J. Plant Biotechnology (2006) Vol. 8 (1). pp. 43~50

Journal of Plant Biotechnology

# Development of Cryopreservation System using Shoot-Apex in Yam (*Dioscorea batatas*)

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## Abstract

**The goal of this research was to develop an efficient cryopreservation protocol for germplasms of yam (*Dioscorea batatas*), that were cultivated in Korea. Comparative studies with four other cryogenic techniques and subsequent experiments for shoot regrowth were conducted. *In vitro*-grown shoot-apices of the *D. batatas* were successfully cryopreserved by encapsulation-dehydration. The maximum survival of shoot-apices could be achieved when the precultured (with 0.3 M of sucrose for one day) and encapsulated (with a 3%(w/v) Na-alginate solution) apices were dehydrated for 3.5~4 h prior to direct immersion in LN (liquid nitrogen). The frequency of regrowth rate of cryopreserved apices was not decreased during 3-month storage period. The thawing method markedly affected survival of the cryopreserved apices, and thawing at 40℃ for 3 min produced the best results. When cryopreserved apices were post-cultured on the post-culture medium (MS), supplemented with 0.2 mg $l^{-1}$ of BA ($N_6$-benzyladenine) and 0.2 mg $l^{-1}$of kinetin, they showed direct shooting without callusing.**

**Key words:** Encapsulation-dehydration, cryopreservation, yam

## Introduction

Yam plants (*Dioscorea* spp.) are monocots and usually form a single, large underground tuber at the lowest node of main, viny stem and many aerial tubers at axillary buds. In South Korea, *Dioscorea batatas* Decne. has been cultivated widely to produce edible tubers. Several wild *Dioscorea* species have been used for medicinal drugs. The cultural area and productivity of the yam has increased every year. More than 449 M/T of edible yam tubers are produced a year in Korea. Yam cultivation and storage require a high labour input. Heavy equipment, such as an excavator, is necessary to harvest tubers because yam tubers grow in soil depths of 15~100 cm with cultivars. In addition, the storage facility maintained at around 4℃ and subsequent management are also needed for the storage of their large and soft tubers. Cultural areas of the yam, however, have increased every year since it is kept at a stable price. Yam germplasm conservation also requires a high labour input for efficient maintenance. Yams, being clonally propagated, are conventionally preserved in the field gen banks as living collections. Field maintenance, however, is expensive and presents a high-risk loss of materials due to biotic and abiotic stress. The genetic resources are exposed to pests, diseases and other natural hazards such as drought, weather damage, human error and vandalism. *In vitro* conservation by tissue culturing also has several risks. Problems of *in vitro* conservation include the high cost of maintaining stock cultures, the risk of losing materials by contamination, and the accumulation of somaclonal variations over time (Scowcroft, 1984). Therefore, the development of an efficient and reliable protocol for the cryopreservation of yams is essential for the long-term conservation of its germplasm. Cryopreservation is becoming recognized as an effective tool for the long-term storage of vegetatively propagated plant material, offering a technology which requires minimum space, minimum maintenance and genetic stability (Gonzalez Arnao et al. 1993; Pence, 1995; Schafer-Menuhr, 1996; Malaurie et al. 1998).

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Received Feb. 9, 2006; Accepted Mar. 25, 2006

Mandal et al. (1996a, b) and Malaurie et al. (1998) have investigated the effects of factors on the survival of cryopreserved shoot-tips of the *in vitro* plantlets of tropical yam species. In both studies, however, the frequency of survival of the frozen apices and subsequent shoot regeneration were not uniformly adequate with all species. More importantly, many of the shoots that recovered from the frozen apices were regenerated through an intermediary callus phase. Also, there are many previous studies for *in vitro* micropropagation of yam species (Uduebo, 1971; Grewal et al. 1977; Mantell et al. 1978; Chaturvedi and Sinha, 1979; Ammirato, 1984; Sylvia et al. 1995). The genus *Dioscorea*, however, showed large differences in its response depending on the type of explants, cultivars and concentration levels of plant growth regulators.

