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Original Research Article

Regeneration of Cryopreserved Pear Shoot Tips Grown *in Vitro* by Encapsulation-Dehydration

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Abstract - The preservation of pear germplasm, like that of other clonal germplasms, is difficult because it requires conservation of whole plants or their tissues. Among the currently available methods for long-term conservation of clonal germplasm, cryopreservation of shoot tips is the most reliable and cost- and space-effective option. Alginate-coated axillary shoot tips from *in vitro*－grown pear were conserved successfully in liquid nitrogen (LN) following dehydration. Shoot recovery from cryopreserved shoot tips was improved greatly after 8 weeks of cold acclimation, but recovery decreased slightly after then. The highest regeneration rate was observed when *in vitro* shoot tips were preincubated in MS (Murashige and Skoog) medium with 0.3 M sucrose for 48 h, and when alginate-coated shoot tips were precultured in MS medium with increasing sucrose concentrations (0.5 M and 0.7 M) for 8 and 16 h, respectively. When the encapsulated beads were dehydrated for up to 7 h [25% water content (fresh weight basis)] under laminar flow, the highest regeneration rate was observed in "BaeYun No. 3" (55.7%) and "Whanggeum" (43.3%) after warming from LN. This technique is useful as a practical procedure to cryopreserve plant material that is sensitive to freezing of the surrounding cryoprotectant medium. Therefore, this technique appears to be promising for the cryopreservation of shoot tips from *in vitro*－grown plantlets of pear germplasm.

Key words - Cryopreservation, Encapsulation-dehydration, *in vitro*, Pear, Shoot tip

Introduction

Pear (*Pyrus* species) is an important horticultural germplasm grown widely in South Korea. Fruit tree germplasms, including that of pear, are generally preserved in field gene banks, but the procedures are very costly and the material can be exposed to pathogens. Cryopreservation is the most reliable and cost- and space-effective option among currently available methods for long-term *ex situ* conservation of clonally propagated germplasm (Reed and Yu, 1995). Among several cryopreservation techniques, the encapsulation-dehydration method is easy to perform and simplifies the dehydration process. The encapsulation-dehydration method, originally described for the cryopreservation of *Solanum* shoot tips (Fabre and Dereuddre, 1990), has also been applied successfully to the cryopreservation of shoot tips of several fruit tree species (Gonzalez-Arnao and Engelmann, 2006), including *Pyrus* (Niino and Sakai, 1992), *Prunus* (Shatnawi *et al.*, 1999), and *Malus* (Niino and Sakai, 1992; Paul *et al.*, 2000; Wu *et al.* 2001). This new technique involves encapsulation of the shoot tips in alginate beads and their dehydration at room temperature before freezing of the tissues in liquid nitrogen (LN). This method is environmentally and user friendly because it eliminates toxic cryoprotectant media and the need for a costly programmable freezer. In this technique, tolerance to dehydration and freezing is induced by cold hardening and preculturing of the encapsulated shoot tips in medium enriched with sucrose. Cold acclimation is used as a pretreatment for cryopreservation of many *in vitro*－grown plants. Freezing tolerance is induced by low but above-freezing temperatures (Thomashow, 1998). Most hardy plants, such as woody perennials, cannot tolerate -3℃ during the growing season, but, when fully cold acclimated in winter, they can tolerate temperatures as low as -196℃ (Yelenosky and Guy, 1989). Generally, survival and regrowth of cryopreserved plant material are dependent on the preculture duration and residual water content of explants after desiccation.

In this study, encapsulated shoot tips from *in vitro*-grown

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plantlets of two pear cultivars ("BaeYun No. 3" and "Whanggeum") were cryopreserved and recovered successfully. Shoot tip resistance to dehydration and freezing was optimized by applying cold acclimation, preincubation, preculture progressively increasing sucrose concentration, and water contents.

