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CRYOPRESERVATION OF PLANT GENETIC RESOURCES

TECHNICAL ASSISTANCE ACTIVITIES FOR GENETIC RESOURCES PROJECTS

JAPAN INTERNATIONAL COOPERATION AGENCY

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ABBREVIATIONS

ABA	Abscisic acid
BA	Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
DMSO	Dimethyl sulfoxide
DSC	Differntial scanning calorimeter
EG	Ethylene glycol
FDA	Fluorescein diacetate
GA3	Gibberellic acid
IBA	Indole-3-butyric acid
LN	Liquid nitrogen
NAA	α-Naphtalene acetic acid
PG	Propylene glycol
PVS1	Vitrification solution containing 22%(w/v) glycerol, 13%(w/v) propylene glycol, 13%(w/v) ethylene glycol and 6%(w/v) DMSO in basal medium
PVS2	Vitrification solution containing 30%(w/v) glycerol, 15%(w/v) ethylene glycol and 15%(w/v)DMSO in basal medium
PVS3	Vitrification solution containing 50%(w/v) glycerol and 50%(w/v) sucrose in basal medium
SHC	Sodium Hypochloride
Td	Devitrification temperature
Tg	Glass transition temperature
Th	Homogeneous nucleation temperature
Tm	Melting temperature

COMPOSITION OF PLANT CULTURE MEDIA

Constituents	Media (amount in mg l^{-1})			
	MT[a]	MS[b]	B5[c]	NLN[d]
Inorganic				
NH_4NO_3	1,650	1,650	–	–
KNO_3	1,900	1,900	2,527.5	125
$CaCl_2 \cdot 2H_2O$	440	440	150	–
$MgSO_4 \cdot 7H_2O$	370	370	246.5	125
KH_2PO_4	170	170	–	125
$(NH_4)_2SO_4$	–	–	134	–
$NaH_2PO_4 \cdot H_2O$	–	–	150	–
$Ca(NO_3)_2 \cdot 4H_2O$	–	–	–	500
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8	27.8
$Na_2EDTA \cdot 2H_2O$	37.3	37.3	37.3	37.3
$MnSO_4 \cdot 4H_2O$	22.3	22.3	–	25.0
$MnSO_4 \cdot H_2O$	–	–	10.0	–
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	2.0	10.0
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.025
$CoSO_4 \cdot 6H_2O$	–	–	–	0.025
KI	0.83	0.83	0.75	–
H_3BO_3	6.2	6.3	3.0	10.0
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25
Organic				
Myo-inositol	100	100	100	100
Thiamine-HCl	10.0	0.1	10.0	0.25
Nicotinic acid	5.0	0.5	1.0	5.0
Pyridoxine-HCl	10.0	0.5	1.0	0.5
Biotin	–	–	–	0.05
Folic acid	–	–	–	0.5
Glycine	2.0	2.0	–	2.0
Glutamine	–	–	–	800
Serine	–	–	–	100
Glutachione	–	–	–	30
Sucrose	5%	3%	2%	13%

[a] Murashige and Skoog (1962)
[b] Murashige and Tucker (1969)
[c] Gamborg *et al.* (1968)
[d] Polsoni *et al.* (1988)

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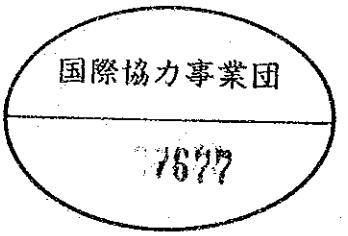

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Introduction
(Cryopreservation of Plant Genetic Resources in Japan)

Tissue culture methods are commonly used for mass propagation of a wide variety of plant species. Meristem culture technique is being used not only for clonal propagation, but also for the supply of virus-free plants. Furthermore, the progenies regenerated by *in vitro* culture of meristems have, so far, displayed a genetic stability to a larger extent compared to other methods of *in vitro* plant regeneration. A possible exception would be the stability attained through somatic embryogenesis. These unique attributes make plant meristems ideal candidates for use for the preservation of germplasm.

Germplasm preservation includes short- to medium- term preservation and long-term preservation by cryogenic methods. *In vitro* storage of plant materials, reduced growth or minimum conditions of growth have been achieved in several ways. The advantages of using *in vitro* storage techniques are considerable, especially for the germplasm of vegetatively propagated woody plants. In spite of the advantages, *in vitro* storage methods can only fulfill short- to medium- term objectives. Problems arising from the loss of vigor of plantlets, progressive changes of the genome, microbial contamination, and the labor required for maintaining valuable *in vitro* collections cannot be ignored.

