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Application of Slow-Freezing Cryopreservation Method for the Conservation of Diverse Potato (*Solanum tuberosum* L.) Genotypes

Mei-Ai Zhao, Shambhu P. Dhital, Yi-Lan Fang, Dong-Man Khu, Ye-Su Song, Eung-Jun Park, Chang-Won Kang and Hak-Tae Lim*

Department of Plant Biotechnology, Center for the Korea Potato Genetic Resources (KPGR), and Potato Valley Co., Ltd., Kangwon National University, Chunchon, 200-701, Korea

Abstract

Cryopreservation has been recognized as a practical and efficient tool for the long-term storage of vegetatively propagated plants. This study was conducted to investigate the effects of slow-freezing techniques on the cryopreservation of potato. *In vitro* plantlets of the potato genotypes of 'Atlantic', 'Superior', 'Namseo', 'J138', and 'CTO5-5' were cold acclimated, and the excised axillary buds were precultured, osmoprotected, exposed to plant vitrification solution, frozen slowly to -40℃ and then rapidly plunged into liquid nitrogen, thawed and finally plated on the regeneration medium. It was found that the higher the sucrose concentrations in the subculture medium of donor plantlets, the higher the survival rates of shoot tips after cryopreservation, and the highest survival (20%) was observed in the medium added with 0.25 M sucrose. As for the effect of cooling, 0.3℃/min cooling speed showed the highest survival (25%). Different varieties showed different responses over different cryopreservation treatments. Survival rate was increased by slow-freezing technique method as compared with that of the basic cryopreservation method of vitrification alone in the diverse potato genotypes. Leaf and tuber morphologies of potatoes regenerated after cryopreservation using slow freezing technique were similar to those derived from the *in vitro* stock plantlets.

Key words: cold hardening, cooling speed, osmoprotection, regeneration, thawing, vitrification

* Corresponding author, E-mail: potatokorea@empal.com
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Introduction

Cultivated potatoes are tetraploids and highly heterozygous (Hawkes 1978). Maintenance of potato germplasm in the field requires plenty of man-power 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 and Benson 1994). Cryopreservation appears to be a logical choice for the long-term storage of potato germplasm with minimum space and maintenance requirements and low genetic instability (Bajaj 1987; Harding and Benson 1994). Cryopreservation has been applied to more than 80 plant species and considerably less labor-intensive method than conventional procedures for in vitro germplasm maintenance (Bajaj 1987; Niino et al. 1992; Palasz et al. 2000; Reed and Lagerstedt 1987). As the number of cultures maintained in laboratories increases, the techniques for cryopreservation become to be more important. Recent improvements in cryopreservation techniques make it possible to maintain germplasm through base (long-term back-up) storage in liquid nitrogen. Among the various cryopreservation techniques, slow-freezing method seems to be more common, and this method requires low level expertise and is easy to handle the whole process with high survival rates. The overall process for cryopreservation consists of three phases; first, conditioning of the stock plants, second, cryogenic conditions including solution application, cooling and warming rates, and third, recovery processes. We described here some aspects of the first two phases in order to generate a useful shoot-tip cryopreservation protocol for potato germplasm. The objectives of this study were to evaluate the

sucrose concentrations for subculture of donor plantlets, cooling speed, genotype responses and subculture medium for the survival of shoot tips after cryopreservation by slow freezing method.

Materials and Methods

Plant materials

In vitro plantlets of the potato (*Solanum tuberosum.* L) genotypes of 'Atlantic', 'Superior', 'Namseo', 'J138', and 'CT05-5' were used in this study. *In vitro* plantlets were multiplied and maintained over several weeks through shoot cuttings on MS (Murashige and Skoog, 1962) medium at 24℃ ± 2℃ under a 16-h light/day photoperiod.

Sucrose concentrations of the subculture medium of donor plantlets

Shoot buds of potato plantlets were sub-cultured on MS solid medium containing 0.09 M, 0.19 M, 0.25 M, or 0.50 M sucrose and incubated under a 16-h light/day photoperiod at 24 ± 2℃. When the plantlets were 10 cm in height or after 2-3 weeks of subculture, they were transferred to an incubator for cold-hardening at 10℃ under a 16-h light/day photoperiod for three weeks. Then, axillary shoot tips with five leaf primordia (about 1-1.5 mm in diameter) were excised for cryopreservation experiments (Sarkar and Naik 1998).

Cryopreservation procedure

Excised shoot tips were pre-cultured on MS medium containing 0.3 M sucrose and 0.8% agar (w/v) for 24 h under a 16-h light/day photoperiod at room temperature. Then shoot tips were transferred to each cryotube (2.0-ml size) containing osmoprotectant solution with a mixture of MS and 1.2 M sucrose and held for 30 min. The cryotubes were cooled slowly from ambient temperature to 0℃ at the cooling rate of 2.5℃/min in 2.0 ml of plant vitrification solution-2 (PVS-2) (Sakai et al. 1990) containing MS, 0.4 M sucrose, 30% glycerol, 15% dimethyl sulfoxide (DMSO), and 15% ethylene glycol and then to -40℃ in a programmable, controlled temperature, freezing chamber, followed by immediate plunging of the cryotubes into liquid nitrogen (LN) in a dewar flask and held for at least 1 h. After 1 h of freezing, the cryotubes were rapidly thawed for 90 sec at 38℃ in a water bath, and the cryoprotectant solution was replaced by loading solution (washing solution) containing MS with 1.2 M sucrose and held for 30 min as described by Sakai et al. (1990) and Sakar and Naik (1998). Control shoot tips were not frozen.