Therefore, in the present study, in order to develop an efficient cryopreservation protocol yam (*D. batatas*) cultivated in Korea, comparative studies with four other cryogenic techniques and a subsequent experiments for shoot regeneration were conducted.

## Materials and Methods

### Plant materials, surface sterilization and *In vitro* plant induction from the shoot-apices of *D. batatas*.

The stock plants of *Dioscorea batatas* cv. 'Db037' (round tubers) were grown at field of the institute. Vine cuttings, with several axillary buds, were collected in late July, and then prepared by removing the leaves. The explants were surface sterilized by dipping the tissue in 70% (v/v) ethanol for 30 sec and then by immersing them in a 2% (v/v) NaOCl solution for 15 min. The explants were then thoroughly washed 3 times with sterile, distilled water. Shoot-apices excised from the axillary buds were cultured in a MS (Murashige and Skoog, 1962) medium with 0.2 mg $l^{-1}$ of kinetin and 0.2 mg $l^{-1}$ of BA. The MS medium, which had an additional 30 g $l^{-1}$ of sucrose and 2 mg $l^{-1}$ of gelrite was used as the basal medium. The medium pH was adjusted to 5.8 before autoclaving at 121℃ and 1.2 kg $cm^{-3}$ for 20 min. The cultures were incubated under a 16 h photoperiod (provided by cool-white fluorescent lamps at a photon flux density of 1200 lux) at 26±1℃.

The plant materials consisted of the *in vitro* plantlets of *D. batatas* cv. 'Db037', which were being maintained in the tissue culture repository of the Institute of Bioresources, Gyeongbuk Provincial Agricultural Technology Administration, Andong, Korea. *In vitro* plantlets were maintained for more than one year by nodal segment culturing, with sequential sub-culturing at 6-week intervals onto a standard proliferation medium consisting of MS mineral salts and vitamins supplemented with 30 g $l^{-1}$ of sucrose and 2 g $l^{-1}$ of gelrite. Shoot-apices measuring 0.5～2.0 mm, depending on the procedure and/or apecies, were dissected from 6 to 8-week-old plantlets and were used as explants for cryopreservation.

### Cryopreservaion methods using shoot-apices

**Desiccation technique:** Shoot-apices were collected from yam plantlets grown under *in vitro* conditions. Harvested apices were placed in open empty petri-dishes, 60 apices to a 9-cm diameter dish and desiccated in the air stream of a laminar flow cabinet for 40 min. The half of the desiccated apices were then transferred to a MS medium containing 0.2 mg $l^{-1}$ of kinetin plus 0.2 mg $l^{-1}$ of BA, and the other half were placed in 2 ml cryovials (30 apices to a vial), which were immediately plunged into LN. After thawing (3～5 min in a 40℃ water-bath), they were placed on a MS medium containing 0.2 mg $l^{-1}$ of kinetin and 0.2 mg $l^{-1}$ of BA.

**Preculture-dehydration technique:** Harvested shoot-apices were cultured for a day in a 9-cm diameter petri-dishes, containing 25 ml of a MS medium which contains 0.3 M of sucrose with 0.2 mg $l^{-1}$ of kinetin and 0.2 mg $l^{-1}$ of BA. The half of the precultured apices were transferred to a MS medium containing 0.2 mg $l^{-1}$ of kinetin plus 0.2 mg $l^{-1}$ of BA, and the other half were placed in 2 ml cryovials (30 apices to a vial), which were immediately plunged into LN. After thawing (3～5 min in a 40℃ water-bath), they were placed on a MS medium containing 0.2 mg $l^{-1}$ of kinetin and 0.2 mg $l^{-1}$ of BA.

