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Survival of cultured cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification

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Abstract. Cultured cells and somatic embryos derived from the mesophyll tissue of asparagus (Asparagus officinalis L.) were cryopreserved by vitrification. The vitrification solution (PVS) contains (w/v) 22% glycerol, 15% ethylene glycol, 15% propylene glycol and 7% DMSO in Murashige-Skoog medium enriched with 0.5M sorbitol. After initial cryoprotection with sorbitol supplemented MS medium containing 12% ethylene glycol, cells or embryos were exposed stepwise to 85% PVS at 0°C. They were loaded into 0.5 ml transparent straws, and were then plunged directly into liquid nitrogen. After rapid warming, PVS was removed and diluted stepwise. The highest survivals of vitrified cells and embryos were about 65 and 50%, respectively. Surviving embryos developed into plantlets.

Keywords: Cryopreservation - Vitrification - Plant germplasm - Asparagus - Asparagus officinalis

Abbreviations: DMSO, dimetyl sulfoxide; PVS, vitrification solution; LN, liquid nitrogen; DSC, differential scanning calorimeter; MS, Murashige-Skoog salt medium; NAA, naphthalene acetic acid; BA, 6-benzyladenine.

Introduction

Winter hardy mulberry cortical cells which were directly immersed into LN from room temperature remained alive without cryoprotectant when rewarmed rapidly (Sakai 1958, 1966, 1971). The same hardy tissue sections suspended in 2M solutions of DMSO, ehtylene glycol and glucose, respectively, remained alive by vitrification without any freeze-dehydration. However, in less or nonhardy cultured plant cells and meristems, successful vitrification seems difficult without partial freeze-dehydration in the presence of cryoprotectants prior to immersion into LN (Sakai et al.1978; Uemura and Sakai 1980; Kartha et al. 1982).

Cultured plant cells and meristems exhibit high levels of survival when frozen slowly at 0.5 to 1.0 °C min^{-1} to about -40°C prior to immersion into LN in the presence of suitable cryoprotectants and when rewarmed rapidly (Withers 1985). However, such slow freezing is time consuming and requires a sophisticated biological freezer. The main advantages of a vitrification method for the cryopreservation are that(1) controlled-rate freezing equipment is not necessary and that (2) the time required for cooling is considerably reduced. Recently, successful vitrification for the cryopreservation of animal cells(Takahashi et al. 1986) and embryos (Rall and Fahy 1985) was accomplished using a low toxic vitrification solution (VS1).

Regeneration of plant somatic embryos cryopreserved by the vitrification process has not been reported. Thus, to develop a simple and effective method for cryopreserving plant germplasm, some factors contributing to the survival of cells cooled to -196°C by vitrification were investigated using asparagus cells and embryos.

Materials and methods

Single cells of asparagus (Asparagus officinalis L. line 873) were mechanically isolated mainly according to Jullien (1972) as follows. Fully expanded cladophylls were surface-strilized and washed with autoclaved distilled water. One gram of cladophylls was gently ground in a grinding solution consisting of 0.3M sucrose and 1mM $CaCl_2$, and then filtered through a 34-µm nylon mesh. The resulting cell suspension was centrifuged at 100xg for 3 min and then resuspended in MS medium (Murashige and Skoog 1962) with half in strength of NH_4NO_3 and KNO_3,and containing 0.3M sucrose, $5x10^{-5}$M NAA, 10^{-6}M BA, 500 mg/l L-glutamine. The cells were cultured in Petri dishes (9 cm in diameter) at a density of $3x10^5$ cells per ml. The plates were incubated at 25°C under continuous cool white fluorescent light ($37\mu Em^{-2}s^{-1}$).

Somatic embryos were initiated from single cell cultures of asparagus line "KBFx3-9". Four-week old colonies were transferred to MS medium without phytohormone. After 2 to 4 weeks, a small number of the colonies formed globular somatic embryos. These embryos were subcultured to and maintained as embryogenic callus in solidified MS medium containing $5x10^{-5}$M NAA and 10^{-6}M BA. About 1g of embryogenic callus was cultured in liquid MS medium without phytohormone in an Erlenmeyer flask and shaken (100 rev.min^{-1}) at 22°C under diffuse light. After 10 days, the culture was

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filtered through a 500-μm nylon mesh before being used for experiments. In all experiments, cells and embryos were precultured in MS medium supplemented with 0.3M sorbitol and 0.2M sucrose for 16 hr at 22°C prior to cryoprotection (Chen et al. 1984; Kartha et al. 1988) The thirteen to 23 day-old cultured cells and the early globular stage of embryos were used for experiments.