Cooling speed during cryopreservation

After osmoprotection, the shoot tips were frozen in 200 ml of the cryoprotectant at the rate of 0.1℃/min, 0.3℃/min, or 0.5℃/min to -40℃ in a programmable, controlled-temperature, cooling chamber and then immediately plunged into liquid nitrogen in a dewar flask and held for at least 1 h.

Plant re-growth and regeneration

After thawing, the shoot tips were plated on a MS medium containing 3% sucrose, 0.7% agar, 0.5 mg l^{-1} zeatin, 0.1 mg l^{-1} IAA, 1.0 mg l^{-1} GA_3 and incubated at 24 ± 2℃ under a 16h light/day photoperiod. Ten shoot tips were incubated in each of the treatments and repeated three times. Shoot tips survival rates were observed after four weeks of subculture. When the shoots were 10 cm in height, they were transferred to the pots containing sterilized soil-based potting medium.

Results and Discussion

Effect of sucrose concentration in the subculture medium of donor plantlets

Sucrose concentration in the subculture medium of donor plantlets has been shown to be very important for improving the survival of cryopreserved shoot tips in several plant species (Chen et al., 1996). In the present study, donor plantlets subcultured on the MS medium containing 0.25 M sucrose gave the highest survival (20%), but none of the shoot tips survived in the medium with very low (0.09 M) sucrose added after cryopreservation (Table 1). The results

Table 1. Effect of sucrose concentration in the subculture medium of donor plantlets on the survival of cryopreserved shoot tips of the potato genotype 'Superior' by basic method (vitrification)[z]

Sucrose concentration (M)	Survival (%)
0.09	0.0
0.19	13.3 ± 1.7
0.25	20.0 ± 3.2
0.50	5.0 ± 1.4

[z]Subculture medium and culture conditions; MS medium supplemented with 0.25 M sucrose and 0.8% agar and incubation at 10℃ under a 16-h light/day photoperiod for 3 weeks. Basal vitrification solution (PVS-2): MS, 0.4 M sucrose, 30% glycerol, 15% DMSO, and 15% ethylene glycol. Shoot tip size: 1-1.5 mm in diameter.

were also in agreement with the finding of Chen et al. (1996), who reported that high concentration of sucrose was the most important aspect for successful cryopreservation.

The subculture of plantlets in the medium containing high sucrose concentrations dramatically increased the intercellular sugar concentration, which enhanced the survival and regrowth of cryopreserved tissue (Chen et al. 1996). Compared with the control plants grown on a standard concentration (3%) of sucrose, the plantlets subcultured at the high concentration of sucrose appeared to be shorter and more compact. They possessed larger cormels, shorter petioles, thicker leaf bases, smaller leaf blades, and more rigid tissue. These characteristics made the dissection of tiny, fragile meristems easier, resulting in less damage during the cryopreservation process. Moreover, meristem tips dissected from sub-cultured *in vitro* plantlets looked morphologically more uniform and much less watery than those of the control plants. Analysis of shoot parts showed that subculture significantly reduced the water content but enhanced the accumulation of stress-responsive solutes (sugars and free proline). After LN storage, the post-thaw survival rates of sub-cultured apices were significantly higher than that of the control in grapevine, and similar effects were also observed in banana and orchids. Thus, subculture at high sucrose concentrations appears to be a promising step to be included in the cryopreservation process as described by Sakai et al. (2003). In the present study, it was also found that the *in vitro* plantlets subcultured at the high sucrose concentrations (0.25 M) produced strong, rigid shoots and showed resistance to cold temperature during cold acclimation compared to the plantlets grown at the very high or low sucrose concentration. Preculture step along with sucrose also has been shown to be very important in improving the survival of cryopreserved apical meristms of wasabi (Matsumoto et al. 1994).

Effect of cooling speed during cryopreservation

Cooling speed is a major factor of the slow-freezing method of cryopreservation (Chang and Reed 2000; Benson et al. 1996). Slow cooling of shoot tips may cause cell injury by exposure to high concentrations of solution, whereas cooling at high speeds causes injury by intracellular ice formation. The effect of cooling speed on tissue survival was highly significant for cold hardening, with the slower cooling rates producing the higher survival rates (Chang and Reed 2000). The response of individual species to cooling speed varied, but in general, the slower the cooling rate, the higher the survival rate (Table 2).