Materials and Methods

Preparation of plant material and cold hardening

Proliferating pear (*Pyrus* L. cv. BaeYun No. 3 and Whanggeum) shoots were subcultured every 21 days on medium consisting of a mixture of MS (Murashige and Skoog, 1962) with vitamins, 1 ㎎/L 6-benzylaminopurine (BA), and 0.1 ㎎/L indole-3-butyric acid. The pH was adjusted to 5.7 before sterilization, and the cultures were maintained at 23± 1℃ under an 16 h L/8 h D photoperiod and light intensity of 40 μmol/m^2/s^1 provided by cool white fluorescent tubes (Philips TL-D, Beijing, China). Plantlets grown *in vitro* for 3 weeks after the last subculture were cold acclimatized for 0–20 weeks (5℃ under a photoperiod of 16 h L/8 h D with light intensity of 10 μmol/m^2/s^1). Cultures were not transferred to new medium during acclimation. Subsequently, the shoot tips (2–3 ㎜ long) were excised aseptically in a laminar airflow cabinet (Korea Scientific Technique Industry, Korea). A control was kept under standard culture conditions.

Preincubation, encapsulation, and preculture

The excised shoot tips were preincubated for 1–2 days on solid MS medium containing 0.3 M sucrose on a rotary shaker in the dark at 23±1℃. Shoot tips were suspended in calcium-free liquid MS medium with 3% (w/v) low-viscosity Na-alginate (100–300 cP; Sigma-Aldrich, St. Louis, MO, USA) in liquid MS medium, pH 5.7. The shoot tips were pipetted with some alginate solution, which was dispensed dropwise into liquid MS medium containing 0.1 M calcium chloride, where the alginate formed solid beads by polymerizing with calcium. The beads were left for 30 min to polymerize. The encapsulated beads were precultured in liquid MS medium containing 0.5 and (or) 0.7 M sucrose, with gentle shaking (80 rpm) for different periods under a light intensity of 40 μmol/m^2/s^1.

Air desiccation

The precultured encapsulated shoot tips were desiccated under laminar flow for 2–30 h, plunged into LN for 1 h, rewarmed, and plated. Three sets of 10 desiccated beads were used to determine moisture content, regrowth after air dehydration, and regrowth after air dehydration and LN exposure. The beads were weighed and dried at 103℃ for 18–22 h. Moisture content (%) was calculated as: [(fresh weight - dry weight)/fresh weight] × 100. Fig. 1 shows the changes in water content (%) of the beads during dehydration under the laminar airflow cabinet after preculture. After dehydration for various periods, three replicates per treatment were placed in 2-㎖ cryotubes (10 beads/cryotube) and rapidly immersed in LN.

Thawing and plant regeneration

The samples were maintained in LN for at least 1 h and then thawed at room temperature for 15 min. The encapsulated shoot tips were placed directly on hormone-free solid MS medium in plastic Petri dishes in the dark, and incubated for 1 week at 23±1℃ for recovery from freezing. The beads were transferred to fresh semi-solid MS medium containing 1.0 ㎎/L BA and 0.1 ㎎/L gibberellic acid for an additional week (Table 1), and then placed under standard growth conditions. In this study, we used micropropagation system for pear germplasm established in previous study (Yi *et al*., 2015). Regrowth was

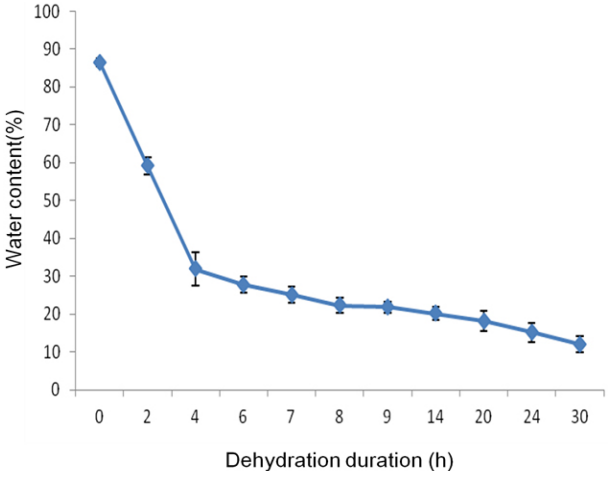

Fig. 1. Changes in water content (%) of beads during dehydration under a laminar flow cabinet after preculture with 0.7 M sucrose. Bead moisture content is expressed on a fresh weight basis (10 beads/replicate).

