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Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification

A. Sakai [1], S. Kobayashi [2], and I. Oiyama [2]

[1] Asabucho 1-5-23, Kitaku, Sapporo, Japan 001
[2] Akitu Branch, Fruit Tree Research Station, Akitu, Hiroshima, Japan 729-24

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Abstract. The nucellar cells of navel orange(Citrus sinensis Osb. var. brasiliensis Tanaka) were successfully cryopreserved by vitrification. In this method, cells were sufficiently dehydrated with highly concentrated cryoprotective solution(PVS2) prior to direct plunge in liquid nitrogen. The PVS2 contains(w/v) 30% glycerol, 15% ethylene glycol and 15% DMSO in Murashige-Tucker medium(MT) containing 0.15 M sucrose. Cells were treated with 60% PVS2 at 25°C for 5 min and then chilled PVS2 at 0°C for 3 min. The cell suspension of about 0.1 ml was loaded in a 0.5 ml transparent plastic straw and directly plunged in liquid nitrogen for 30 min. After rapid warming,the cell suspension was expelled in 2 ml of MT medium containing 1.2 M sucrose. The average rate of survival was about 80%. The vitrified cells regenerated plantlets. This method is very simple and the time required for cryopreservation is only about 10 min.

Key words: Cryopreservation, Vitrification, Nucellar cells , Navel orange, Citrus sinensis.

Abbreviations: DMSO, dimethyl sulfoxide; PVS2, vitrification solution; LN, liquid nitrogen; DSC, differential scanning calorimeter; BA, 6-benzylaminopurine; MT, Murashige-Tucker basal medium. NAA, naphthaleneacetic acid.

Introduction:

Cryopreservation of plant cells, meristems and organs has become an important tool for the long-term preservation of plant germplasm and experimental material without genetic alteration. However, cryopreservation of cultured cells and organs is still far from being a routine laboratory method. Simple and reliable techniques would allow a much more widespread use of frozen cells if they exhibited a sufficient survival comparable to that of cells frozen by conventional slow rate freezing.

Winter hardy mulberry cortical cells which were ultra-rapidly cooled by a direct immersion into liquid nitrogen from room temperature(cooling rate:10°C/min) remained alive without cryoprotectant when rewarmed rapidly(Sakai 1966,1986). 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 ultra-rapid cooling in LN(Sakai 1986, Uemura and Sakai 1980, Kartha et al 1982). Such approaches to avoide intracellular ice formation through the imposition of ultra-rapid cooling rates have not been adequate as a routine laboratory method. An alternative approach relies on the ability of highly concentrated aqueous solutions of cryoprotective agents to supercool to sufficiently low temperatures to achieve the vitreous state (Rall and Fahy 1985). At sufficiently low temperatures, highly concentrated aqueous solutions of cryoprotective agents become so viscous that they solidify into a metastable glass at a practical cooling rate, without the formation of ice, a process termed vitrification.

In a previous report(Sakai's group), cultured cells and somatic embryos of asparagus were first cryopreserved by vitrification (Uragami et al,1989). However, the survival rate was not acceptable, and the procedure was very complicated . Thus, to improve the survival and to develop a more simple and suitable vitrification procedure, the present research was carried out using new vitrification solution(PVS2) and nucellar cells of navel orange. The nucellar cells have a high ability to regenerate plants via embryogenesis and a high phenotypic stability(Kobayashi et al 1985, Kobayashi 1987). We intend to develop a simple and reliable procedure for cryopreservation applicable to a wide range of plant material.

Materials and Methods:

Callus derived from nucellar tissue of navel orange(Citrus sinensis Obs. var.

