Cryopreservation of *in vitro*-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification

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Introduction

Maintenance of potato germplasm in the field is a major consumer of time, manpower and space aside from diseases and environmental stresses. Conservation of *in vitro*-cultured potato also entails high maintenance costs, risks of somaclonal variation and genetic instability, especially when growth retardants are used (Harding 1991). Thus, cryopreservation appears to be a logical choice for long-term storage of potato germplasm, with minimum space and maintenance requirements without genetic instability (Bajaj 1978, 1985; Harding and Benson 1994). However, the conventional slow freezing method, which is very complicated and time-consuming, produced low rates of shoot formation and growth recovery took place through callusing (Towill 1983, 1984; Benson *et al.* 1989). For long-term conservation, the availability and development of safe and cost-effective techniques and subsequent high plant regeneration are the basic requirements. Recently, some simplified and reliable cryogenic procedures such as vitrification, encapsulation-drying and encapsulation-vitrification have been developed and the number of species or cultivars that have been cryopreserved has sharply increased (Sakai 1997).

We found that encapsulated potato meristems that were osmoprotected and then dehydrated with PVS2 solution at 0°C prior to a plunge into LN produced high levels of shoot formation without intermediary callus formation. Thus, we report an effective cryogenic procedure, the encapsulation-vitrification method, for long-term conservation of potato germplasm using meristems.

Materials and methods

Plant materials

Potato (*Solanum tuberosum* L.) cv. Danshakuimo was mainly used. Apical buds of plantlets were subcultured every 10 days (each plantlet had 3 to 4 nodes and apical bud) on MS medium (Murashige and Skoog 1962) with 0.5 g/L casamino acids, 30 g/L sucrose, 2.5 g/L gellan gum at 23°C under 16-h light/8-h dark photoperiod under a light intensity of 96 µmol m^{-2} s^{-1}. This medium was used as a basal medium. Nodal segments were transferred on basal medium in plastic dishes (90 x 20 mm) and cultured to induce axillary buds. These segments were cold-hardened at 4°C for 3 weeks under a 12-h light/12-h dark photoperiod with a light intensity of 20 µmol m^{-2} s^{-1}. Non-hardened nodal segments were cultured for 2 to 10 days under the previous conditions. Then, axillary meristems with five leaf primordia (about 1 mm size) were excised for experiments. The excised meristems were precultured on basal medium supplemented with 0.3M sucrose, 1 mg/L GA_3 (gibberellic acid), 0.01 mg/L BAP (6–benzylaminopurine) and 0.001 mg/L NAA (1–naphthaleneacetic acid) at 23°C for 16 h. Eleven other varieties were also used in this study.

Encapsulation, osmoprotection and dilution

Precultured meristems with or without cold-hardening were encapsulated in 2% Na–alginate gel beads (Hirai *et al.* 1998) and then osmoprotected with MS medium (the same plant hormones as the preculture medium) supplemented with 0.4–1.6M sucrose ± 2M glycerol (LS solution) for 90 min at 25°C (50 rpm on a rotary shaker) before dehydration with the PVS2 solution. Encapsulated dehydrated meristems were placed in a 1.8-ml cryotube with 1 ml PVS2 solution, then plunged into LN (liquid nitrogen). The cryotubes were rapidly rewarmed in a water-bath at 38°C and the PVS2 solution was drained from the cryotubes and replaced twice with 1 ml of 1.2M sucrose solution and held for 10 min.

Encapsulation-dehydration procedure

Meristems with three leaf primodia (about 0.5 mm size) were used for this experiment. Encapsulated meristems were treated in MS medium with 0.75M sucrose for 48 h at 90 rpm on a rotary shaker at 25°C. They were subjected to air-drying for 3.5 h in Petri dishes containing 50 g of dried silica gel. The water content of beads after air-drying was about 23% (FW basis). Dried, encapsulated meristems were placed in a 1.8-ml cryotube and directly plunged into LN and held for at least 1 h. Cryotubes were rewarmed in a water-bath at 38°C for 3 min.

Viability and plant regrowth

The cryopreserved meristems were plated on basal medium in plastic dishes with the same plant hormones as the preculture medium and cultured for 1 d (encapsulation-vitrification) or 7 d (encapsulation-dehydration) under the conditions described before. Then they were transplanted on basal medium supplemented with 0.0005 mg/L GA_3. The rate of shoot formation was expressed as a percentage of the total number of meristems forming normal shoots 2 weeks after plating. In every experiment approximately 10 meristems were treated for each of three replicates.