Table 1. Compositions of subculture, recovery, multiplication, and rooting media used in this study

Medium	Composition (㎎/L)	Remarks
SUM[z]	BA[v] 1.0 + IBA[u] 0.1	Subculture medium
REM[y]	BA 1.0 + GA_3[t] 0.1	Recovery medium
MUM[x]	BA 2.0 + IBA 0.2	Multiplication medium
ROM[w]	NAA[s] 0.2 + IBA 0.2	Rooting medium

[z,y,x,w]4.4 g/L MS containing a vitamin mixture, 30 g/L sucrose, and 2.1 g/L phytagel.
[v]BA; 6-benzylaminopurine.
[u]IBA; indole-3-butyric acid.
[t]GA_3; gibberellic acid.
[s]NAA; 1-naphthaleneacetic acid.

defined as the percentage of shoot tips resuming normal growth 8 weeks after they had been plated out. Necrotic basal parts were removed as necessary, and the tips were placed directly on hormone-free MS medium.

Data analysis and statistical procedures

The results were obtained as average percentages with standard deviations. Each experiment consisted of three replicates per treatment, and each cryovial held 10 samples. The results were assessed by analysis of variance, and the means were separated using Duncan's multiple-range test. P values < 0.05 were considered to be significant.

Results and Discussion

Effects of cold hardening on recovery

The alginate-coated axillary shoot tips from *in vitro*-grown pear were conserved successfully in LN following dehydration. Shoot recovery was studied as a function of the duration of cold acclimation. Cold acclimation (5℃) lasted 0-20 weeks. Cold hardening greatly improved the regeneration rates of cryopreserved pear shoot tips (Fig. 2). The regeneration rate of "BaeYun No. 3" increased progressively during the cold treatment (to 21.2% and 55.7% after 4 and 8 weeks, respectively), and the recovery rate then decreased slightly during weeks 16 and 20 (to 47.7% and 48.1%, respectively). The recovery rate of "Whanggeum" increased during cold acclimation up to 8 weeks (43.3%) and then decreased slightly to 37.8%. During the first 4 weeks of cold acclimation, the percentage shoot

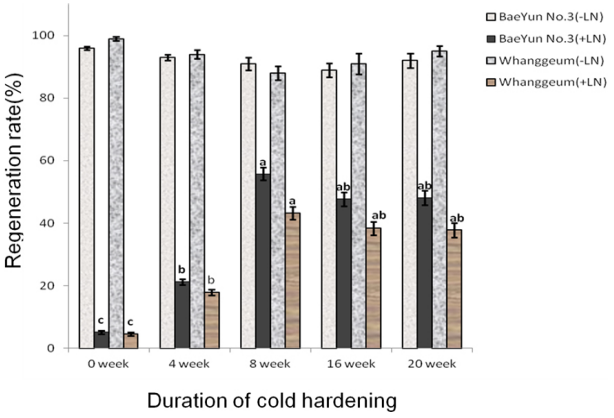

Fig. 2. Effect of cold hardening on regeneration rates of pear shoot tips cryopreserved following immersion in liquid nitrogen.

recovery increased sharply from 5.1% and 4.5% (for "BaeYun No. 3" and "Whanggeum" without cold treatment, respectively) to 21.2% and 17.9%, reaching 55.7% and 43.3% by 8 weeks, and then decreased slightly during the following weeks. High shoot recovery rates have been obtained previously using conventional techniques with shoot tips from cold-acclimated *in vitro* cultures (Reed, 1990). The regeneration rates of cold-hardened shoot tips were considerably higher in the two cultivars evaluated in this study than in controls, suggesting that cold hardening is required to cryopreserve shoot tip germplasm. Cold hardening of donor plants tends to induce an intrinsic tolerance to low temperature and desiccation by triggering genes responsible for cold stress (Takagi, 2000). Tahtamouni and Shibli (1999) demonstrated that cold hardening of the mother stock for 3 weeks at 4℃ under dark conditions followed by vitrification treatment improves survival and regrowth of cryopreserved wild pear shoot tips. Strawberry shoot tips that were cold hardened at 4℃ for 2 weeks and then cryopreserved by encapsulation-vitrification formed more shoots than did non-hardened shoot tips (Hirai *et al*., 1998). In this study, cold acclimation seemed to be essential for survival and regeneration from encapsulated shoot tips of pear, which can withstand prolonged exposure to low temperatures. Cold acclimation is used as a pretreatment for cryopreservation in many *in vitro*-grown plants (Niino *et al*., 1992; Reed, 1989; Reed, 1990). However, no report has directly compared the effects of these different cold acclimation conditions.

