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Cryopreservation of *In Vitro*-Grown Apical Shoot Tips of Strawberry by Vitrification

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Abstract

After the strawberry shoots on culture medium were cold-hardened at 5 ℃ for 20-30 days, shoot tips were dissected and precultured at 5 ℃ for 1 day on MS medium containing 2 M glycerol and 0.3 M sucrose. Precultured 10 shoot tips were placed in 2.0 ml cryotubes each, and treated with a mixture of 2 M glycerol and 0.3 M sucrose for 20 min at 25 ℃. They were then treated with PVS2 solution for 50 min at 25 ℃, and immersed into LN. After rapid rewarming in water at 35 ℃, they were transferred onto the MS medium containing 1 gl^{-1} PVP and 0.2 mgl^{-1} BA and grown under standard conditions. By this protocol, survival rate of cryopreserved "Donner" shoot tips reached 93%. This protocol was successfully applied to 7 other races or lines of strawberry with high survival rates. These results prompt us to setting up large-scale strawberry LN storage system.

Key words: cryopreservation, preculture, shoot tips, strawberry, vitrification.

Abbreviations

BA, benzyl aminopurine; LN, liquid nitrogen; MS medium, Murashige and Skoog medium; PVP, polyvinyl pyrrolidone; PVS2, plant vitrification solution 2.

Introduction

Strawberry is an important economic and nutritious crop in many countries. Strawberry germplasm is commonly preserved as plants either in field or in insect-proof screen houses. In these gene banks, the plants are subject to risks of losses caused by biological and natural hazards. For strawberry preservation, there are some disadvantages, such as periodic replanting, possibility of contamination by runners from other clones and naturally spread viruses or virus-like diseases. An alternative method is *in vitro* preservation of strawberry cultures at refrigerated temperature for medium-term storage. Over 350 Fragaria accessions are stored in gas-permeable tissue-culture bags at 4 ℃ in National Clonal Germplasm Repository, Corvallis (Reed and Hummer, 1995).

Cryopreservation is one of the ideal and suitable options for long-term storage of plant germplasm. Cryopreservation techniques are now advanced at the stage where they can be implemented for useful storage of germplasm (Reed, 2001). Techniques of controlled freezing, vitrification, encapsulation-dehydration, encapsulation-vitrification, dormant bud preservation and combinations of these are now available for use. Although a few papers have been reported for strawberry cryopreservation (Sakai *et al.*, 1978; Kartha *et al.*, 1980; Reed and Hummer, 1995; Wu *et al.*, 1997; Hirai *et al.*, 1998), there has been no application on a large-scale to germplasm lines.

In Japan, about 700 accessions of strawberry germplasm (*Fragaria* species) have been maintained as field collections. The objective in this study was to establish a reliable protocol for the cryopreservation of strawberry shoot tips using

vitrification. This method would then be used for a large-scale cryopreservation project of *Fragaria* species.

Materials and Methods

Plant material

Tissue-cultured shoots of strawberry (*Fragaria* × *ananassa* Duch.) cv. Donner were used for most experiments. Seven other races or lines of strawberry, Nyoho, Hokowase, Sachinoka, Tochiyutaka, Toyonoka, Cirano and VC-10 were also tested. *In vitro* grown strawberry shoots were multiplied from runner tips and periodically subcultured on solid Murashige and Skoog medium (1962) supplemented with 0.2 mgl^{-1} BA, 2.5% sucrose and 8 gl^{-1} agar at pH 5.8. Cultures were incubated at 25 ℃ with a 16-h photoperiod under white fluorescent light (52 $\mu mol\ m^{-2}\ s^{-1}$).