As a result of our preliminary experiments with plant samples, a low-toxicity combination that allowed the contents of the straw to remain transparent without crystallization during cooling as well as during warming was adopted as vitrification solution(PVS). This contains 22%(w/v) glycerol, 15%(w/v) ethylene glycol, 15%(w/v) propylene glycol and 7%(w/v) DMSO in MS medium containing 0.5M sorbitol and is adjusted to pH 5.8. The cell suspensions containing 80 to 100% of PVS remained transparent after plunging into LN and during subsequent warming. And the highest survival was obtained at 85% PVS. Thus, an 85% concentration of stock solution was mainly used.

Low temperatures are needed to reduce the injurious effects of the high osmolarity of PVS, but they also reduce cell permeability and thus increase the length of time that cells are exposed to osmotic stress. These two requirements are in conflict. This was avoided by a prior cryoprotective treatment with ethylene glycol at room temperature for 5 min before exposure to 85% PVS at 0°C. In preliminary experiments, ethylene glycol and DMSO were observed to easily permeate the cells, which deplasmolysed within 5min at 22°C. Thus, 0.5M sorbitol-MS supplemented with 12% ethylene glycol was used as the initial cryoprotective solution. This solution was added to sedimented cells placed in a screw capped pyrex tube (20 cm in length and 2 cm in diameter) at 22°C and the cell suspension was equilibrated for 5 min. After initial cryoprotection, the cell suspension was centrifuged at 100xg for 30 sec and the sedimented cells were transferred to an ice bath. Chilled 85% PVS was added dropwise to the sedimented cells at 0°C, over a period of 5min using a pipette whose tip had been narrowed to deliver about 10μl per drop, so that the concentration of PVS in the cell suspension reached about 42.5%, and then was allowed to equilibrate for an additional 5min. After removing the supernatant, 0.5 ml chilled 85% PVS was added to the packed cells, and the resulting cell suspension was equilibrated for 5 min at 0°C. The cell suspension of about 50 μl was then loaded in the middle part of a 0.5 ml straw separated by air bubbles from the diluting medium(about 200μl) consisting of 1.2M sorbitol-MS. After sealing the top end of each straws by a heat sealer, straws were cooled by plunging directly into liquid nitrogen followed by storing there for 30 or 60 min. The cooling and warming rates of the cell suspension loaded within straws were determined by thermocouples. The mean cooling rate between 0 and -196°C was about 990°C min^{-1}. The straws were warmed rapidly by transfer from LN into a water bath at 22°C. The warming rate was about 900°C min^{-1} at the temperature range between -120 and -40°C. After warming, the contents of the straw were mixed by flipping the central part of the straw with fingers to dislodge the air bubbles. The cell suspension mixed with the diluting solution was centrifuged at 100xg for 30 sec and the supernatant was removed. The sedimented cells in straws were resuspended in 1.2M sorbitol-MS containing 8% ethylene glycol and mixed well using a pipette. After centrifugation, the resulting cell sediment was further diluted at 3min intervals with 0.8M and 0.5M sorbitol-MS, respectively.

For differential scanning calorimeter (DSC) measurements, solutions of 5 to 6mg were placed in sealable aluminum pans of a DSC (Perkin-Elmer DSC-7, Norwalk, Conn.), weighed, and then cooled at 80°C min^{-1} and warmed at 10°C min^{-1}, respectively.

Viability of cells or embryos after dilution was determined by fluorescein diacetate(FDA) vital staining (Widholm 1972). In each treatment, three straws were used and about one thousand cells in each straw were examined for their viability. The viability of cells or embryos after treatment was expressed as per cent survival over the nontreated, unfrozen control. Regrowth of vitrified somatic embryos was achieved by transferring them onto a 34μm nylon mesh over hormone-free MS medium with 0.5M sucrose solidified with 0.2% gerlite. After 16hr, the nylon mesh with embryos was transferred to solidified fresh MS medium containing 0.3M sucrose. In the case of cultured cells, solidified MS medium supplemented with 0.3M sucrose, $5x10^{-5}$M NAA and 10^{-6}M BA was used.

Results

Effect of cryoprotectants on the survival of cells

Initial cryoprotection of the cells was compared with various cryoprotectants which dissolved in 0.5M sorbitol-MS. As shown in Table 1, the highest cryoprotection was obtained with 12% ethylene glycol in 0.5M sorbitol-MS. Only a slight difference was observed in the cells treated with 12% ethylene glycol for 5 or 10 min. Cryoprotection by DMSO was less effective than that by ethylene glycol, but was more effective than propylene glycol (data not shown). Thus, in all experiments, cells and embryos were previously cryoprotected with 0.5M sorbitol-MS supplemented with 12% ethylene glycol for 5 min at 22 °C.