Embryogenic cell cultures are of considerable importance since they are currently a major source of totipotent cells and protoplasts for genetic transformation, as well as for efficient germplasm conservation without undergoing genetic changes. Embryogenic cell cultures are maintained and multiplied by serial subculture, a process which requires time and labor. Thus, as it is essential to develop techniques available for long-term preservation of germplasm, in recent years, attention has been focused on the application of cryogenic techniques.

The technique of cryopreservation, in which cells and meristems are frozen under controlled conditions and stored in liquid nitrogen is a reliable method for long-term preservation of germplasm. Cryopreservation is based on the reduction and subsequent interruption of metabolic functions of biological materials by the decrease of the temperature to the level of that of liquid nitrogen (−196℃), while viability is maintained. At the temperature of liquid nitrogen almost all the metabolic activities of the cells are at a standstill and the cells can be preserved in such a state for a long period of time. Bull spermatozoa

have been successfully stored in LN for 20 years without any decrease in the survival and fertilization ability (Shiroyama and Iritani, 1987).

Cryopreservation of plant cells and meristems has become an important method for the long-term preservation of germplasm since it requires minimum space and maintenance and genetic alterations do not occur. However, the availability or development of efficient and reliable protocols for regeneration of plants from cryopreserved cells or meristems is one of the basic requirements prior to cryopreservation of cultured plant cells and meristems. This consideration may obviously narrow the choice of the plant materials currently suitable for cryopreservation unless efforts are made to include many other plant species. Successful cryopreservation has generally been achieved by slow prefreezing to about −40℃ prior to immersion into LN in the presence of suitable cryoprotectants. This conventional slow freezing method which is basically the major protocol, requires a controlled freezing equipment and complicated cryoprotective procedures. Thus, the development of a simple and reliable method for cryopreservation would allow for wider use of cryopreserved cells, meristems and embryos. Recently, some simple and reliable protocols, especially vitrification methods have been developed, and citrus (nucellar callus), mulberry (winter buds and *in vitro*-grown shoots), pear and apple (*in vitro*-grown shoots), white clover (shoot tips and meristemoids), asparagus (embryogenic cells), garlic and tea plant (*in vitro* shoot tips) have been successfully cryopreserved by vitrification in Japan.

In this report, we describe some efficient cryogenic protocols and successful cryopreservation methods for cultured cells, meristems and embryos developed in Japan in recent few years.

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Cryogenic Strategies for Survival of Plant Cultured Cells and Meristems Cooled to − 196°C

by

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1. Introduction

For successful cryopreservation, it is necessary to avoid lethal intracellular freezing, which occurs during rapid cooling in LN. Thus, cells and meristems have to be sufficiently dehydrated or concentrated before being immersed into LN. Extracellular freezing is an effective method of dehydration of hardy cells. Sakai (1956) first demonstrated that winter hardy tissues or twigs prefrozen at −30°C remained alive after immersion into LN even when thawed slowly in the air. Winter willow twigs prefrozen at −30°C were successfully cryopreserved in LN for 1 year and subsequently developed shoots and roots (Sakai, 1960). This was the first experiment carried out for the cryopreservation of non-dehydrated plant materials in LN. This method was successfully applied to very hardy woody plants (Sakai, 1965 a,b), fruit buds (Sakai and Nishiyama, 1978; Tyler and Stushnoff, 1988; Suzuki *et al.*, 1988; Oka *et al.*, 1991), and mulberry buds (Yakuwa and Oka, 1988; Niino *et al.*, 1992 a).

This prefreezing method is also widely used as a routine method for cryopreservation of less hardy cultured cells and meristems, with prior application of cryoprotectants. Conventional cryopreservation of cultured cells and meristems has been achieved by slow prefreezing to −40°C in the presence of suitable cryoprotectants (Sugawara and Sakai, 1974; Uemura and Sakai, 1980; Sakai, 1985; Kobayashi *et al.*, 1990).

