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Preservation of *In Vitro* Grown Shoot Tips of Potato (*Solanum tuberosum* L.) by Different Methods of Cryopreservation

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Abstract

Cryopreservation has been recognized as a practical and efficient tool for long-term storage of vegetatively propagated plants. This study was conducted to investigate the effects of sucrose concentration, hardening temperature and different cryopreservation methods on the survival rate of potato shoot tips after cryopreservation. Excised shoot tips of *in vitro* plantlets of potato cultivars, Atlantic and Superior were cryopreserved by vitrification, encapsulation-vitrification and encapsulation-dehydration. Cryopreservation by vitrification method was used to determine the optimum concentration of sucrose and cold hardening temperature during sub-culturing period to the donor plantlets. Nine-percent sucrose gave 46.7% survival in Atlantic and 40% in Superior. The most optimum hardening temperature for 50% survival in Atlantic and 43.3% in Superior was 10°C. In the case of comparative study of three different cryopreservation methods, the highest survival (52%) as well as regeneration (46%) were observed when the shoot tips were cryopreserved by encapsulation-vitrification method, and the lowest survival (36%) and regeneration (28%) from the vitrification. Plant and tuber morphology of potato regenerated after cryopreservation were similar to those of the non-cryopreserved *in vitro* plantlets (control). Thus, this study demonstrated that encapsulation-vitrification method was the most effective one among other methods for higher survival as well as regeneration in *in vitro* shoot tips of potato.

Key words: cryopreservation, dehydration, encapsulation, potato, regeneration, vitrification

Introduction

Maintenance of potato germplasm in the field demands more manpower and space aside from diseases and environmental stresses. *In vitro* conservation of potato germplasm also entails high maintenance cost, risks of somaclonal variation, and genetic instability (Harding & Benson 1994). Cryopreservation has been recognized as a practical and efficient tool for long-term storage of potato germplasm with minimum space and low genetic instability. Conservation at ultra-low temperature, usually -196°C, allows the long-term and contamination-free storage of plant genetic resources (Bajaj 1987, Helliot *et al*. 2002). Low rate of survival of plant materials after cryopreservation has become major problem in most of the plant species**.** For this reason, various protocols have been developed to solve this problem. In conventional cryopreservation, plant materials are slowly frozen to different temperature up to -30ºC and plunged into liquid nitrogen. Sakai *et al.* (1990) developed glycerol-based cryopreservation method by vitrification. Later, Hirai and Sakai (1999) modified the vitrification method by encapsulation which has been successfully applied to many plant species with higher survival rate. Then, encapsulation dehydration method was developed (Sakai *et al*. 2000) for higher survival rate and regeneration of cryopreserved plant materials.

The cryopreservation process includes three phases. First is the growing condition of the donor

plants, which include plant species, culture medium and hardening temperature. Second is the cryogenic condition, including solution application, cooling and warming. Third is the recovery processes after cryopreservation. We studied some aspects of the first two phases in order to evaluate different growing conditions for donor plantlets, and different cryopreservation methods for higher regeneration of *in vitro* grown shoot tips of potato.

Materials and Methods

Plant materials

In vitro plantlets of potato cultivars Atlantic and Superior were received from the Center for the Korea Potato Genetic Resources (KPGR), Kangwon National University, Chunchon, Korea. Plantlets were further propagated under *in vitro* conditions through single nodal cuttings on MS medium (Murashige & Skoog 1962) supplemented with 3% sucrose and 0.8% agar for four weeks under 16 h photoperiod. This research work was carried out at Molecular Laboratory, Department of Biotechnology, Kangwon National University, Chunchon, Korea in 2005.

Sucrose concentration and cold hardening temperature to the donor *in vitro* plantlets

Four concentrations (3, 6, 9, and 12%) of sucrose were evaluated for the regeneration of shoot tips after cryopreservation to determine the optimal concentration of sucrose on MS medium for the donor *in vitro* plantlets. Similarly, to find out the maximum regeneration of the shoot tips after cryopreservation, *in vitro* donor plantlets were hardened under three different temperatures (0, 10, 25°C) for preliminary studies. Proliferated shoots were again sub-cultured on MS medium supplemented with 9% sucrose and 0.8% agar for four weeks under 16 h photoperiod (Sakai *et al*. 2003). After four weeks, the subcultures were incubated at 10°C for three weeks and shoot tips (1–1.5 mm) were excised from cold hardened *in vitro* plantlets for cryopreservation by vitrification. The best sucrose concentration (9%) and hardening temperature (10°C) were applied for further studies.

