds at 36°C and 1.5 minutes at 74°C. A final elongation step of 5 minutes at 74°C was included. The PCR products were separated on 1.5% agarose gels stained with ethidium bromide. The gels were visualized with a UV transilluminator and photographed using Polaroid 667 film. A DNA ladder (Lambda/Hind deg. C) was used as a size marker. The number of bands were varied according to the random primer.

5. Statistical analysis

The data shown represent the mean ± S.E. of three independent experiments. An analysis of significant dif-

ference were carried out using Duncan's Multiple Range Test (DMRT) using SPSS 10.0 (SPSS Inc. USA).

Results

1. Pretreatment and vitrification methods

The effects of cold-hardening on the survival of axillary buds immersed in LN are shown in the Figure 1. Survival rate of cryopreserved axillary buds was correlated with the cold-hardening duration. Pretreatment for 2 days gave the worst survival rate regardless PVS2 treatments. In each cold hardening, buds were more tolerant to cold treatment when they were treated with PVS2 for 60 minutes, except 2 day cold-hardening that gave better survival at PVS2 90 minutes treatment than 60 minutes. There was no difference on survival rate between 28 and 60 days of cold hardening. Over 90% of survival was obtained when the axillary buds were cold-hardened for 28 or 60 days with PVS2 60 minutes treatment.

Figure 2 represents the effects of PVS2 solution on shoot formation of axillary buds by cold hardness. Non-cryoprevered axillary buds were proliferated and developed to shoots giving almost 100% shoot formation rate in all treatment. However, cryopreserved buds were obserbed the variation of shoot formation rate. 30 minutes exposure to PVS2 solution was not enough to protect axillary buds from freezing in LN. It gave the lowest rate of shoot formation giving approximately 20% rate. Shoot formation increased in PVS2 60 minutes treatment after freezing in LN. However, the rate decreased gradually by longer PVS2 exposure, 90 and 120 minutes. The highest shoot formation was approximately 65% in PVS2 60 minutes treatment. There was the significnat difference at 5% among each exporsure time of PVS2.

The size of axillary buds along with loading time was found as important factors for promoting shoot formation. The frequency of shoot formation was in direct proportion to the axillary bud size and loading time (Table 2). The buds of 1-2 mm size were more suitable for increasing shoot formation rate than other size of buds (3-4 and 5-6 mm size). In all size of buds, 15 minutes loading time gave rise to higher shoot formation rate, and longer loading time (25 and 35 minutes) were better than shorter time (0 and 5 minutes) for shoot formation. The highest frequency of shoot formation (67%) was obtained with 1-2 mm size of bud and 15 minutes loading time.

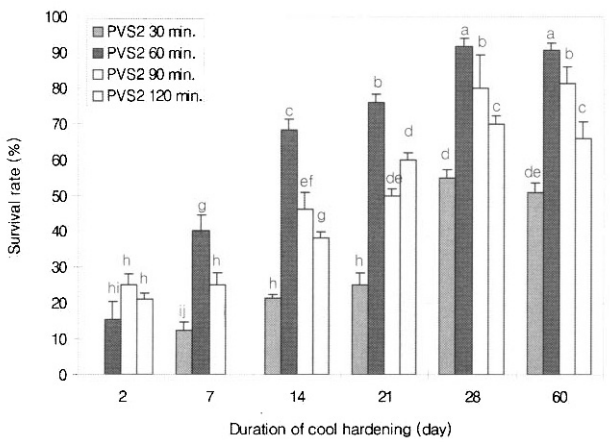

Figure 1. The effects of the cold hardening duration on the survival of axillary buds immersed in LN. Plantlets were acclimated at 10°C for 2 to 60 days. Mean followed by a different letter on the bar given factor 5% differ significantly by Duncun's multiple test. The bars represent standard deviation.

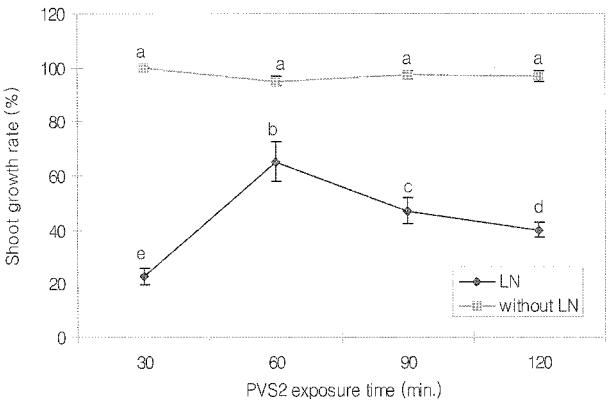

Figure 2. The Effect of exposure time to PVS2 solution at 0°C on the shoot formation of axillary buds cold-hardened. LN-immersed in liquid nitrogen; Without LN-not immersed in liquid nitrogen. Mean followed by a different letter on the bar given factor 5% differ significantly by Duncun's multiple test. The bars represent standard deviation.