Recent work has focused on the procedures that would eliminate the need for controlled freezing and enable cells and meristems to be cryopreserved by direct transfer into LN. An approach, called vitrification, enables cells and meristems to be cooled to −196°C without ice formation. Vitrification refers to the physical process by which a highly concentrated solution is supercooled to very low temperatures and finally solidifies into metastable glass without crystallization. Vitrification has long been proposed as a method for the cryopreservation of biological materials, because the potentially detrimental effects of extracellular and intracellular freezing might be avoided in this way (Luyet, 1937). Successful cryopreservation procedures by either partial (cytoplasm alone) or complete vitrification (both cytoplasm and suspending solution) at a practical cooling rate can be divided into four different categories based on the dehydration method prior to rapid cooling into LN (Fig. 1).

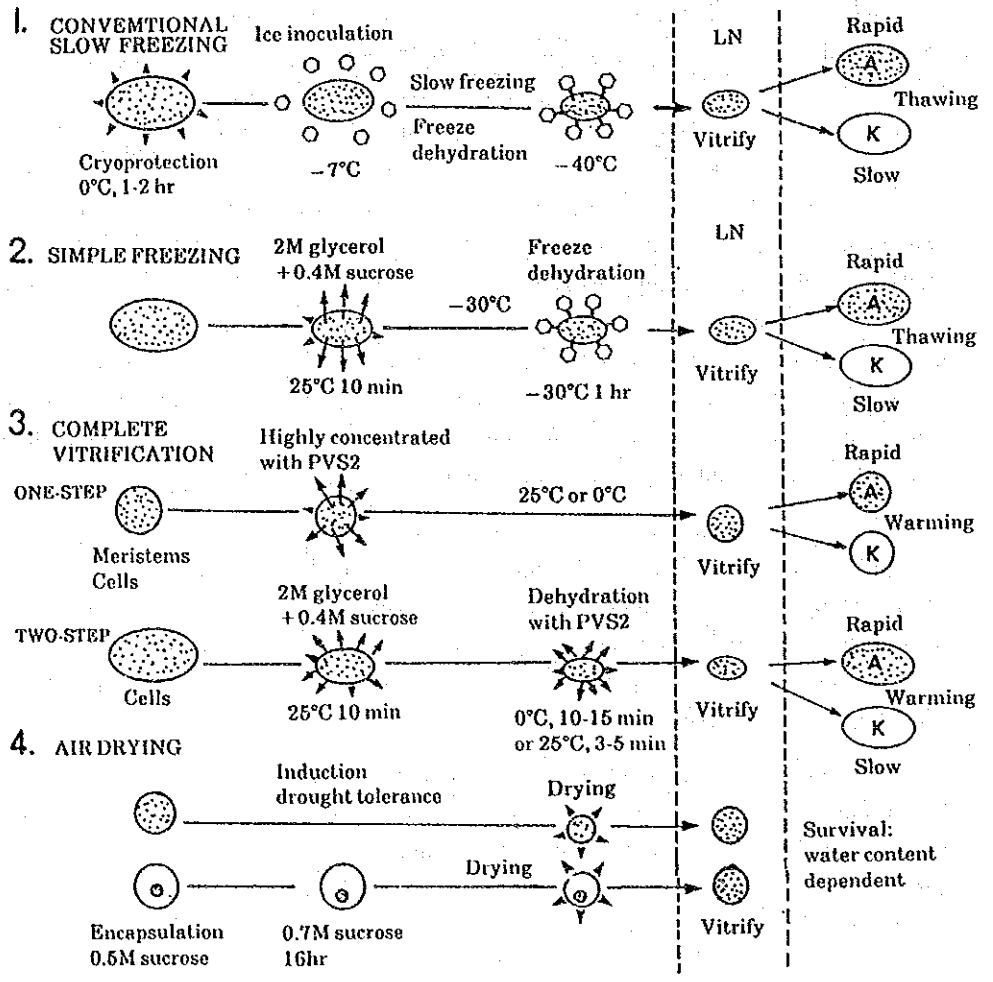

Fig. 1 Cryogenic strategies for survival of cultured cells and meristerms cooled to −196°C.
A: alive; K: killed.

2. Crystallization and Vitrification

Ice formation is the passage of water from the random arrangement of a liquid state to an ordered one. Many liquids, including water, do not invariably freeze at the melting point of the solid phase. Such liquids can be supercooled to several degrees below the melting point of the solid phase and will freeze only

upon the spontaneous formation of, or addition of, a substance that acts as a catalyst for the liquid-solid phase transition. Catalysts for the water-ice phase transition are referred to as ice nuclei. There are two types of ice nucleation: homogeneous and heterogeneous nucleation. In homogeneous nucleation, the nuclei are formed spontaneously in the liquid without intervention of foreign bodies at a very low temperature approaching −40°C. The freezing process involves two different phenomena, formation of ice nuclei and growth of crystal units.