Cryopreservation by vitrification

Excised shoot tips (1–1.5 mm diameter) were pre-cultured in petri-dishes (9 cm) containing MS medium supplemented with 0.3 M sucrose and 0.8% agar with pH adjusted to 5.6. The petri-dishes were incubated for 24 h under 16 h photoperiod at room temperature. After pre-culturing, ten shoot tips were transferred into a cryo-vial with loading solution containing 2 M glycerol and 0.4 M sucrose dissolved in MS medium and left for 30 min at room temperature (Helliot *et al*. 2002, Sarkar & Naik 1998). After that, the loading solution was replaced by ice-cooled and filter-sterilized plant vitrification solution-2 (PVS-2) (Sakai *et al*. 1990) containing 3.26 M glycerol, 2.42 M ethylene glycol, 1.9 M dimethylsulfoxide (DMSO) and 0.4 M sucrose dissolved in MS medium with pH adjusted to 5.6 before autoclaving. Shoot tips were immersed into 1 ml PVS-2 solution in a cryo-vial (2 ml capacity) for 30 min at 0°C and then plunged into liquid nitrogen in a Dewar flask and held for at least one hr (Sarkar & Naik 1998). Figure 1 shows the cryopreservation by vitrification method developed by Sakai and coworker (Sakai *et al*. 1990) used in the present study.

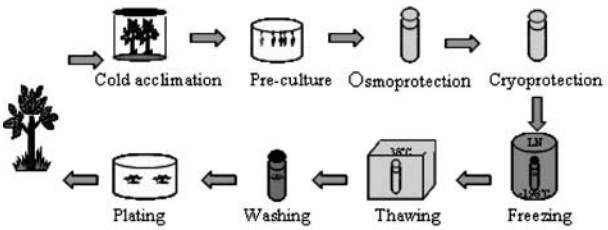

Fig. 1. Cryopreservation by vitrification method (Sakai *et al*. 1990)

Cryopreservation by encapsulation-vitrification

Excised shoot tips were suspended in a half-strength of MS medium supplemented with 3% (w/v) Na-alginate, 2 M glycerol and 0.4 M sucrose in a beaker. The mixture of shoot tips was dispensed with a sterile pipette into a 0.1 M $CaCl_2$ solution containing 2 M glycerol and 0.4 M sucrose at room temperature for 30 min to form beads (about 4 mm in diameter) and each bead contained one shoot tip (Wang *et al*. 2003, Hirai & Sakai 1999). The beads were pre-cultured on MS medium supplemented with 0.3 M sucrose and 0.8% agar, and incubated for 24 h under 16 h photoperiod at room temperature. Ten beads were transferred into a cryo-vial containing loading solution and held for 30 min under room temperature for dehydration. Beads were transferred into ice-cooled and filter-sterilized PVS-2 solution as mentioned earlier and

held for 30 min at 0°C. Then the cryo-vials were plunged into the liquid nitrogen and held at least for one h (Sarkar & Naik 1998).

Cryopreservation by encapsulation-dehydration

Excised shoot tips were encapsulated as mentioned in the above section and dehydrated with loading solution under laminar flow for one hour. The beads were transferred in petri-dishes containing silica gel and air dried for three hour. The beads were then transferred into a cryo-vial and rapidly plunged into liquid nitrogen and held at least for one h (Sarkar & Naik 1998).

Regeneration

After plunged into liquid nitrogen and held at least for one hour, cryo-vials containing shoot tips were rapidly thawed for 90 sec at 38–40°C in a water bath. In the case of vitrified and encapsulation-vitrified, the PVS-2 solution was replaced by deloading solution containing MS medium with 1.2 M sucrose and held for 30 min (Sarkar & Naik 1998) and in the case of cryopreservation by encapsulation-dehydration, shot tips were directly planted on MS solid medium containing 3% sucrose, 0.7% agar, 0.5 mg l^{-1} zeatin, 0.1 mg l^{-1} IAA, and 1.0 mg l^{-1} GA_3 (Zhao 2002). Tips were incubated in the dark at 24 ± 2°C for two days and then transferred under 16 h photoperiod condition. After 2–3 weeks, survived plantlets were again transferred into the hormone-free MS solid medium containing 3% sucrose and 0.8% agar for further multiplication. After three months of cryopreservation, regenerated plantlets were transferred to the disinfected pots containing sterilized commercial soil and placed under glasshouse conditions.