**Vitrification technique:** To induce dehydration tolerance, excised apices from *in vitro* grown plantlets were precultured on a medium with high sucrose concentration levels (0.3 M) for a day and then treated with a mixture of 2 M of glycerol plus 0.4 M of sucrose (loading solution) for 20 min, before dehydration with a PVS2 solution. Then, the explants were placed, 30 to a vial, in 2 ml cryovials, each of which had 1.8 ml of a cryoprotectant mixtures, PVS2, were added. PVS2 consisted of 0.4 M of 30% (w/v) glycerol, 15% (w/v) DMSO, 15% (w/v) ethyleneglycol in a MS medium containing 0.4 M of sucrose. After 30 min at 26℃ in these solutions, the apices were resuspended in 0.6 ml of the same solution and were then plunged into LN and kept there for 1 h, after which they were rewarmed by being immersed in a 40℃ water-bath for 3～5 min and subsequently, the cryoprotectant solution was drained. The apices were then washed in the cryovials with a MS liquid medium supplemented with 1.2 M of sucrose. The liquid medium was changed twice (after 10 min). Then the apices

were placed on a proliferation medium (MS medium contained 0.2 mg $l^{-1}$ of kinetin plus 0.2 mg $l^{-1}$ of BA) gelled with 2 g $l^{-1}$ of gelrite. Uncooled explants treated with the cryoprotectant solutions (treated controls) were also included.

**Encapsulation-dehydration technique:** Excised shoot-apices from *in vitro* grown plantlets were precultured on a medium with high sucrose or sorbitol concentration levels (0.3～0.6 M) for 16 h. Shoot-apices were suspended in a calcium free MS medium supplemented with 3% (w/v) Na-alginate and 0.4 M of sucrose. The mixture including the apices was encapsulated with a sterile pipette into 0.1 M of $CaCl_2$ solution containing 0.4 M of sucrose at room temperature for 1 h, in order to form beads (about 4 mm in diameter). Each bead contains one shoot-apex. In order to increase dehydration tolerance before air drying, these encapsulated apices were treated in a MS medium supplemented with 0.5 M of sucrose and 2 M of glycerol for 1 h at 100 rpm on a rotary shaker at 26±1℃. They were subjected to air drying for 0～8 h in petri-dishes with or without 50 g of dried silica gel. Twenty dried beads were placed in a 2 ml polypropylene sterile cryovials and frozen in LN where they were kept for at least 1 h. The remaining beads were placed in petri-dishes on a MS medium with 0.2 mg $l^{-1}$ of kinetin plus 0.2 mg $l^{-1}$ of BA. The water content of the beads was determined as follows: total fresh weight of 30 alginate beads, with three replicates, was measured after sucrose/glycerol loaded-culturing and during the desiccation period (0～24 h). After the final period of desiccation, the beads were oven-dried at 130℃ for 24 h to determine the dry weight. The water content of the beads on each period was calculated from these values and expressed as a percentage of water weight over dry weight.

### Thawing, post-culture and regeneration of the whole plant

The cryovials, which had been immersed in LN, rapidly thawed in a water-bath at 40℃ for 3～5 min. Cryopreserved shoot-apices, after thawing, were post-cultured on a MS medium containing 0.2 mg $l^{-1}$ of kinetin and 0.2 mg $l^{-1}$ of BA. The shoot-apices were maintained in the above conditions for stock culturing. For the determination of survival, which was defined as the percentage of the total number of explants that formed shoots at 60 days after post-culturing. The effects of slow thawing at room temperature (26℃) for 9 min was compared with the effects of fast thawing at 40～50℃ for 3 min. Experiments were done in a completely randomized design with three or five replicates per treatments. Data were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison. A level of

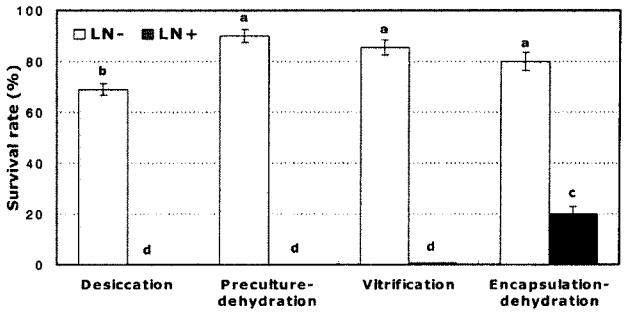


**Figure 1.** Survival rate (%) of apices of *D. batatas* cryopreserved at -196℃ by four different cryopreservation techniques. More than 150 apices were cultured with five replicates per treatment. Data represent mean ± SE (*$P \leq 0.05$).