Table 2. The effects of axillary bud size and loading time on survival rates after LN immersion under room temperature. Different superscripts are significantly different at p<0.05 (Duncan's multiple test).

Axillary bud size (mm)	Frequency of shoot formation (% ± SD)				
	Loading time (minute)				
	0	5	15	25	35
1 - 2	43.5 ± 2.50ef	59.0 ± 4.00bc	67.0 ± 2.00a	65.3 ± 0.70ab	61.0 ± 2.50ab
3 - 4	30.0 ± 2.50g	47.5 ± 1.50ef	55.2 ± 2.30cd	55.0 ± 1.50cd	49.3 ± 2.10de
5 - 6	10.5 ± 2.50jk	22.3 ± 0.64h	21.0 ± 1.50hi	20.6 ± 1.60hi	15.0 ± 3.00ij

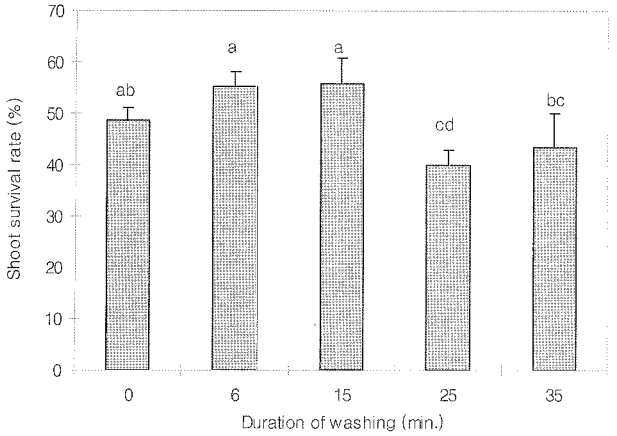

Figure 3. The effects of washing time on survival rate of the *L. chinense* shoot-tips after exposure to PVS2 solution and vitrification. Mean followed by a different letter on the bar given factor 5% differ significantly by Duncun's multiple test. The bars represent standard deviation.

The axillary buds were washed with liquid MS medium containing 1.2 M sucrose for 0 to 35 minutes at room temperature after exposure to PVS2 solution and vitrification. As shown in Figure 3, short time of washing (6 and 15 minutes) was more effective for survival than long time of washing (25 and 35 minutes). No washing also gave higher survival rate than long washing which means that long washing gives negative effect for survival compared with other treatment time. The highest survival was observed with 15 minutes washing time giving over 55%, although both 6 and 15 minutes washing gave similar results.

2. Plant recuperation

Plantlets were recuperated successfully from cryopreserved axillary shoot-tip meristems. The washed axillary shoot-tip meristems were cultured on 3/2 MS medium containing 4% sucrose and 0.15% gelite to enhance bud breaking and proliferation for one day, and then transferred onto 3/2 MS medium containing 4% sucrose and 0.3% gelite. As shown in Figure 4, small leaf tissues were emerged from the buds seven days after culture (Figure 4A) and expanded their size bigger and bigger during one month culture (Figure 4B and C). Roots were developed and reached their length to 20-30 mm when shoots were continuously cultured on the same medium for one more months (Figure 4D).

After 3 months of culturing, the plantlets were transferred to greenhouse. Gelatin was carefully washed off from the roots and planted on a pot filled with vermiculite and perlite (1:1) mixture. The pots were placed in the culture room until they are ready to be moved to a greenhouse condition. Glass covers were used to keep high humidity and uncovered gradually during the acclimation period (Figure 4F). Acclimated plants were transferred to greenhouse, and maintained for further study.

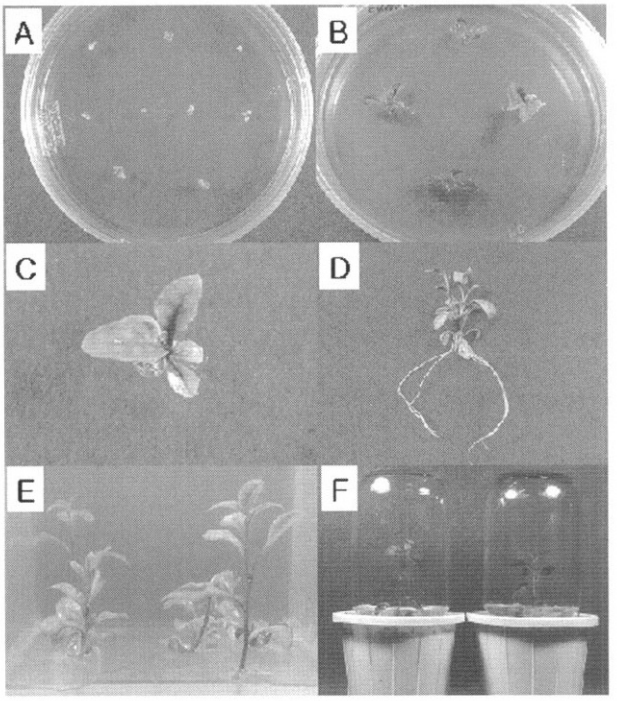

Figure 4. Successful shoot development from cryopreserved axillary shoot-tip meristems of *L. chinense* immerged in LN. A and B-vitrified and thawed buds; C, D and E-shoot and root elongation after transferred onto medium, and F-plants covered with a glass 20 days after acclimation.