Results

Effect of sucrose concentration on the survival of shoot tips of potato by vitrification

Survival was greatly influenced by the sucrose concentrations. The highest survival of cryopreserved shoot tips, 46.7% in Atlantic and 40% in Superior, were obtained when the donor plantlets were sub-cultured on 9% sucrose. None of the shoot tips survived in both of tested varieties when the donor plantlets were sub-cultured on standard sucrose concentration (3%). On the other hand, the survival percent of shoot tips decreased when they were sub-cultured on MS medium containing more than 9% sucrose. Thus, MS medium containing 9% sucrose was used as a sub-culture medium to donor plantlets for the subsequent cryopreservation studies. Atlantic gave higher survival than Superior in all the evaluated sucrose concentrations (Table 1).

Table 1. Effect of sucrose concentration supplemented on the MS subculture medium to the donor *in vitro* plantlets for cryopreservation by vitrification on the survival of shoot tips of potato cvs. Atlantic and Superior under 10°C incubation condition

Sucrose concentration(%)	Survival rate (%)	
	Atlantic	Superior
3	0	0
6	13.2 ± 1.9	10.0 ± 1.0
9	46.7 ± 4.5	40.0 ± 3.8
12	16.7 ± 2.6	20.0 ± 2.0

Ten shoot tips were used in each of three replicates. Data are presented as mean ± standard error.

Effect of hardening temperature on the survival of cryopreserved shoot tips of potato by vitrification

The highest survival of shoot tips, 50% in Atlantic and 43.3% in Superior, were obtained when the donor *in vitro* plantlets were hardened at 10°C for three weeks (Table 2). Survival percent was decreased in both cultivars when the plantlets were hardened either at 25°C or 0°C.

Table 2. Effect of hardening temperature to donor *in vitro* plantlets for cryopreservation by vitrification on the survival of shoot tips of potato cvs. Atlantic and Superior

Hardening temperature (°C)	Survival rate (%)	
	Atlantic	Superior
0	3.3 ± 1.1	6.7 ± 1.2
10	50.0 ± 5.3	43.3 ± 3.8
25	10.0 ± 1.2	13.3 ± 1.2

Ten shoot tips were used in each of three replicates. Data are presented as mean ± standard error.

Effect of different cryopreservation methods on the survival and regeneration of shoot tips of potato

Three cryopreservation methods, (i) vitrification, (ii) encapsulation-vitrification and (iii) encapsulation-dehydration were evaluated for survival and regeneration of shoot tips after cryopreservation. Results showed that survival differed among the cryopreservation methods and the highest survival (52%) was obtained from the encapsulation-vitrification and the lowest (36%) from the vitrification (Table 3).

Table 3. Effect of different methods of cryopreservation on the survival and regeneration of shoot tips of potato cv. Atlantic

Treatment	No of Shoot tip used	Survival (%)	Regeneration (%)	Plant height (cm)	Branching (No)
Cryopreservation by vitrification	50	36.0	28.0	6.5	2.3
Cryopreservation by encapsulation-vitrification	50	52.0	46.0	7.1	2.3
Cryopreservation by encapsulation-dehydration	50	44.0	36.0	7.3	2.1

Regeneration and evaluation of *in vitro* plantlets under glasshouse conditions

In vitro plantlets after cryopreservation from different methods were grown under *in vivo* condition for further study on the regeneration and morphological characters. In the case of survivability, shoot tips were confirmed after one week of post-culture. About five weeks after regeneration of the shoot tips, whole plantlets (Fig. 2A) were transferred to MS solid medium free of plant growth regulators for development of whole plant. Profuse root and normal shoot structure were formed six weeks after transferring to the medium (Fig. 2B). More than 80% of the plants were established under glasshouse conditions (Fig. 2C), using sterilized commercial soil as a growing medium. Leaf and tuber morphology of plants regenerated from cryopreserved shoot tips were similar to those from the normal *in vitro* plantlets. The highest tuber yield was also obtained from the regenerated plants after cryopreservation by encapsulation-vitrification than the other evaluated methods (Table 4). Potato plants regenerated after cryopreservation were morphologically similar to those plants regenerated from the *in vitro* plantlets in the study.