**Desiccation techniques:** Shoot-apices excised from *in vitro* grown plantlets were dried in the air stream of a laminar flow cabinet for 40 min before being plunged into LN.
**Preculture-dehydration techniques:** Shoot-apices precultured in a MS medium containing 0.3 M of sucrose for one day before being plunged into LN.
**Vitrification techniques:** Precultured apices were treated in PVS2 for 30 min before being plunged into LN.
**Encapsulation-dehydration techniques:** Shoot-apices were precultured on a solidified MS medium with 0.3 M of sucrose for one day. The encapsulated shoot-apices were dehydrated by air drying in a laminar flow cabinet at room temperature for 4 h before being plunged into LN.

$P<0.05$ was accepted as being statistically significant. Tukey's test was used to determine inter-group differences. The regrowth rates of the cryopreserved explants were also recorded for all experiments.

## Results and Discussion

### Comparative study with four different cryopreservation technique

The survival of cryopreserved shoot-apices was dependent upon the dehydration methods. The maximum survival rate (20%) could be achieved when the apices were cryoprserved by the encapsulation-dehydration method. Use of preculturing - dehydration, desiccation and vitrification techniques, however, resulted in poor results with very low post-thaw regrowth frequency (Figure 1). These results are different from those of Matsumoto and Sakai (1995). Among the most of the plants tested, the vitrification method, with or without encapsulation, produced more shoots than the encapsulation-dehydration technique under optimal conditions (Matsumoto and Sakai, 1995). The same results were observed with endangered Australian plants (Touchell, 1995;

Touchell and Dixon, 1996). Their results indicate that yam apices can withstand deep-freezing in LN when the materials were processed using the best methods. Therefore, further studies are still required to establish cryopreservation methods for yams (*D. batatas*). Thus, the survival and regrowth of the cryopreserved apices of *D. batatas* can be improved by modifying the cryogenic procedure and/or recovery growth media.

## Cryopreservation procedure of yam apices by encapsulation-dehydration techniques

For successful cryopreservation, excised apices must be in a physiological state suitable for the acquisition of osmotolerance. In addition, the production of vigorous growth recovery and the preconditioning of stock shoots, before preculturing, was an essential step (Niino and Sakai, 1992; Niino et al. 1997). In this study, shoot-apices excised from stock plantlets, grown under 16 and 24 h photoperiods, were used for cryopreservation. The results show that the survival of cryopreserved apices was improved in shoot-apices from the stock shoots preconditioned over 16 h photoperiods, but there was no statistically significant difference at a level of P=0.05 (Figure 2). The bud size in the Na-alginate bead was also an important factor for successful cryopreservation. There was significant difference in the survival of non-cooling apices. The growth of larger apices (1～2 mm) were better than that of smaller one (<1 mm). After cryopreserving, however, the survival rate of the smaller apices was higher than the larger ones. Desiccated and encapsulated small size (below 1 mm) apices showed a 30% survival rate after cryopreserving, while bigger apices (1～2 mm) showed an 8% in the survival rate (Figure 3).

The survival rate of encapsulated, cryopreserved shoot-apices increased approximately from 40% to 45% as the apices were precultured on a 3 M of sucrose or sorbitol medium for one day. There was no significant difference in the survival rate of cryopreserved samples between sucrose and sorbitol treatment (Figure 4).