Cold-hardening and preculture

After the last subculture, 2-week-old normal shoots were cold-hardened at 5 ℃ with 8-h photoperiod (26 $\mu mol\ m^{-2}s^{-1}$) over the period of 0 to 40 days. Shoot tip was dissected from cold-hardened shoot. The shoot tip consisted of apical doom, 2 young leaf bases, 1-2 leaf primordia and basal stem. Also, the cold-hardened clumps (30 days at 5 ℃, about 1.5-2.0 mm long, 1 mm base diameter) were used. The clump tested consisted of 1-2 dooms with 1-2 leaf primordia and basal stems. These were precultured at 5 ℃ for 1-5 days on solid MS media containing either 0.3 M sucrose, 0.3 M sucrose + 1 M glycerol or 0.3 M sucrose + 2 M glycerol with 8-h photoperiod (26 $\mu mol\ m^{-2}\ s^{-1}$).

Vitrification procedure

After preculturing, 10 shoot tips were placed into 2-ml plastic cryotubes and treated or osmoprotected with liquid MS basal medium supplemented with 2 M glycerol and 0.4 M sucrose for 20 min at 25 ℃. After this solution was removed, the shoot tips were then dehydrated with 1 ml PVS2 at 25 ℃ for 30-60 min. During this treatment, PVS2 was replaced with fresh PVS2 once. The shoot tips were suspended finally in 0.5 ml of fresh PVS2 solution in the cryotubes just before immersion in LN. Cryotubes were stored in LN for at least 1 day. The vitrification solution PVS2 (Sakai *et al.*, 1991) was prepared in liquid MS basal medium containing 0.4 M sucrose (pH 5.8) and additives: 30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG) and 15% (w/v), dimethyl sulfoxide (DMSO).

Recovery and survival determination of shoot tips after cryostorage

Cryotubes were rapid warmed in a water bath at 35 ℃. PVS2 was drained from the cryotubes and replaced twice with liquid MS medium containing 1 M sucrose. Shoot tips were blotted and transferred onto solid MS recovery medium with various modifications, such as polyvinyl pyrrolidone (PVP, K-30, Wako, JAPAN) and BA content. Recovery medium tested contained PVP at 0-2 gl^{-1} in regular MS medium supplemented with 0.2 mgl^{-1} BA and 2.5% sucrose. The other test used MS medium supplemented with different concentrations of BA (0 to 2 mgl^{-1}), 1 gl^{-1} PVP and 2.5% sucrose.

Survival was recorded as a percentage of total number of shoot tips forming normal shoots after 4 weeks of plating. Ten shoot tips were tested for each of the three replicates in each experiment.

Results

Two types of shoot tips either from normal shoots or clumps were treated with PVS2 for different periods of time. The highest survival percentages from normal shoots and clumps were obtained 70% for 50 min and 70% for 30 min treatment, respectively (**Fig. 1**). We subsequently used shoot tips from normal shoots, rather than clumps as an experimental material because it was easy to select the size and developmental stage. Also, shoot tips from clumps were too soft and immature for use with vitrification methods. The meristem doom of shoot tip from normal shoot was tightly covered by leaf bases and leaf primordia compared with that of clumps.

Cold-hardening significantly improved the survival of shoot tips of strawberry cooled to -196 ℃

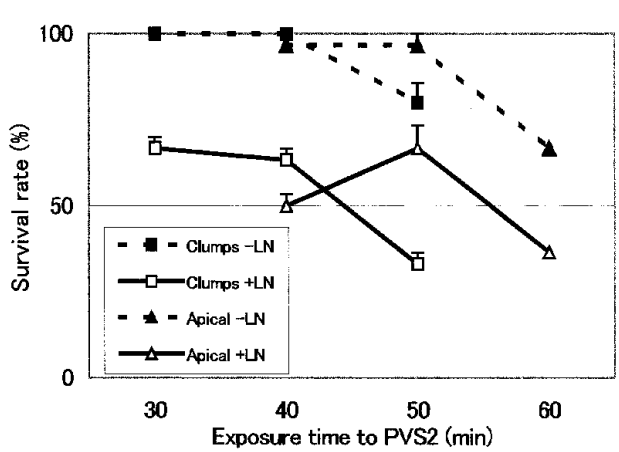

Fig. 1 Effect of materials and exposure time to PVS2 on the survival of cryopreserved strawberry shoot tips. Hardening: 5 ℃, 30 days; Preculture: 5 ℃, 2 days; Post-thaw growth medium: PVP 1 gl^{-1}, BA 0.2 mgl^{-1}; Vertical bar represents SE.

by vitrification. More than 10 days were necessary to obtain high survival values (**Fig. 2**).