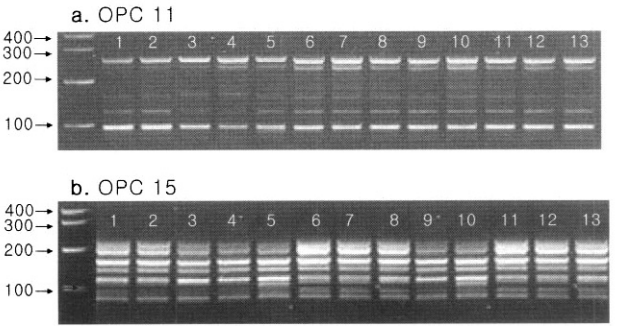

Figure 5. RAPDs band patterning of the plants with the primers OPC 11 and OPC 15. (lane 1-non-cryopreserved plant, lane 2 to 4-cryopreserved plants after PVS2 exposure for 30 minutes, lane 5 to 7-cryopreserved plants after PVS2 exposure for 60 minutes, lane 8 to 10-cryopreserved plants after PVS2 exposure for 90 minutes and lane 11 to 13-cryopreserved plants after PVS2 exposure for 120 minutes).

3. RAPDs analysis

Genetic stability is one of the most important factors for cryopreservation of germplasm. Plants rescued from cryopreservation were analyzed by RAPDs to check genetic stability. Phenotypic variations were not detected

and there was no difference on growth among plants in greenhouse. Genomic DNA was isolated from cryopreserved and non-cryopreserved plants and PCR reaction was performed using 20 random primers. No genetic variation was observed when the RAPDs bands from crypreserved plants were compared with non-cryopreserved one. Fig. 5 shows RAPDs patterning of the plants which were exposed to PVS2 solution in different time. Primer OPC 11 gave rise to less band patterning than primer OPC 15. The bands were distributed from 100 bp to 300 bp with OPC 11 and from 100 bp to 200 bp with OPC 15. Based on the RAPDs results, it seems that cryopreservation does not change genetic background on plants.

Discussion

Success in cryopreservation by vitrification depends on an appropriate vitrification to induce an effective dehydration and cryoprotectant permeation, and to prevent injury caused by chemical toxicity or excessive osmotic stress during treatment. The harmful effects of dehydration can be alleviated or eliminated by adequate preconditionings, such as cold hardening, preculture with high concentration of sucrose and cryoprotective treatment (Reed, 1999). Preculture and cryoprotect loading time are very important factors for cryopreservation. In this study, preculture was carried out in MS medium containing 0.3 M sucrose for one day and loading solution with 2.0 M glycerol and 0.4 M sucrose. This loading solution was reported to be very effective in enhancing freezedehydration tolerance or dehydration tolerance in many cells (Sakai *et al.*, 1991; Nishizawa *et al.*, 1993; Park *et al.*, 2005). Cold acclimation pretreatments are used to trigger a natural resistance mechanisms of plants to cold weather. It increases the amount of starch grains, lipid bodies, sugar content and dry weight (Tanino *et al.*, 1990). Changes in amount and composition of the membrane lipids may protect against freezing-induced injury (Reed, 1999). We described here the optimized vitrification method for the axillary shoot-tip meristems of *L. chinense*. Survival rates after cryopreservation is best when the explant size is 1-2 mm combined with the 15 minutes loading treatment. Cold acclimation of *in vitro L. chinense* plant proved to be essential for the successful cryopreservation with vitrification method. Survival rates were increased by 28 days cold acclimation of axillary bud tips before loading and 60 minutes of dehydration with PVS2 after cryopreservation. Washing time is also an important factor for survival rates. The survival rate was highest when the washing time was between 6-15 minutes, and decreased significantly after 25 minutes.

Cryopreservation of plant tissues has become an indispensible tool of gene conservation. In this area, a lot of works still need to be done. Further studies are necessary, with an emphasis on using other materials like somatic embryos or shoot-tips of *L. chinense*. In addition, different approach is required on cryopreservation methods like simple freezing method or encapsulation method.

Acknowledgement

This research was supported by Kyungpook National University Research Fund, 2002.

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(Received June 27, 2006; Accepted September 1, 2006)