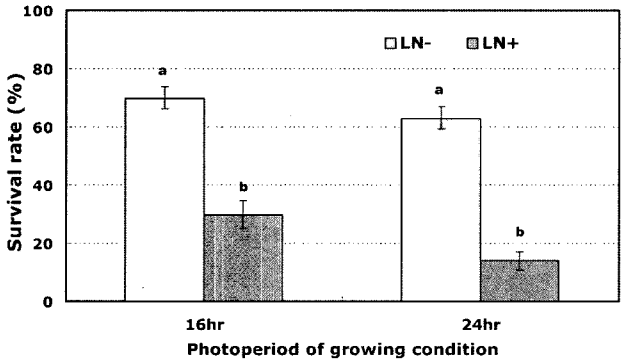


**Figure 2.** Survival rate (%) of control (*-LN*) and cryopreserved (*+LN*) yam shoot-apices excised from plantlets grown under 16～24 h photoperiod conditions. In each treatment, 90 apices were tested with three replicates. Data represent mean ± SE (*P≤0.05). Encapsulated shoot-apices preconditioned with 0.3 M of sucrose were encapsulated with 3%(w/v) Na-alginate. The precultured beads were dehydrated by air drying in a laminar flow cabinet for 4 h at room temperature.

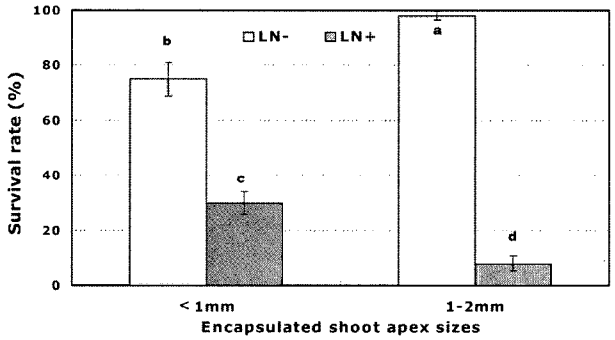


**Figure 3.** Effect of shoot-apex sizes on the survival of the control (*-LN*) and cryopreserved (*+LN*) apices of *D. batatas* by encapsulation-dehydration. In each treatment, 150 apices were tested with three replicates. Data represent mean ± SE (*P≤0.05). Encapsulated shoot-apices preconditioned with 0.3 M of sucrose were encapsulated with 3% (w/v) Na-alginate. The precultured beads were dehydrated by air drying in a laminar flow cabinet for 4 h at room temperature.

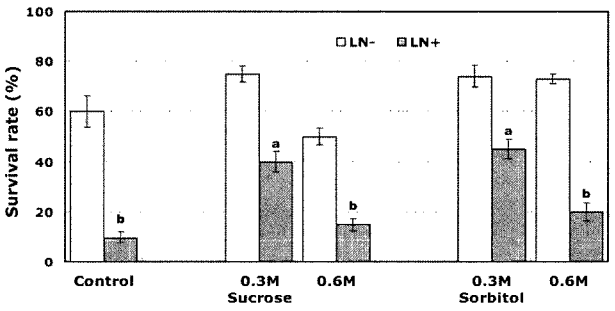


**Figure 4.** Effect of sucrose and sorbitol amounts in a precultured medium for the survival of the control (*-LN*) and cryopreserved (*+LN*) yam apices. In each treatment, 150 apices were tested with three replicates. Data represent mean ± SE (*P≤0.05). Encapsulated apices precultured with 0, 0.3, 0.6 M of sucrose and sorbitol were placed on sterilized filter paper in 9-cm petri-dishes and dehydrated by air drying in a laminar flow cabinet for 4 h at room temperature and then directly immersed in LN for 1 h. Shoot-apices immersed in LN were thawed in 40℃ water-bath for 3 min. Control and cryopreserved apices were post-cultured on a solidified MS medium containing 30 g $l^{-1}$ of sucrose, 0.2 mg $l^{-1}$ of BA and 0.2 mg $l^{-1}$ of kinetin, under light conditions for survival assessment.