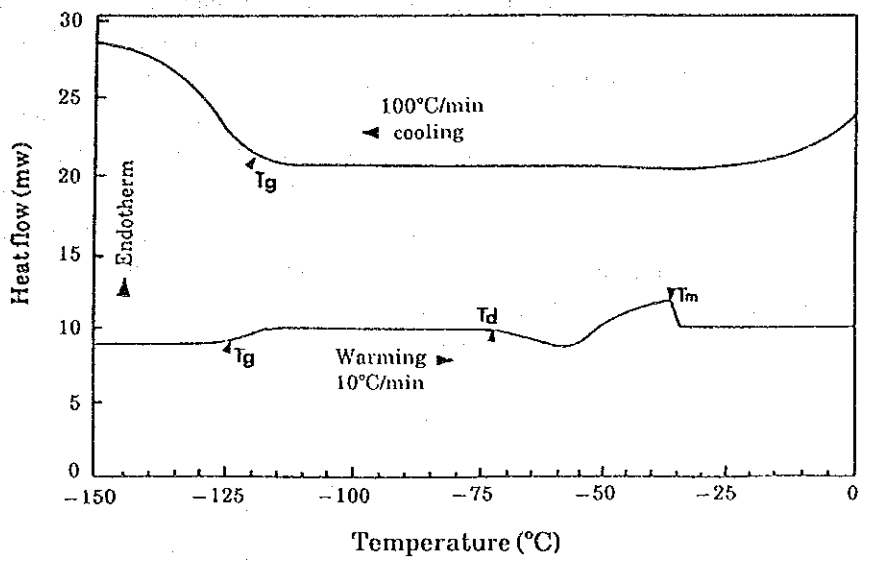

Fig. 2 DSC record of PVS2 in MT-medium containing 0.15 M sucrose.
Tg, about −115°C; Td, about −75°C); Tm, about −36°C (Sakai *et al.*, 1990).

As shown in Fig. 2 (DSC record), a highly concentrated vitrification solution (PVS2) supercooled below −70°C and finally became vitrified at about −115°C (Tg), and glass transition occurred during subsequent slow warming followed by exothermic devitrification (crystallization)(Td) and endothermic melting (Tm). The crystallization during the warming process can be prevented if warming occurs rapidly. If the vitrification solution is more concentrated, the Tg shifts to a higher temperature and Tm to a lower temperature. The basic concept of vitrification is best described by referring to a supplemented phase diagram such as the generalized one presented by Fahy *et al.* (1984) (Fig. 3). Tm corresponds to the equilibrium freezing or melting point curve. Solutions normally supercooled in the region between Tm and Th (the homogeneous

nucleation temperature), before they actually begin to freeze by heterogeneous nucleation, as represented by x in Fig. 3. Tg is the glass transition temperature, at which supercooled liquid becomes vitrified. And finally, Td corresponds to the devitrification curve, at which the previously vitrified solution freezes upon slow warming. There are certain rather well-defined regions in the phase diagram in which different types of vitrification behavior appear. In the relatively dilute Region I, vitrification cannot occur because both heterogeneous and homogeneous nucleation are unavoidable. In the more concentrated Region II, both types of nucleation are inhibited. However, glass formed in this range is unstable. At still higher concentrations (Region III), Th becomes equal to Tg and the value actually falls below that of Tg. In this region it is possible to slowly cool even bulk liquids directly to reach to the Tg value without experiencing any detectable freezing events. The intersection between the Th curve and Tg curve indicates therefore the threshold or lowest possible concentration of cryoprotectant that might be used for vitrification. In this region, based on the Td curve a vitrified sample should be rewarmed rapidly enough to prevent crystallization. Finally, in Region IV, devitrification is prevented, and the system is virtually stable (Fahy *et al.*, 1984).