Effects of preincubation and preculture on recovery

Regrowth of the encapsulated shoot tips depended on the sucrose concentration and preculture duration. No recovery was observed after freezing without preculture in sucrose in either cultivar (Table 2). The highest regeneration rates were observed when *in vitro* shoot tips were preincubated in MS medium with 0.3 M sucrose for 48 h before encapsulation and precultured after encapsulation in MS medium with increasing sucrose concentrations (0.5 and 0.7 M) for 8 and 16 h, respectively (Table 2). Preculturing of shoot tips in high sucrose medium results in the accumulation of sugar in the alginate beads and tissues, which helps to maintain viability of tissues during cryopreservation. This process occurs due to the increased osmotic stress imposed by the medium on the explants, reducing the availability of water and increasing tissue internal solute concentration, which decreases the formation of ice crystals (Benson *et al*., 1996). Preculture is used to increase the tolerance of shoot tips to dehydration and subsequent freezing in LN. Sucrose is generally added to the culture media for this purpose. Sucrose concentrations of 0.75–1.0 M are suitable to ensure high survival rates of cryopreserved *Malus* shoot tips (Paul *et al.*, 2000). However, some plant species are sensitive to high sucrose concentrations and deleterious effects can be observed, such as in *Vitis* shoot tips (Plessis *et al.*, 1991). Progressively increasing the sucrose concentration overcomes this problem (Plessis *et al.*, 1991). According to Wang *et al.* (2004), preculturing of grape vines with high sucrose concentrations (0.75–1.0 M) increases total soluble protein and sugar contents in the treated tissues, resulting in a two-fold increase in the survival of cryopreserved cells compared with preculture in a low sucrose concentration (0.25 M). In this study, preculturing of the pear shoot tips in progressively increasing sucrose concentrations (0.3, 0.5, and 0.7 M) increased regeneration rates of cryopreserved shoot tips grown *in vitro*. The osmotic effect of sucrose allows large quantities to permeate the cells during preculture (Finkle *et al.*, 1985). This accumulation of sugar in the tissue is associated with freezing tolerance, but is not a guarantee of tissue viability after cryopreservation (Gonzalez-Arnao *et al.*, 1996).

Effects of dehydration by air drying on regeneration

As shown in Fig. 1, the initial water content of the precultured beads was 86.4% on a fresh weight basis. Water content decreased to 31.9% within the first 4 h of air drying under a laminar flow cabinet and was 12% after 30 h drying. The highest regeneration rates in "BaeYun No. 3" (55.7%) and "Whanggeum" (43.3%) after warming from LN were observed when water content was 25% (air drying for 7 h; Table 3). In this study, water was removed from within tissues by air drying. The low water content in encapsulated pear shoot tips obtained after dehydration (25%) promoted the resumption of growth. This amount of water is known to increase the tolerance of shoot tips to direct cooling in LN with (Dereuddre *et al.*, 1991) and without (Uragami *et al.*, 1990) encapsulation. The sucrose concentration in the alginate beads increased markedly during the drying process and probably reached or exceeded the saturation point of the sucrose solution, resulting in glass transition during cooling and rewarming in the alginate beads

Table 2. Effects of preincubation and preculture on regeneration rates of pear shoot tips cryopreserved after immersion in liquid nitrogen

Preincubation (hour)	Preculture (hour)	Regeneration rate (%)			
		BaeYun No.3		Whanggeum	
		-LN	+LN	-LN	+LN
No preincubation	No preculture	37.7 b[y]	0 d	34.3 b	0 d
0.3 M Suc.[z] (24)	0.7 M Suc (24)	89.5 a	15.5 c	100 a	19.2 c
	0.5MSuc (8) → 0.7M Suc (16)	91.8 a	38.5 b	100 a	28.1 b
0.3 M Suc. (48)	0.7 M Suc (24)	100 a	45.7 ab	96.4 a	35.1 ab
	0.5MSuc (8) → 0.7M Suc (16)	95.3 a	55.7 a	93.2 a	43.3 a

[z]Suc.; MS (Murashige & Skoog) mixture + sucrose.