To increase dehydration tolerance, cold-hardened shoot tips were precultured by a direct transfer to three different preculture media at 5 ℃ for 1-5 days. As shown in **Table 1**, 5 day preculture with MS medium containing only 0.3 M sucrose gave 80% survival after cryopreservation. However, the vitrified shoot tips precultured with MS medium containing 0.3 M sucrose and 1 M glycerol or 2 M glycerol gave similar levels of survival after 1 or 2 days of preculture at 5 ℃. The highest survival was obtained with preculture on MS medium containing 0.3 M sucrose and 2 M glycerol for 1 day at 5 ℃.

Recovery medium of vitrified shoot tips affected survival percentage. PVP could be a controller and an adsorbent of oxidation products in the tissue culture medium. A recovery medium containing 1 gl^{-1} PVP resulted in significantly higher survival of shoot tips cooled to -196 ℃ compared to the regular MS medium containing no PVP (**Fig. 3**).

The growth regulator BA is also important for high survival percentage. A recovery medium containing 0.2 mgl^{-1} BA gave significantly higher

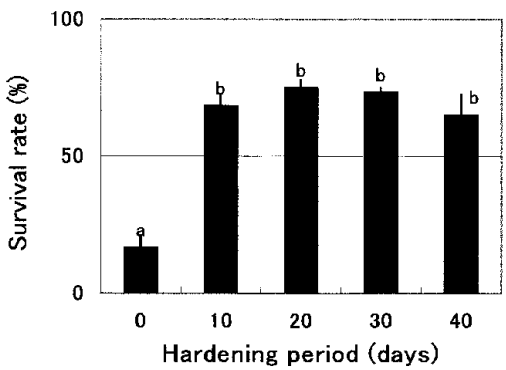

Fig. 2 Effect of hardening period at 5 ℃ on the survival of cryopreserved strawberry shoot tips. Preculture: 5 ℃, 2 days; Exposure time to PVS2: 50 min; Post-thaw growth medium: PVP 1 gl^{-1}, BA 0.2 mgl^{-1}; Means with different letters indicate significant differences ($P<0.05$) by Tukey's test.

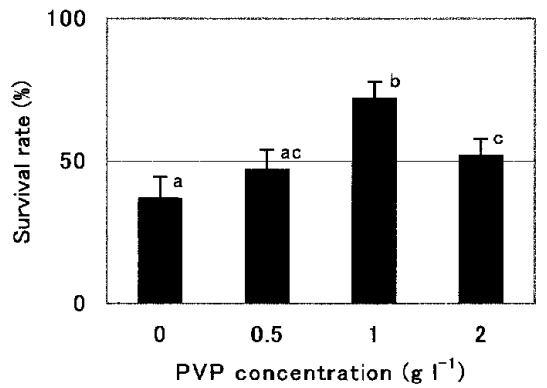

Fig. 3 Survival of cryopreserved strawberry shoot tips recovered on three different concentrations of PVP in the medium. Hardening: 5 ℃, 20 days; Preculture: 0.3 M Suc. 2 days; Exposure time to PVS2: 50 min; Post-thaw growth medium: PVP 0-2 gl^{-1}, BA 0.2 mgl^{-1}; Means with different letters indicate significant difference ($P<0.05$) by Tukey's test.