Effect of holding temperature on survival of vitrified cells

To test the effect of devitrification on cell survival, cell suspensions were vitrified and then transferred to a bath at -70°C, close to the temperature at which devitrification occurs, and held there for 2 or 60 min. As shown in Table 2, in the cells supercooled at -70°C or held at -70°C for 2 min after rapid cooling to -196°C, little or no damage was observed. However, holding cells at -70°C for 60 min or -50°C for 10 min caused serious damage, indicating that devitrification proceeded at -70°C with increasing length of time, but more rapidly at -50°C than at -70°C.

In order to investigate further the detrimental effect of devitrification during slow warming, cells were transferred to a bath at -70°C after removal from LN, and then rewarmed at 3.5°C min^{-1}. Immediately after reaching various temperatures, cell suspension

was rapidly rewarmed in a bath at 22°C. Cell survival decreased with increasing temperatures. The relative rates of survival were as follows: 100(at -70°C), 76.3(-60°C), 36.4(-50°C) and 12.0(-40°C), respectively.

Table 1. Effect of cryoprotectants on the survival of cells cooled to -196°C by vitrification

Cryoprotectant	Survival(%)
Experiment 1	
7% ethylene glycol (5min)	29.0±2.8
10% ethylene glycol (5min)	35.7±1.0
12% ethylene glycol (5min)	44.9±4.7
15% ethylene glycol (5min)	40.5±4.5
Experiment 2	
12% ethylene glycol (5min)	37.2±5.0
5% DMSO (5min)	25.6±2.6
7% DMSO (5min)	33.2±3.4
10% DMSO (5min)	31.1±4.2
Experiment 3	
12% ethylene glycol (5min)	47.9±2.0
12% ethylene glycol (10min)	48.5±4.1
7% DMSO (5min)	39.4±3.4
7% DMSO (10min)	34.2±6.6

Survival was expressed as per cent over the nontreated, unfrozen controls. Cells were treated with 0.5M sorbitol-MS supplemented with different concentrations of cryoprotectants at 22°C prior to exposure to 85% PVS at 0°C.

Table 2. Effects of holding temperature on the survival of vitrified cells

Treatment: holding temperature and period held at that temperature	Survival(%)
Experiment 1	
Treated control[a]	47.4±3.2
Vitrified cells[b]	49.9±0.9
Experiment 2	
Supercooled at -70°C for 20 min	42.8±2.2
-70°C for 2 min[c]	41.1±3.9
-70°C for 60 min[c]	6.1±0.7
-50°C for 10 min[c]	9.5±1.3

a: Cell suspension was treated with 85% PVS and diluted without cooling to -196°C; b: Vitrified cells were rewarmed directly at a bath of 22°C; c: Vitrified cells were rapidly transferred to a bath at -70 or -50°C and held there before being rewarmed.

Thermal properties of vitrification solution

The thermal behavior of the vitrification solution was observed during cooling and warming in a differential scanning calorimeter, and a representative thermogram during warming is presented in Fig.1. During cooling at 80°C min^{-1}, no freezing exotherm was observed, but a glass was seen to form at about -115°C (data not shown). On warming, the glass transition was observed at -115.3°C. The metastable solution initiated devitrification at -76.2°C and showed a maximum exotherm at -67.2°C due to devitrification. The ice began to melt at about -56°C in equilibrium with the concentrated solution and the melting point was -31.6°C.

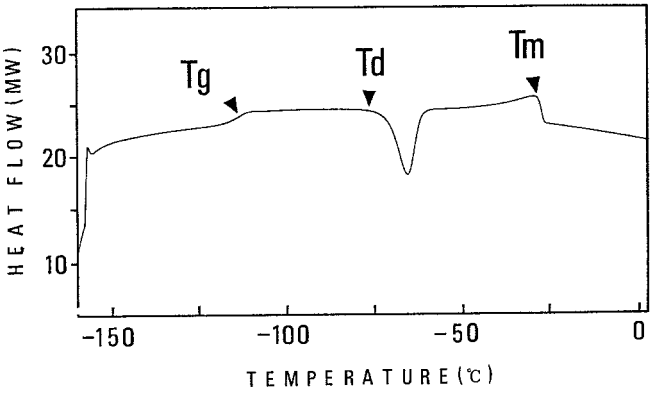
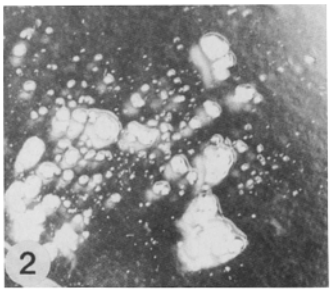


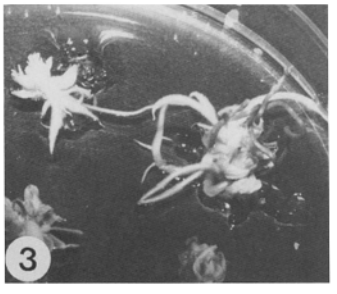
Fig.1. DSC record of 85% PVS in 0.5M sorbitol MS medium.
The warming rate was 10°C min^{-1}. Tg: temperature of the glass transition(-115.3°C); Td: temperature of the devitrification (-76.2°C); Tm: melting point(-31.6°C).