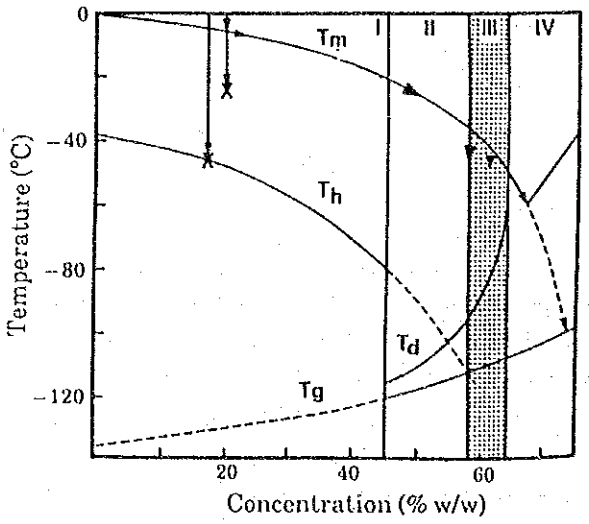

Fig. 3 Supplemented phase diagram of a hypothetical cryoprotectant.
Tm: equilibrium freezing or melting curve; Th: homogeneous nucleation temperature; Tg: glass transition curve; Td: devitrification curve (Fahy *et al.*, 1984; with permission).

Glass fills spaces in a tissue, and during dehydration may contribute to stop the prevention of additional tissue collapse, solute concentration, and pH alterations. Operationally, glass is expected to exhibit a lower water vapor pressure than the corresponding crystalline solid and thereby prevent further dehydration. As glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation may lead to dormancy and stability over time (Burke, 1986).

3. Conventional Slow Prefreezing Method

Cultured cells or meristems in the presence of a suitable cryoprotectant are slowly prefrozen to about −40°C prior to being immersed into LN (Sugawara and Sakai, 1974; Uemura and Sakai, 1980) (Fig. 4). Slow freezing to about −40°C results in sufficient concentration of the unfrozen fraction of the suspending solution and of the cytosol to enable vitrification upon rapid cooling into LN (Fig. 1,1). In the slow freezing method, the cells frozen extracellularly tend to follow the Tm curve and reach the threshold concentration for vitrification (Region III in Fig. 3) before being immersed into LN.

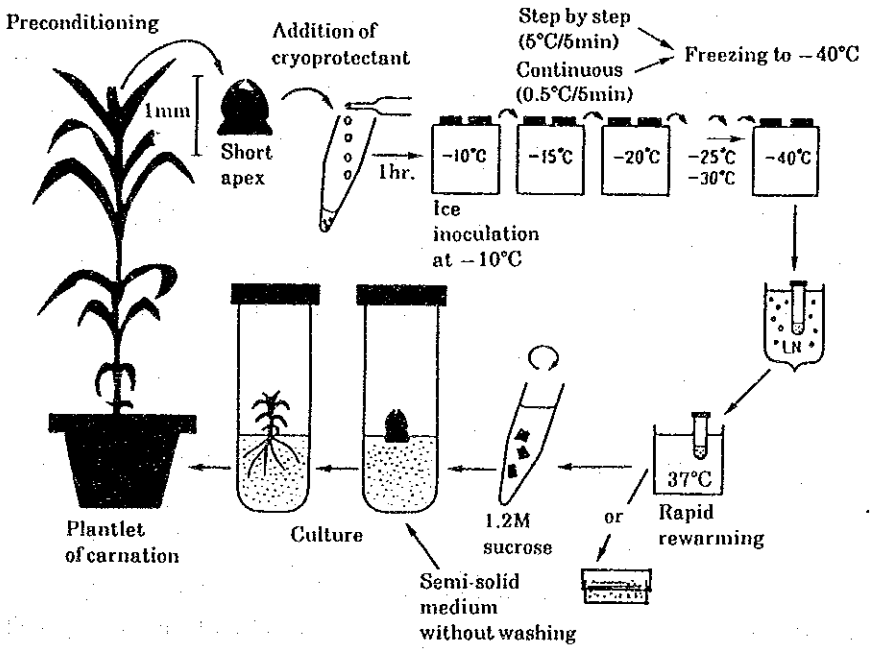

Fig. 4. Procedure for cryopreservation by slow prefreezing method (Uemura and Sakai, 1980; modified).

Conventional slow prefreezing method is still important for the cryopreservation of cultured cells (Van Iren *et al.*, 1991; Dussert *et al.*, 1991; Ogawa *et al.*, 1991; Fukai, 1991). However, this method which requires a controlled freezing equipment and complicated cryoprotective procedures is time-consuming and laborious.