Table 4. Morphological evaluation and tuber yield of potato cv. Atlantic under glasshouse conditions after cryopreservation

Treatment	No. of *in vitro* plantlets transplanted	Regeneration (%)	Flowering	Plant height (cm)	Tuber/plant	
					No.	Yield (g)
Vitrification and cryopreservation	31	77.4	Yes	30.3	4.1	56.5
Encapsulation-vitrification and cryopreservation	40	87.5	Yes	35.1	4.4	52.6
Encapsulation-dehydration and cryopreservation	40	80.0	Yes	31.4	4.0	50.0
In vitro plantlets (Control)	50	94.0	Yes	36.0	5.3	60.2

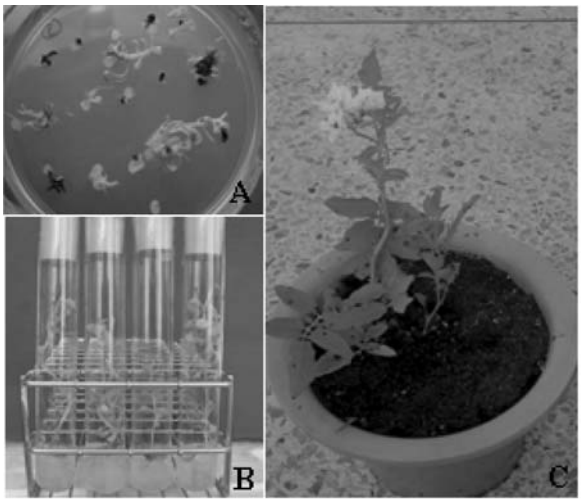

Fig. 2. Surviving shoot tips, regenerated *in vitro* plantlets and established plantlets of potato cv. Atlantic after cryopreservation by encapsulation-vitrification under glasshouse. A. Elongated root and shoot tips after five weeks of cryopreservation; B. Profuse root and normal shoot after six weeks of cryopreservation; C. Plantlet established under net-house conditions eight weeks after transferring to pot.

Discussion

Different protocols are available for cryopreservation of different plant species. However, many reports have been focused mainly for higher survival. Most commonly used methods for cryopreservation are vitrification (Sakai *et al*. 1990), encapsulation-vitrification (Hirai & Sakai 1999) and encapsulation-dehydration (Sakai *et al*. 2000). There are several factors affecting survival of post-thawing shoot tips after cryopreservation. The major factors are hardening of donor plantlets, sub-culture medium, pre-culture medium, loading solution, vitrification solution, thawing, regeneration medium, and plant species. The sub-culture of cells in the medium containing high sucrose concentration increased intercellular sugar concentration, which enhanced the survival and re-growth of cryopreserved tissue (Chen *et al*. 1996). Sakai and coworkers also reported that plantlets grown on a high concentration of sucrose appeared to be shorter and more compact, possessed larger cormels, shorter petioles, thicker leaf bases, smaller leaf blades, and more rigid tissue (Sakai *et al*. 2003). These changes in plant structures make the dissection of the tiny, fragile meristems easier, resulting in less damage during the cryopreservation process. Besides strong and rigid plantlets due to high sucrose concentration, the present study also showed that *in vitro* plantlet sub-cultures in the medium containing high sucrose concentration (9 and 12%) were more resistant to low temperature than the plantlet sub-cultures in the medium containing standard sucrose concentration (3%).

Osmotic potential of leaves and stems decreased significantly after cold acclimatization (Chang & Reed 2000). During cold acclimatization many changes occur in the cellular structure, physiology, biochemistry of cells and tissues (Chen *et al*. 1996, Guy 1990). Under cold conditions, plants grow slowly with compact and dormant buds (Chang & Reed 2000).

Low water content induced by low temperature is correlated with an increase in freezing tolerance (Fahy 1996). In 1999, Vandenbusssche and co-workers also reported that cold hardening of sugar beet increased the survival of encapsulated-dehydrated shoot tips. This type of response was associated with increased concentrations of sugars and unsaturated fatty acids in membrane lipids. The present study also found that higher the incubation temperature faster was the plant growth with succulent plant and lower the temperature (10°C or less), slower the plant growth with compact and rigid structure.

Cryopreservation by encapsulation-vitrification protocol has successfully been applied to numerous plant species (Hirai & Sakai 1999). In the present study, the lowest survival of shoot tips were obtained from cryopreservation by vitrification, but higher survival rate in cryopreservation by encapsulation-vitrification. This might be due to toxic effects of PVS-2 solution with the vitrification and cryopreservation, whereas encapsulation and vitrification process with Na-alginate could protect tissues from direct contact with PVS-2. Hirai and Sakai (2003) reported that encapsulation-vitrification method made vitrification easily applicable in the gene bank context, where large number of samples were to be treated routinely.

In conclusion, high concentration (9%) of sucrose in the subculture medium and lower incubation temperature (10°C) were found better for higher survival of shoot tips after cryopreservation. Among the evaluated methods, cryopreservation by encapsulation-

vitrification was found the most effective for the higher rate of survival and regeneration in potato. Thus, this method could be considered as the most effective and reliable for long-term preservation of potato germplasms under contamination free conditions.

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