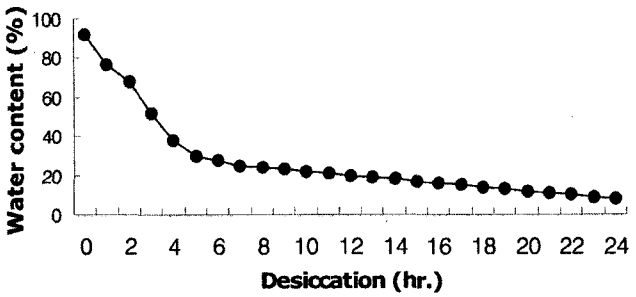


**Figure 5.** Changes in the water content of precultured beads during desiccation.
Encapsulated shoot-apices were loaded on to a liquid MS medium containing 2 M of glycerol and 0.5 M of sucrose for 1 h. The precultured beads were then placed on sterilized filter paper in 9-cm petri-dishes and dehydrated by air-drying in a laminar flow cabinet at room temperature.

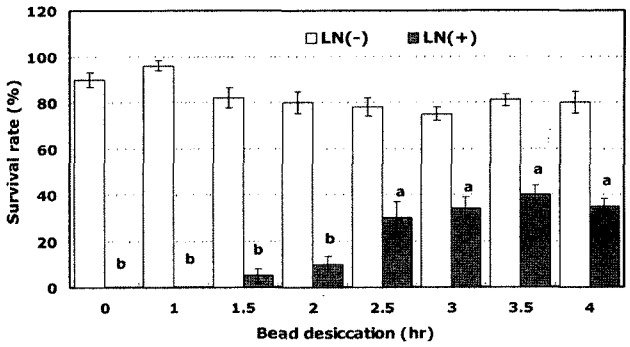


**Figure 6.** Effect of bead desiccation on the survival of control (*-LN*) and cryopreserved (*+LN*) apices of *D. batatas*. In each treatment, 150 apices were tested with three replicates. Data represent mean ± SE ($^*P \leq 0.05$).
Encapsulated apices, precultured with 0.3 M of sucrose were placed on sterilized filter paper in 9-cm petri-dishes and dehydrated by air-drying in a laminar flow cabinet for 0~4 h at room temperature and then directly immersed in LN for 1 h. Shoot-apices immersed in LN were thawed in 40℃ water-bath for 3 min. Control and cryopreserved apices were post-cultured on a solidified MS medium containing 30 g $l^{-1}$ of sucrose, 0.2 mg $l^{-1}$ of BA and 0.2 mg $l^{-1}$ of kinetin under light conditions for survival assessment.

High sucrose concentration amounts may increase tolerance to desiccation; and it is possible that increased sugar content is involved, not only in the effects of sucrose itself, but also in those of desiccation, which may stimulate the accumulation of certain sugars. Two mechanisms have been postulated for the beneficial effects of increased sugar content for cell integrity: a decrease in cell volume due to osmotic processes and the direct stabilization of membranes (Crowe et al. 1987). For most plant cell and organ cultures, injury during freezing is associated with intracellular ice formation in highly vacuolated cells, therefore, the survival rate of cryopreserved shoot-apices could be increased by reducing the cell water content. Hitmi et al. (1999) demonstrated that sucrose decreased the water content of *Chrysanthemum cinerariaefolium* shoot-tips and thus, enhanced their freezing tolerance. Sucrose has been reported to maintain plasma membrane integrity by being a substitute for water on the membrane surface and thus, stabilizing protein under dry and freezing conditions (Santarius, 1973; Crowe et al. 1987). In a number of cases, it has been found that sucrose treatment by itself was sufficient to offer some protection in order to allow moderate recovery rates. This has been reported for oil palm somatic embryos where this high-sucrose pre-culture contributes to the maintenance of tissue viability during the dehydration treatment, by improving both desiccation and cryopreservation tolerance (Dumet et al. 1992, 2000).