RAPD analysis

Genomic DNA was isolated using an ISOPLANT kit (Nippon Gene, Tokyo, Japan) from cryopreserved and non-treated (control) plantlets. DNA amplifications using 17 primers of 10 bases each (Operon technologies, CA, USA) were performed according to Williams *et al.* (1991). Differential bands were detected by staining with SYBR Green I nucleic acid gel-stain.

Results

In preliminary experiments, little or no differences were observed in cryopreservability among the nodal segments (1st to 3rd rank from the top of the plantlets) and the days of culture of nodal segments (2 to 10 d).

The optimal duration of exposure to PVS2 solution was 3 h for both non-hardened and cold-hardened meristems. Cold-hardened and non-hardened meristems treated with PVS2 solution for up to 4 h without cooling (treated control) retained high levels of shoot formation. However, after 4 h, shoot formation decreased in line with increasing exposure time.

The effects of cold-hardening and osmoprotectant on shoot formation of encapsulated vitrified meristems cooled to –196°C were investigated. As shown in Figure 1, non-hardened meristems osmoprotected with a mixture of 2M glycerol plus 0.6M sucrose for 90 min before dehydration with PVS2 solution produced 70.0% of shoot formation, while the percentage of shoot formation of cold-hardened meristems was 62.8%. When osmoprotected with sucrose alone, the meristems with or without cold-hardening produced much lower rates of shoot formation than those treated with 2M glycerol in combination with any concentration of sucrose.

Successfully encapsulated vitrified meristems cooled to –196°C resumed growth within 3 days and developed shoots and leaves within 10 days after plating without intermediary callus formation. All these shoots formed roots on basal MS medium and successfully produced microtubers. No morphological abnormalities were observed during the regrowth period and no differences in RAPD analysis were detected between cryopreserved and control plants for 17 primers used in this study. The encapsulation-vitrification protocol established in the present study was successfully applied to 12 other varieties tested (Fig. 2).

The rate of shoot formation and fresh weight of plants was compared for two different cryogenic procedures (Fig. 2). The encapsulated vitrified meristems gave much higher shoot formation and much faster growth than the encapsulated dried meristems in all varieties.

Discussion

In the vitrification procedure, meristems should be sufficiently dehydrated osmotically by exposure to a highly concentrated vitrification solution (PVS2). However, the exposure of meristems to the PVS2 solution without osmoprotection causes harmful osmotic stress. Thus, the key of success for cryopreservation by vitrification is to precondition meristems to induce osmotolerance to PVS2. In the present study, this limitation was almost overcome by preculturing meristems with 0.3M sucrose for 16 h, followed by osmoprotection with a mixture of 2M glycerol plus 0.6M sucrose for 90 min. However, without glycerol, no concentration of sucrose increased the osmotolerance of potato meristems to the PVS2 solution. Recently, the LS solution (a mixture of 2M glycerol plus 0.4M sucrose) was reported to be very effective in increasing osmotolerance to PVS2 solution of taro (Takagi *et al.* 1997), banana, orchid and pineapple (Thinh 1997), and strawberry (Hirai *et al.* 1998). For potato meristems, 0.6M sucrose in combination with 2M glycerol was needed to significantly enhance osmotolerance.

The cells of meristems which were immersed in a mixture of 0.6M sucrose plus 2M glycerol were osmotically dehydrated and plasmolyzed to a considerable extent for 90 min. These cells were then successively dehydrated with PVS2 solution. Under these conditions, the apical meristems remained osmotically concentrated and the increase in the cytosolic concentration required for vitrification was attained by osmotic dehydration. In the vitrification procedure, plasmolysis may play an important role in dehydration by mitigating its injurious effects (Steponkus *et al.* 1992; Jitsuyama *et al.* 1997; Matsumoto *et al.* 1998).

It is particularly important that cryopreserved meristems directly produce plants identical to the non-treated controls (Haskins *et al.* 1980; Kartha *et al.* 1980; Towill 1984). In the present study, successfully vitrified and rewarmed meristems vigorously developed shoots directly within a week after plating. No morphological abnormalities and callus formation were observed during recovery growth. The same results were reported in many plants. Benson *et al.* (1996) also reported that vitrification and encapsulation dehydration exceeded the conventional freezing method in terms of shoot formation and recovery growth in *Ribes*. Thus, the vitrification method with or without encapsulation certainly offers a considerable advantage over slow freezing method.