Offprint requests to: A. Sakai

brasiliensis Tanaka) was used for cryopreservation. For nucellar callus induction, ovules were excised from flower buds that were just about at the flowering stage and placed on the Murashige and Tucker(1969) basal medium containing 0.15M sucrose,supplemented with 10 mg/l BA, and 0.8% agar. The cultures were kept at 25°C under 16h/day illumination with a cool fluorescent light(25 $\mu Em^{-2}s^{-1}$). The nucellar callus has been maintained for 2 years by subculturing at 30 day intervals on the same medium. About 1g of nucellar callus was inoculated into 50 ml of liquid MT medium supplemented with 10 mg/l BA and cultured on a shaker(110 rpm) at 25°C under continuous light(25 $\mu Em^{-2}s^{-1}$). Serial transfer of the cells was done every two weeks.

Vitrification procedures:
As a result of our preliminary experiments with nucellar cells, a low-toxicity combination that allowed the contents of straw to remain transparent without ice crystallization during cooling and warming was adopted as vitrification solution (designated PVS2). This contains 30%(w/v) glycerol, 15%(w/v) ethylene glycol and 15%(w/v) DMSO in MT medium containing 0.15 M sucrose. An 8 to 10 day-old cultured cell suspension was placed in a spitz tube(15 mm diameter,110 mm long). After removing the supernatant, 1 ml of 60% concentration of PVS2 which was diluted with MT medium containing 0.4 M sucrose was added to the packed cells of 1 ml at 25°C and replaced twice with 60% PVS2 and held for 5 min. After removing the 60% PVS2, fresh prechilled PVS2 was added and replaced twice and held at 0°C for 3 min. Then the cell suspension(0.1 ml) was loaded in a 0.5 ml transparent plastic straw and the top end of each straw was sealed by a heat sealer. The straws were plunged directly into LN and stored for 30 min. The mean cooling rate was about 1800°C/min between -30 and -160°C. Straws were then warmed rapidly in a water bath at 25°C. After cutting the ends of each straw, the cell suspension was expelled in 2 ml of a diluting solution containing 1.2 M sucrose in MT medium at 25°C.

Viability Test and Regrowth:
The rewarmed cells were used for assessing the viability and regrowth capacity . The rate of viability of vitrified cells was measured with the controls by means of double staining with FDA and phenosafranin(Widholm 1972). About 100 to 200 cell clusters(20-50 cells/cluster) were observed for their viability for each treatment. The survival of vitrified cells was expressed as per cent survival over the nontreated, unfrozen control. To assess regrowth capacity, samples of 0.5 ml cell suspension were placed over 2 pieces of sterile filter paper(5 cm in diameter) which were placed over 20 ml MT medium containing 5 mg/l BA and 0.8% agar in a Petri dish(8 cm in diameter). After 4 to 5 h, filter papers with cells were transferred to other Petri dishes containing the same medium(Chen et al 1984). The dishes were then sealed with Parafilm strips. They were incubated in a growth chamber at 25°C, and 16 h photoperiod(25 $\mu Em^{-2}s^{-1}$). The weight of wet filter paper plus cells was measured during the growth period of about 25 days.

DSC Measurement:
For differential scanning calorimeter (DSC) measurements, PVS2 solution of 5 to 6 mg was placed in sealable aluminum pans of a DSC(Perkin-Elmer DSC-7, Norwalk,Conn.), weighed, cooled at 80°C/min and warmed at 10°C/min.

Results:

Effect of cryoprotective treatments on the survival of cells cooled to -196°C by vitrification was compared. As shown in Table 1, the highest survival was obtained in the cells treated with 60% PVS2 at 25°C for 5 min and subsequently treated with chilled PVS2 for 3 min. Initially cryoprotected cells with 12% ethylene glycol for 5 min at 25°C were directly exposed to chilled 8M ethylene glycol solution dissolved in MT-medium containing 0.4M sucrose. Limitted survival was observed in these cells following immersion into LN.

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

Cryoprotective treatment	Survival(%)
50%PVS(5min,25°C) ---PVS(0°C,5min)	65.4±1.41
60%PVS(5min,25°C) ---PVS(0°C,3min)	83.5±0.85
PVS(5min,25°C) ---PVS(0°C,5min)	49.1±1.60
PVS(10min,0°C) ---PVS(0°C,5min)	18.1±0.80
12%EG(5min,25°C) ---8MEG(0C,5min)	3.7±0.10

After cryoprotection, cell suspensions loaded in straws were plunged in LN and held there for 30 min before being rewarmed in a water bath at 25°C. In each treatment, two straws were used and about 100 cell clusters in each straw were examined for their viability. EG:ethylene glycol dissolved in MT-medium containing 0.4M sucrose.