4. Simple Freezing Method

Alternative cryogenic strategies reduce or eliminate the need for cellular dehydration during slow freezing by osmotically dehydrating cells or meristems prior to direct transfer to a freezer at −30°C (simple freezing method) (Fig. 1, 2, Fig. 5).

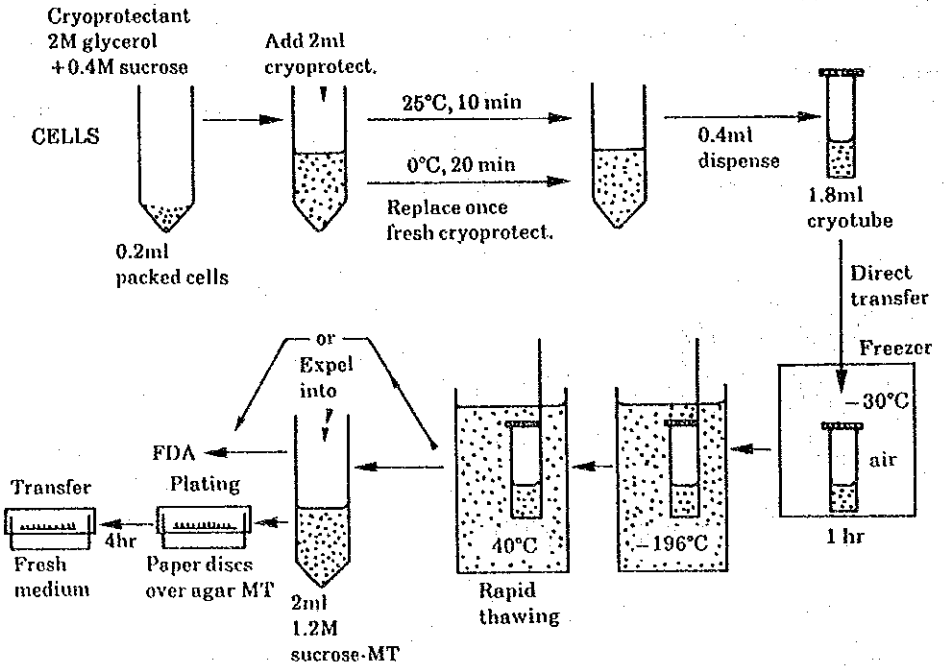

Fig. 5 Procedure for cryopreservation by simple freezing method (Sakai *et al.*, 1991a).

The cryoprotective effect in this method was compared for 2 or 3 M solutions of various solutes dissolved in the culture medium supplemented with 0.4 M sucrose. As shown in Fig. 6, the survival rate was highest for the cells treated with 2 M glycerol and 0.4 M sucrose at 25 °C for 10 min. All the

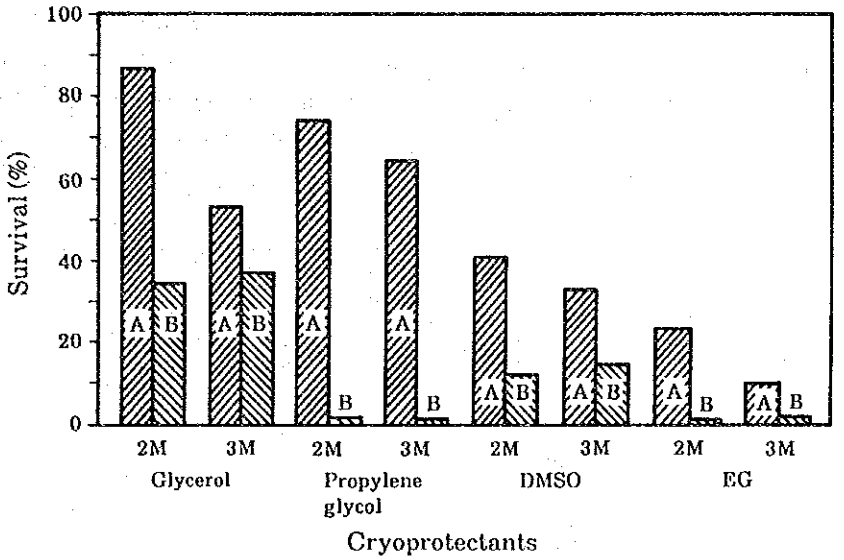

Fig. 6 Effect of cryoprotectants on the survival of cells cooled to −196°C after prefreezing at −30°C.