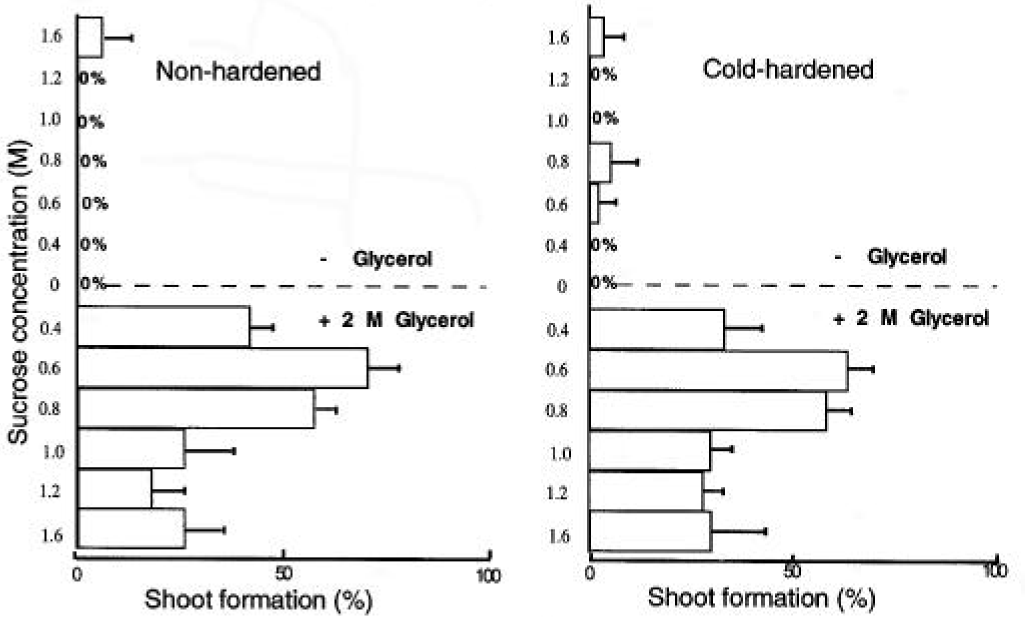

Fig. 1. Effect of osmoprotectant(s) and cold-hardening on the rate of shoot formation of encapsulated vitrified meristems cooled to –196°C. Material: cultivar Danshakuimo. Cold-hardened (right side) and non-hardened (left side) meristems were encapsulated in alginate beads, osmoprotected at 25°C for 90 min and treated with PVS2 solution at 0°C for 3 h prior to a plunge into LN. Approximately 10 meristems were used for each of three replicates. Bars represent the standard errors.