To test the effect of devitrification on cell survival, vitrified cells in LN were then rapidly transferred into a bath held at -70 or -50°C at which devitrification proceeds. And the treated cells with PVS2 were supercooled in a bath held at -70 °C. As shown in Table 2, the vitrified cells held at -70°C for 20 min caused serious damage, indicating that devitrification proceeded at -70 or -50 °C, respectively. However, no decrease in survival was observed in the cells supercooled at -70°C for 10 min.

The thermal behavior of PVS2 was observed during cooling and warming in a differential scanning calorimeter. Representative thermograms during cooling(80°C/min) and warming(10°C/min) are presented in Fig.1. During cooling at 80°C/min, no freezing exotherm was observed, but a glass

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]	63.5±0.99
Vitrified cells[b]	60.9±1.33
Experiment 2	
Supercooled at -70°C[c]	63.3±1.29
-70°C for 20 min[d]	7.5±0.10
-50°C for 20 min[d]	2.0±0.40

a: Cell suspension was treated with PVS2 and diluted without cooling to -196°C; b: Vitrified cells were rewarmed directly in a bath at 25°C; c: Treated cells with PVS2 were supercooled in a bath held at -70°C for 10 min. d: Vitrified cells were rapidly transferred to a bath at -70 or -50°C and held there for 20 min before being rewarmed in a bath held at 25°C.

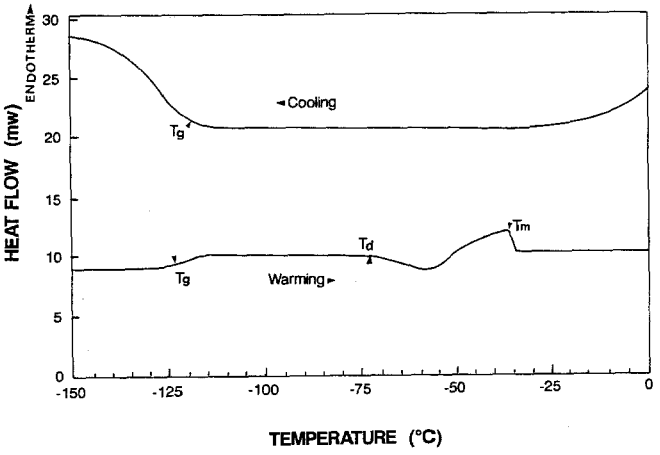

Fig.1. DSC record of PVS2 in MT-medium containing 0.15M sucrose.
Tg: temperature of the glass transition(-115°C); Td: temperature of the devitrification (-75°C); Tm: melting point(-36°C).

transition (T_g) was observed at about -115°C(Fig.1). On warming(10°C/min) the glass transition was observed at nealy the same temperature. The metastable solution initiated devitrification at about -75°C and showed a maximum exotherm at -60°C due to crystallization. The ice began to melt about -50°C in equilibrium with the concentrated solution and melting point was -36°C.

The nucellar cells were treated with 50% PVS2 for 5 min at 25°C and subsequently treated with chilled PVS2 for 5 min prior to plunge in LN. The survival rate was 61%(refer Table 1). The vitrified cells were grown on filter paper discs over agar medium. The control resumed growth 2 days after platings, but the regrowth rates of treated and vitrified cells were lower than that of control until about two weeks after plating, then followed a similar pattern as the control(Fig.2). The vitrified cells regenerated plantlets via embryogenesis.