[y]Means within columns followed by the same letter do not differ significantly at the 5% level.

Table 3. Effects of dehydration duration and water content on the regeneration rate of pear after liquid nitrogen exposure

Dehydration duration (hour) or water content (%FW)	Regeneration rate (%)			
	BaeYun No.3		Whanggeum	
	-LN	+LN	-LN	+LN
4h (30%)	97.4 a	36.1 b[z]	95.3 a	30.3 b
7h (25%)	100 a	55.7 a	98.4 a	43.3 a
14h (20%)	93.3 a	48.5 ab	100 a	38.3 ab
24h (15%)	94.5 a	14.3 c	94.1 a	10.2 c

[z]Means within columns followed by the same letter do not differ significantly at the 5% level.

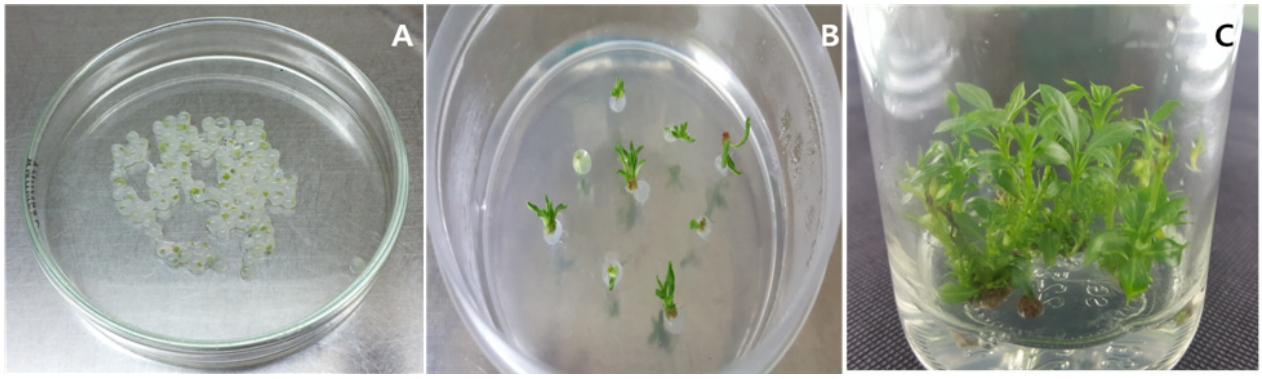

Fig. 3. Cryopreservation of pear shoot tips using encapsulation-dehydration. (A) Encapsulation of pear shoot tips grown *in vitro* using sodium alginate. (B) Regeneration of pear shoot tips 8 weeks after cryopreservation. (C) *In vitro* proliferation of pear regenerants from cryopreserved tissues.

and explants (Dereuddre *et al.*, 1991).

The encapsulation-dehydration technique was developed as a substitute for the dehydration of shoot tips during prefreezing in a programmable freezer, which eliminates the need to use most of the cryoprotectants included in cryoprotectant mixtures (Plessis *et al.*, 1991). In this study, progressive preculture in media with increasing sucrose concentrations was also used to enhance freezing resistance in the presence of cryoprotectants. This method is faster, requires less handling, and produces similar or better recovery than do other methods. It is useful as a practical procedure to cryopreserve plant material that is sensitive to freezing of the surrounding cryoprotectant medium. Therefore, this technique appears to be a promising technique for the cryopreservation of shoot tips from *in vitro*–grown pear plantlets. The recovery rate is still low, so additional studies to optimize this method should be performed using additional pear clones.

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