Table 3. Survival rate of vitrified somatic embryos

Treatment	Survival(%)
85% PVS	48.4±6.0
100% PVS	40.3±4.5

Survival rates were determined by FDA staining after warming and dilution. Somatic embryos:early globular stage.





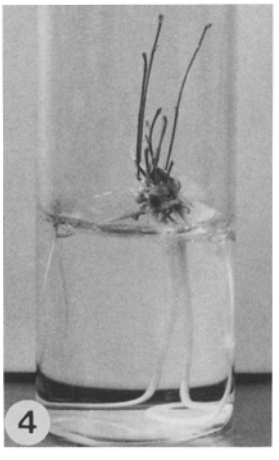


Fig.2. Vitrified somatic embryos 10 days after rewarming (x3.4)
Fig.3. Development of roots and shoots from vitrified somatic embryos (x1.1)
Fig.4. Plantlet developed from vitrified somatic embryos after 60 days (x2.2)

Development of vitrified embryos into plantlets

The survival rate of vitrified somatic embryos is presented in Table 3.

On regrowth medium, the viable embryos after vitrification resumed growth in about 1 week(Fig.2). The cultured embryos were then transferred to fresh medium. Embryos developed roots and shoots in 2 or 3 weeks (Fig.3) and developed into plantlets (Fig.4). The vitrified embryos followed the same pattern of development as the controls. The cultured cells also resumed growth and developed many green spots in about one month.

Discussion

Fahy et al.(1984) have developed a cryopreservation medium (VS1) containing 20.5%(w/v) DMSO, 15.5%(w/v) acetamide, 10%(w/v) propylene glycol and 6%(w/v) polyethylene glycol which is vitrified at 1 atm. We adopted PVS as vitrification solution for asparagus cultured cells and somatic embryos. The survival rate after addition and removal of 85% PVS without cooling to -196°C, was 49.9% of the untreated control(Table 2). And survival of the cells vitrified and subsequently diluted was 47.4%, indicating that the actual vitrification and subsequent warming of cells did not cause any additional loss beyond that produced by the addition and removal of 85% PVS. Thus, it is clear that the vitrification itself is not harmful to the cells, but the addition and removal of vitrification solution can be. Toxicity and osmotic injury by the vitrification solution are influenced by the concentration, time and temperature, and subsequent dilution procedures. We controlled these factors, but the maximal survival rates(about 65% in cultured cells and about 50% in embryos) were not sufficiently high, probably due to procedures of dehydration by PVS. In our experiments, the survival rate varied considerably from 65 to 20% according to the growth stage of the cell culture. Thus, to improve the survival, the optimal cell growth stage (Sala et al. 1979) and more suitable procedures for vitrification must be determined.

Normally, when a liquid is quenched in LN and becomes a transparent solid, it is considered to have vitrified; the whitening that occurs during subsequent warming due to the growth of ice crystals, or devitrification, is considered to be the proof. Meryman(1958) demonstrated that a transparent solid could consist of ice crystals too small to diffract light so that the whitening would have to be interpreted as mostly crystallization. The most compelling evidence for an amorphous solid is a change in heat capacity, or glass transition during warming, of the sort presented by MacKenzie(1977). A 2-min exposure to -70°C after quick cooling in LN did not cause any decrease in survival, but a 60-min at -70°C or a 10-min at -50°C resulted in a remarkable decrease in the survival. And the survival rate decreased considerably between -60 and -40°C during slow rewarming. These results are interpreted as that a large number of intracellular fine ice crystals (innocuous intracellular ice) formed during devitrification can grow to a damaging size in the temperature zone of ice crystal growth, and that a rapid increase in the rate of crystal growth occurs between -60 and -40°C, as reported in very hardy cells by Sakai et al.(Sakai 1966; Sakai and Yoshida 1967; Sakai et al. 1968).

Many normal asparagus plants regenerated from vitrified somatic embryos. In somatic embryos of carrot (Withers 1979) and orange(Marin and Duran-Vila 1988) which were cooled to -196°C by a conventional freezing method, recovery was not due to the survival of a whole embryo, but due to the recovery of proliferating structures from surviving cells by secondary embryogenesis. In vitrified asparagus embryos, plantlets were developed from revived embryos(Fig.2) which is critical for germplasm preservation.

Thus, cryopreservation of somatic embryos by vitrification seems promising for plant germplasm preservation. Further studies are needed to establish more suitable and simple procedures for successful vitrification for a wide range materials.

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