A standard curve of changes in the water content of precultured beads during desiccation is presented in Figure 5. The initial water content was 92% on a dry-weight basis; it rapidly decreased to 20~40 % within the first 5 h and then gradually dropped after 6 h. The survival of both control (-LN) and cryopreserved (+LN) shoot-apices was greatly influenced by the water content of the beads (Figure 6). The survival of the non-cryopreserved (-LN) apices remained above 70%, as the water content decreased from 91% to 40%. The survival of cryopreserved apices was detected when the water content was lower than 70%, with maximal survival (40%) being achieved at a 40% water content (dry weight basis). Three to four h of bead desiccation changed water content to around 40%. In a previous study, Cho (2001) reported similar results from the cryopreservation of citrus embryos by encapsulation-dehydration. Air-drying of the beads for three hours provided the best survival of frozen citrus embryos with a water content of 29.6%.

The freezing method markedly affected the survival rate of cryopreserved shoot-apices (Table 1). Their survival rate was 40% when the samples were directly plunged into LN. The apices that were cryopreserved by slow freezing, however, showed a very low survival rate.

Thawing methods also affected the survival of cryopreserved materials (Table 2). Thawing at room temperature for 9 min provided a 17.8% survival rate, compared with 26.5 and 39% survival rate obtained by thawing at 30 and 40℃ for 3 min respectively. In previous studies, rapid thawing in 37~40℃ water-bath for 1~3 min proved to be suitable for protecting the frozen shoot-tips from the damaging effects of ice crystal formation in the cells, which may occur during slow warming (Niino and Sakai, 1992; Bhojwani and Razdan, 1996). A thorough experiment on the effects of thawing temperatures ranging from 30 to 50℃, on

**Table 1.** Effect of freezing methods on survival of cryopreserved shoot-apices of *D. batatas*

| Freezing method | Procedure | Survival rate (%) | |
|---|---|---|---|
| | | LN(-) | LN(+) |
| Rapid | Control (-196℃, $LN_2$ ) | 75 ± 3.2 | 40 ± 4.0 |
| Slow | 4℃→$LN_2$ (-196℃) | 56 ± 7.6 | 4 ± 2.7 |
| | 4℃→-20℃→$LN_2$ (-196℃) | 20 ± 2.5 | 0 ± 0.0 |

Encapsulated apices, precultured with 0.3 M of sucrose, were dehydrated by air- drying in a laminar flow cabinet for 3.5 h at room temperature and then slowly frozen or directly immersed in LN. Samples remained for 30 min for each step. In each treatment, 90 apices were tested with three replicates. Data represent mean ± SE.

**Table 2.** Effect of thawing methods on the survival of cryopreserved shoot-apices of *D. batatas*

| Thawing method | Survival rate (%) |
|---|---|
| Slow thawing | |
| room temp., 9 min | 17.8 ± 2.8 bc |
| 30℃, 3 min | 26.5 ± 3.9 ab |
| Rapid thawing | |
| 40℃, 3 min | 39.0 ± 4.0 a |
| 50℃, 3 min | 4.2 ± 2.6 c |

Encapsulated apices, precultured with 0.3 M of sucrose, were dehydrated by air- drying in a laminar flow cabinet for 3.5 h at room temperature and then directly immersed in LN for 1 h. Shoot-apices immersed in LN thawed at room temperature for 9 min or in a water bath at 30, 40 or 50℃ for 3 min. In each treatment, 150 apices were tested with three replicates. Data represent mean ± SE (*$P \leq 0.05$).

the survival of cryopreserved shoot-tips of the white poplar (*Populus alba* L.), was recently reported by Lambardi et al. (2000). They observed a much higher survival rate under fast thawing at 30～40℃ than under slow thawing at room temperature. In previous studies with *Vitis vinifera* by Plessis et al. (1991, 1993), however, slow thawing was applied at room temperature for 15 min and in their opinion, the survival of encapsulated shoot-tips was independent of the thawing rate. Apparently, our results are consistent with those of Lambardi et al. (2000) and support the suggestions of Bhojwani and Razdan (1996) and Niino and Sakai (1992).