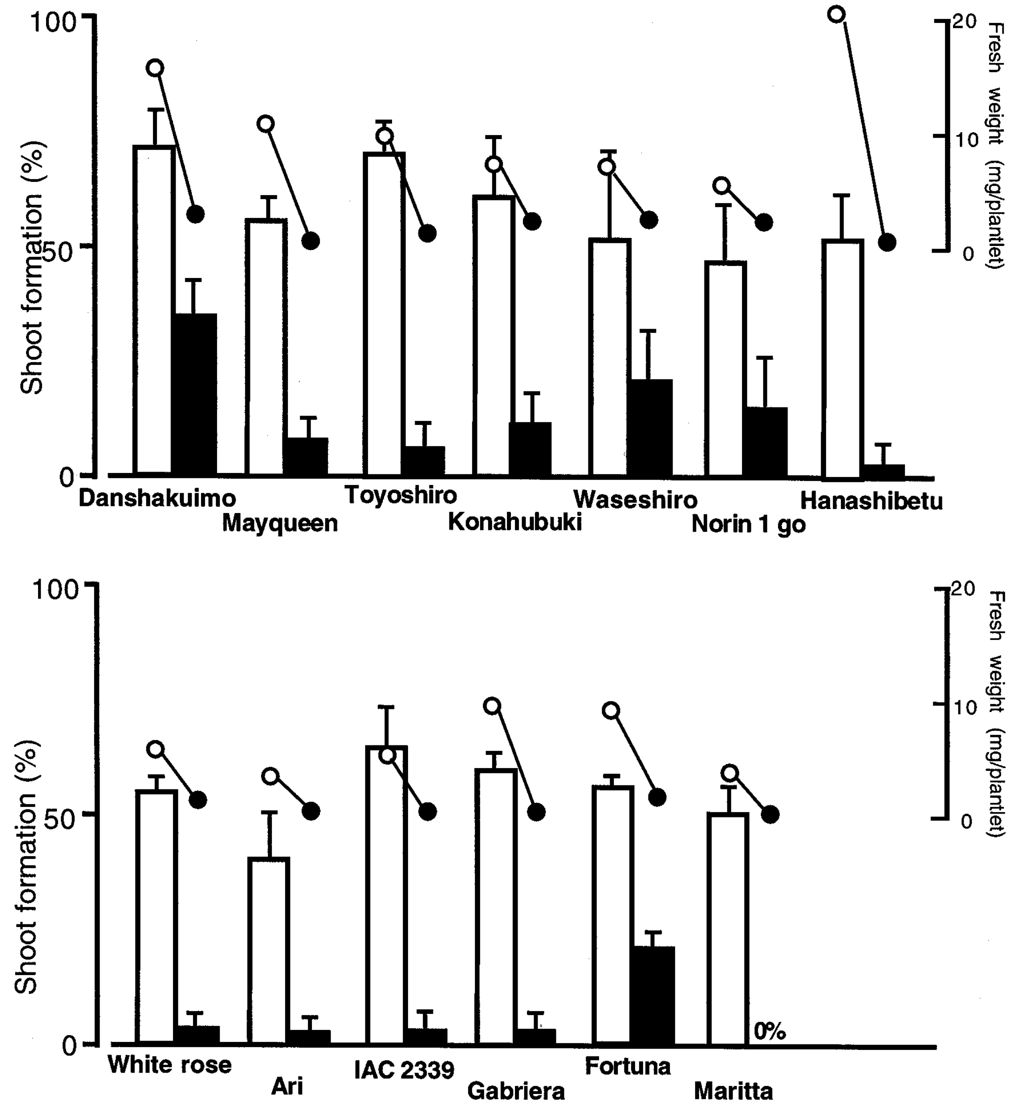

Fig. 2. Rate of the shoot formation (□, ■) and recovery growth (○, ●) of encapsulated vitrified or encapsulated dried meristems of 13 varieties cooled to −196°C. **Encapsulation-vitrification** (□, ○) : Encapsulated meristems in alginate gel beads were osmoprotected with a mixture of 0.6M sucrose plus 2M glycerol in MS medium at 25°C for 90 min and dehydrated with PVS2 at 0°C for 3 h prior to a plunge into LN. **Encapsulation-drying** (■, ●): Encapsulated meristems in alginate gel beads were suspended at 90 rpm in MS liquid media with 0.75M sucrose for 48 h at 25°C before air-drying in silica gel for 3.5 h prior to a plunge into LN. Approximately 10 meristems were treated for each of three replicates. Bars represent standard errors.

Harding (1996) reported that PCR and RAPD technology are useful for the detection of genetic changes and the assessment of the genetic stability of plants recovered from *in vitro* cultures. Within the primers used in this study, no differences were observed in RAPD analysis between cryopreserved and non-preserved (control) plantlets. However, further study is necessary to confirm their genetic stability by other analyses.

The encapsulation-dehydration method has been successfully applied to a wide range of materials. However, the problems are a lower rate of shoot formation and later recovery growth compared with the meristems cryopreserved by vitrification (Matsumoto *et al.* 1995; Hirai *et al.* 1998). In this study, the encapsulation-dehydration method also showed the same tendency. Thus, the treatment to induce dehydration tolerance before air-drying appears to be insufficient to produce a higher level of recovery growth.

The encapsulation-vitrification method is easy to handle and can be used with a large number of meristems at the same time. Besides, regrowth is achieved much earlier than with the encapsulation-dehydration method. Thus, the encapsulation-vitrification method appears to be a potentially valuable cryogenic protocol for large-scale cryopreservation of potato germplasm.

Acknowledgements

The authors wish to thank Ms Noriko Otsuki for her technical assistance and Mr Takeshi Itoh, Kitami A.E.S., Mr Akihiro Arihara, Hokuren A.R.I. and Dr Takeshi Matsumura, Hokkaido Green-bio institute for supplying potato varieties. We also thank Mr Tadahiko Kiguchi, Chuo A.E.S., for teaching the principles and technique of PCR and RAPD analysis. I am especially grateful to Dr Hiroko Takagi-Watanabe, JIRCAS, for giving the opportunity to present this article.

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