Table 2. Effect of cooling speed during cryopreservation by basic method (vitrification) on the survival of cryopreserved shoot tips of the potato genotype 'Superior'.[z]

Cooling speed (℃/min)	Survival (%)
0.1	18.3 ± 2.4
0.3	25.0 ± 2.2
0.5	17.5 ± 2.1

[z]Subculture medium and culture conditions: MS medium supplemented with 0.25 M sucrose and 0.8% agar and incubation at 1 0℃ under a 16-h light/day photoperiod for 3 weeks. Basal vitrification solution (PVS-2): MS, 0.4 M sucrose, 30% glycerol, 15% DMSO, and 15% ethylene glycol. Shoot tip size: 1-1.5 mm in diameter.

Response of potato genotypes

High success of cryopreservation by slow-freezing of *in vitro* plant materials has been found to be the most dependent on the specific genotypes being used. Among the five tested genotypes, 'Atlantic' showed the highest survival of 90% after cryopreservation by slow freezing, whereas 58% of the shoot tips survived from the control cryopreservation method which was based on vitrification alone. The lowest survival rate was 30% of 'CTO5-5' by slow freezing and the 10% survival rate was found in the genotype of 'J138' by vitrification method alone (Fig. 1).

Plant regeneration (regrowth) and establishment of plants under glasshouse condition

Survival of shoot tips was recognized after three days of post-culture (Fig. 2A). About four weeks after survival of the shoot tips, rooted and shooted plantlets (Fig. 2B) were

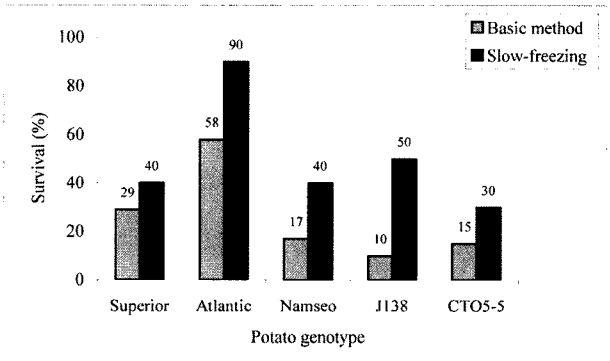

Figure. 1. Response of potato genotypes on the survival of shoots tips after cryopreservation by basic method (vitrification) and slow-freezing techniques. Regeneration medium: MS, 3% sucrose, 0.7% agar, 1.0 mg l^{-1} GA_3, 0.5 mg l^{-1} zeatin, and 0.1 mg l^{-1} IAA.

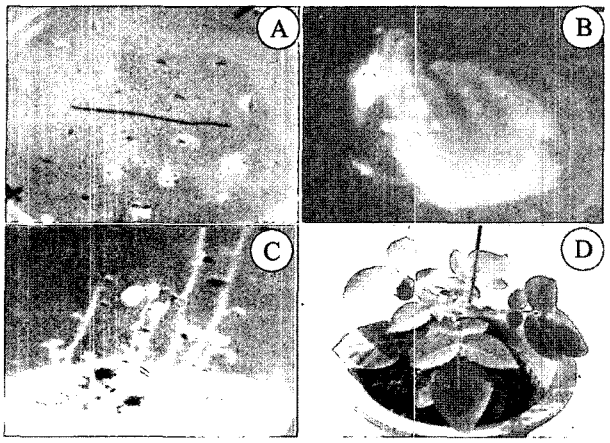

Figure. 2. Surviving shoot tips, elongated shoots, in vitro plantlets and plants established under glasshouse condition of the potato genotype 'Superior' after cryopreservation by slow freezing. A: Surviving shoot tips on MS medium containing 0.25 M sucrose after 3 days of subculture. B: Elongated shoots 4 weeks after survival assessment. C: Regenerated in vitro plantlets 8 weeks after transfer to regeneration medium. D: Plants established under glasshouse conditions 6 weeks after transferring to pots. Regeneration medium: MS, 3% sucrose, 0.8% agar, 1.0 mg l^{-1} GA_3, 0.5 mg l^{-1} zeatin, and 0.1 mg l^{-1} IAA.

transferred to MS medium containing 3% sucrose, 0.7% agar, 0.5 mg l^{-1} zeatin, 0.1 mg l^{-1} IAA, 1.0 mg l^{-1} GA_3 and incubated at 24 ± 2℃ under a 16-h light/day photoperiod for whole plant regeneration. A profuse root system and normal shoot structures were formed 8 weeks later (Fig. 2C). For the morphological study, plantlets regenerated after cryopreservation were grown under glasshouse condition, and nearly 90% of them established normal-looking plants (Fig. 2D). Leaf and tuber morphologies of the potatoes regenerated after cryopreservation using slow freezing technique were similar to those derived from the *in vitro* stock plantlets.

Slow-freezing technique of cryopreservation found to be very efficient for the conservation of the potato germplasm in several reasons. First, it worked well for many genotypes of potato. Second, it was easy to use with large numbers of samples. Third, the cryoprotectant was less toxic to the plants, resulting in high survival rates. Thus, cryopreservation by slow-freezing technique has been recognized as a highly efficient technique for the conservation of diverse potato genotypes.

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