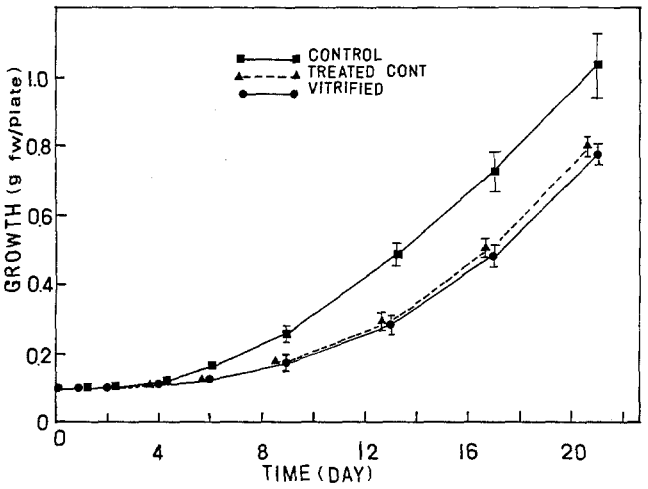

Fig.2 Recovery growth of vitrified nucellar cells.
Samples of 0.5 ml cell suspension were placed over 2 pieces of sterile filter paper which were placed over 20 ml agar culture medium in a Petri dish. After 4 h, filter papers with cells were transferred to other Petri dish containing the same culture medium. The weight of wet filter paper plus cells was measured for 25 days. Control: cultured without any treatment; Vitrified: treated with 50% PVS2 at 25°C for 5 min and then chilled PVS2 at 0°C for 5 min prior to plunge in LN(survival:65%,refer Table 1); Treated control: same as vitrified cells, without cooling to LN. The SE are shown as vertical bars.

Discussion:

The survival rates of nucellar cells after addition and removal of PVS2 , without cooling to -196°C, was 63% of the untreated control. The survival of cells cryopreserved by vitrification was %, indicating that the actual vitrification did not cause additional loss beyond that produced by the addition and removal of PVS2. Little or no decrease was observed in the supercooled cells at -70°C for 20 min. However, a 20-min holding at -70°C after quick cooling in LN resulted in a remarkable decrease in the survival. Also, a noticeable decrease was observed in the survival of vitrified cells between -70 and -40 °C during slow rewarming(Uragami et al 1989). These results indicate that devitrification (crystallization) proceeds in the vitrified cells and causes cell death in the slow rewarming process as reported for very hardy cells (Sakai 1966, Sakai et al 1968, Sakai 1988).

Careful control of the procedures used to expose cells to vitrification solution is necessary to produce the necessary degree of cytoplasmic dehydration and to prevent injury due to chemical toxicity or excessive osmotic stresses during equilibration and dilution. Although some permeation appears necessary for successful cryopreservation by vitrification, excess permeation increases the injury due to chemical toxicity.

A stepwise equilibration procedure is used in animal cells and embryos(Rall and Fahy

1985, Takahashi et al 1986). First, the cells and embryos are treated with 25% VS1 at 20°C and sufficient time is allowed for the cryoprotectants to permeate into cytoplasm. Then the cells and embryos are transferred into 50 and 100% VS in two steps at 4°C. However, a high level of survival was not obtained with such a stepwise method in plant cultured cells(Uragami et al 1989). Thus, we adopted a new vitrification solution(PVS2) suitable to nucellar cells and employed simple procedures for dehydration and dilution. Our strategy controls the extent of cryoprotectant permeation and proceeds the necessary cytoplasmic dehydration by exposing cells to 60% PVS2 at 25°C for 5 min, followed by exposure to chilled PVS2 for 3 min prior to plunge in LN. This enabled cells to survive at a high level of approximately 80 %. The main advantages of this vitrification method are very simple and the time required for the procedure is about 10 min. This procedure eliminates the needs for controlled freezing and for equilibration time in cryoprotectants.

The nucellar cells cryopreserved by both conventional slow freezing method(survival rate:73%) and vitrification maintained an embryogenic potential identical to that of unfrozen controls. Plants regenerated via embryogenesis from these cryopreserved cells were morphological uniform and had characteristics of navel orange(Kobayashi et al unpublished).

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