Table 1 Effect of preculture medium and preculture period on the survival of strawberry shoot tips

Preculture period (days)	Preculture medium (MS medium +)		
	0.3 M Sucrose	0.3 M Sucrose + 1 M Glycerol	0.3 M Sucrose + 2 M Glycerol
	Survival rate (% ± SE)		
1	53.3 ± 3.3 a	70.0 ± 10.0 a	93.3 ± 3.3 a
2	66.7 ± 3.3 ab	80.0 ± 0.0 a	86.7 ± 3.3 a
3	60.0 ± 5.8 ab	53.3 ± 3.3 b	20.0 ± 5.8 b
5	80.0 ± 5.8 b	20.0 ± 5.8 c	23.3 ± 6.7 b
Analysis of variance	Period (A)	**	
	Medium (B)	NS	
	A x B	**	

The shoots of strawberry were cold-hardened at 5 ℃ for 20 days. The excised shoot tips were precultured under each condition at 5 ℃. They were treated with a mixture of 2 M glycerol and 0.4 M sucrose for 20 min at 25 ℃, then dehydrated with PVS2 solution for 50 min at 25 ℃ prior to immersion in LN. Cryopreserved shoot tips were plated on MS medium supplemented with 0.2 mgl^{-1} BA and 1 gl^{-1} PVP.

** Significant differences between means at the 1% level of probability. NS, non-significant at P=5%. Means with different letters indicate significant differences ($P<0.05$) by Tukey's test.

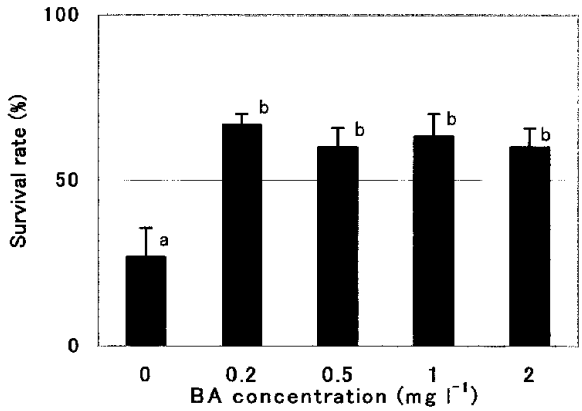

Fig. 4 Survival of cryopreserved strawberry shoot tips recovered on four different concentrations of BA in the medium. Hardening: 5 ℃, 20 days; Preculture: 0.3 M Suc. 2 days; Exposure time to PVS2: 50 min; Post-thaw growth medium: PVP 1 gl^{-1}, BA 0-2 mgl^{-1}; Means with different letters indicate significant differences ($P<0.05$) by Tukey's test.

Table 2 Survival of cryopreserved shoot tips from 7 other strawberry races or lines

Races and lines	Survival rate (% ± SE)
Nyoho	80 ± 0
Hokowase	74 ± 6
Sachinoka	80 ± 0
Tochiyutaka	80 ± 4
Toyonoka	85 ± 5
Cirano	50 ± 10
VC-10	71 ± 4

The shoots of each strawberry were cold-hardened at 5 ℃ for 20 days. The excised shoot tips were precultured on the MS medium containing 0.3 M sucrose and 2 M glycerol for 1 day at 5 ℃. They were treated with a mixture of 2 M glycerol and 0.4 M for 20 min at 25 ℃, then dehydrated with PVS2 solution for 50 min at 25 ℃ prior to immersion in LN. Cryopreserved shoot tips were plated on MS medium supplemented with 0.2 mgl^{-1} BA and 1 gl^{-1} PVP. About 10-20 shoot tips were tested for each of the 2 or 3 replicates.

survival percentage of shoot tips cooled to -196 ℃ compared to the medium devoid of BA (**Fig. 4**).

We tested our improved vitrification procedure on seven other strawberry races or lines (**Table 2**). The survival rates were relatively high in all, ranging from 50-85%.