A microscopic study of morphological patterns performed during the recovery growth phase of the thawed apices revealed that the surviving apices sustained a certain amount of the damage portion. The extent of damage perhaps played a significant role in the regeneration of a shoot either directly or through callus formation. The apices that were damaged whole or in the meristemic region, couldn't become to normal plants (Figure 7-A, B). The apices that had larger portion damaged also couldn't develop normally or formed callus (Figure 7-C), whereas, the apices of non- or partially damaged bract tissue developed normal plantlets (Figure 7-D, E, F).

Though the pattern of recovery growth, i.e. direct or callus-mediated regeneration, depends largely on the concentration and composition of growth hormones of the culture medium, as shown with a potato (Harding and Benson, 1994), it may also depend on the extent of damage that occurs in the thawed apices (Mandal et al. 1996b). Therefore, further studies are required to establish if there is any relation between the use of a particular cryogenic procedure, the extent of damage that occurs during cryopreservation and the recovery growth patterns of the thawed

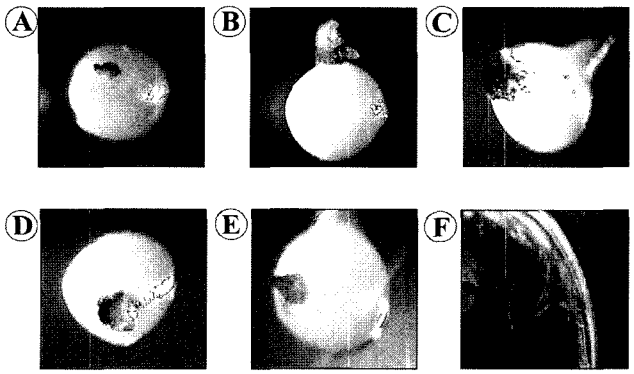


**Figure 7.** Morphological patterns of thawed apices. Damaged (A), partially damaged (B-D), non-damaged apices (E) and well developed plantlet (F) of *D. batatas* cv. Db037 after cooling and thawing. Na-alginate bead sizes were 4～5mm.

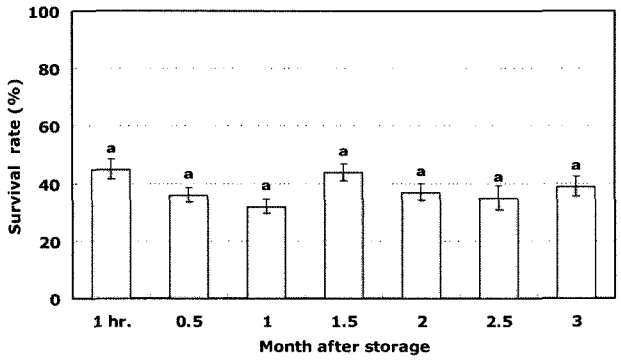


**Figure 8.** Effect of storage duration on the survival rate (%) of shoot-apices of *D. batatas*, cryopreserved at -196℃ by encapsulation- dehydration. More than 150 apices were cultured with five replicates per treatments. Data represent mean ± SE (*P ≤0.05).
Encapsulated apices, precultured with 0.3 M of sucrose, were dehydrated by air-drying in a laminar flow cabinet for 3.5 h at room temperature and then directly immersed in LN for a period between 1 h and 3 months. Shoot-apices immersed in LN were thawed in 40℃ water-bath for 3 min.

apices.

Cryopreservation offers long-term storage capability. In the present study, the survival rate of cryopreserved apices was not affected by storage duration in LN. The survival rate did not decrease while in storage during a 3-month period (Figure 8).

## Acknowledgments

This Study was Supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

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