Discussion

There are several key factors that enhance post-thaw survival in cryopreservation by vitrification. High survival of *in vitro*-grown materials is determined not only by the cryogenic protocol itself, but also by the physiological conditions of materials to be cryopreserved such as growth stage, preconditioning and post-thaw medium. For increasing the chances of positive and uniform response to treatment with PVS2, homogeneous specimens in terms of size, cellular composition, physiological state and growth response are employed for vitrification. Thin *et al.* (1999) reported with *in vitro*-grown shoots of banana that for cryopreservation by vitrification the apical domes must be partially covered by 1 to 2 leaf primordia to produce a high level of regrowth. Niino *et al.* (2000) reported with *in vitro*-grown lateral buds of innala that the meristems must be fully covered by the outer leaf primordia to produce a high level of regrowth. In this study, we chose apical meristems or shoot tips rather than meristematic clumps or axillary buds as an experimental material because it was easy to select the size and developmental stage. The apical dooms were fully covered by 1 to 2 leaf primordia and base of leaves. This tight structure around the dome may have protected the apical doom from the direct damage by PVS2 and handling.

Preconditioning to increase the dehydration tolerance of shoot tips was the most important factor for a successful cryopreservation by vitrification. Cold hardening and preculture of shoot tips with sucrose-enriched media was effective for improving post-thaw survival of some temperate and tropical species (Takagi, 2000). During preculture on sucrose-enriched medium, sugar, starch and proline were greatly increased in the shoot tips and may enhance the stability of membranes under conditions of severe dehydration (Matsumoto, 2002). Also, a cryoprotective or osmoprotective treatment with a mixture of 2 M glycerol and 0.4 M sucrose appeared promising as to enhance dehydration tolerance of shoot tips of several species (Matsumoto, 2002). The protective effect of this solution in the cell's peri-protoplasmic space may be due to mitigation of the large osmotic stress with exposure to PVS2 in addition to some mechanisms that minimize the injurious membrane changes from severe dehydration (Crove *et al.*, 1988, Steponkus *et al.*, 1992). In this study, both cold hardening and preculture were effective. Preculture on different glycerol concentrations resulted in different survival patterns depending on preculture durations. Optimal preculture

durations on 0.3 M sucrose, 0.3 M sucrose + 1 M glycerol and 0.3 M sucrose + 2 M glycerol were 5 days, 2 days and 1 day, respectively. Glycerol treatments shortened the preculture duration and imparted dehydration tolerance to shoot tips quickly. This high concentration of glycerol may be applicable to a limited number of species, but may act by concentrating cytosolic cryoprotectants accumulated during preculture in the same way that the solution containing 2 M glycerol and 0.4 M sucrose did. Though its mode of action is not well understood, glycerol seems to be a significant factor in achieving high post-thaw survival.

Research with sweet potato culture demonstrated the composition of the recovery medium such as pluronic F-68 for a cell protecting agent could influence post-thaw survival (Pennycook and Towill, 2001). Growth regulators in the recovery medium also influence post-thaw survival. Lambardi *et al.* (2000) reported that post-thaw survival of white poplar shoot tips was markedly decreased when cryopreserved shoot tips were cultured on hormone-free medium and greatly enhanced on the medium containing cytokinin and GA_3. In this study, PVP (polyvinyl pyrrolidone) and BA supplementation in the recovery medium increased the survival significantly compared with no supplementation. It is uncertain as to how PVP functions but it may be involved in adsorbing the phenolics produced by dead cells.

Based on these results, the optimal protocol for the cryopreservation of strawberry collections was as follows. After the strawberry shoots were cold-hardened on culture medium at 5 ℃ for 20-30 days, shoot tips were dissected and precultured at 5 ℃ for 1 day on MS medium containing 0.4 M sucrose and 2 M glycerol. Precultured shoot tips were placed in 2.0 ml cryotubes, and treated or osmoprotected with a mixture of 2 M glycerol plus 0.3 M sucrose for 20 min at 25 ℃. They were then treated with PVS2 solution for 50 min at 25 ℃, and immersed into LN. After rapid warming in water at 35 ℃, they were transferred onto the MS medium containing 1 gl^{-1} PVP and 0.2 mgl^{-1} BA and grown under standard conditions. By this protocol, survival of cryopreserved "Donner" shoot tips reached 93%. This protocol was successfully applied to 7 different races or lines of strawberry with high survival values. These results prompt us to setting up a large-scale strawberry LN storage system. We are going to cryopreserve about 100 races or lines of strawberry as a pilot project.

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