Cryoprotection was compared for different solutes dissolved in a medium supplemented with (A) or without (B) 0.4 M sucrose at 25°C for 10 min. Cryoprotected cells were directly transferred to a freezer at −30°C for 1 hr prior to immersion into LN. Material: Asparagus embryogenic cells (4-day-old culture) (Nishizawa *et al.*, 1992).

cryoprotectants tested led to very low survival rates when the cells were frozen without sucrose (Sakai *et al.*, 1991 a; Nishizawa *et al.*, 1992).

Effect of the terminal freezing temperature prior to immersion into LN on survival was examined. Cryoprotected cells were prefrozen by direct transfer to freezers at −15, −20, −30 and −40°C, respectively and maintained there for 1 hr prior to immersion into LN. Prefrozen cells at −30°C showed the highest survival rate (Fig. 7). The prefrozen cells at −40 °C by direct transfer from 25°C showed a very low survival rate before or after immersion into LN. However, the prefrozen cells at −30°C which were additionally frozen at −40°C for 1 hr showed a high survival rate(Fig. 7). It was also observed that the cells cooled slowly to −30 or −40°C at 0.5°C min^{-1} using a programmable freezer showed nearly the same high rate of survival as those frozen in a freezer at −30°C. These results indicates that osmotic dehydration by a mixture containing 2 M glycerol and 0.4 M sucrose for 10 min at 25°C prior to freezing at −30°C led to

high rates of survival and the simplification of complex cryoprotective and freezing procedures (Sakai *et al.*, 1991 a; Nishizawa *et al.*, 1992). This simple freezing method does not require a controlled freezing equipment and ice inoculation to extracellular medium. The temperature of −30°C corresponds to that of ordinary freezers. This method has been successfully applied to cultured cells of several plants such as orange, rice, tobacco, carrot, asparagus, and meristemoids of white clover.

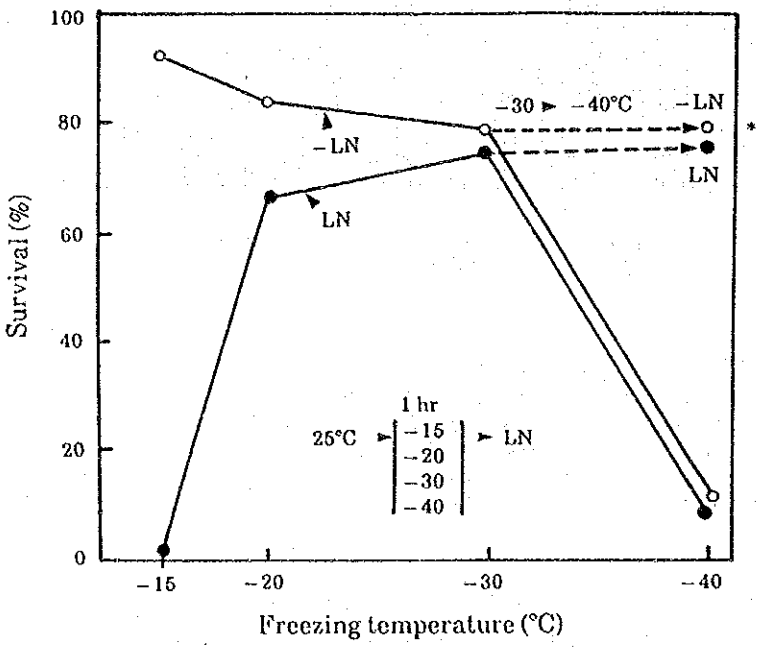

Fig. 7 Effect of the terminal freezing temperature on the survival of cells cooled to − 196°C by simple freezing method.

Cryoprotected cells were frozen by direct transfer to freezers at −15, −20, −30 and −40°C, respectively. These frozen cells were thawed rapidly before or after being immersed into LN.

* Prefrozen cells were additionally frozen in a freezer at −40°C for 1 hr prior to immersion into LN. Cryoprotection: 2 M glycerol + 0.4 M sucrose at 25°C for 10 min. Material: Asparagus embryogenic cells (4-day-old culture) (Nishizawa *et al.*, 1992).

A mixture of 2 M glycerol and 0.4 M sucrose yields a highly concentrated cryoprotective solution composed of about 50% (w/v) glycerol (Luyet and Gehenio, 1952) and 50% (w/v) sucrose due to freezing at −30°C. More recently

we have found that this highly concentrated solution (designated as PVS3) was suitable for the vitrification solution of cultured cells.

5. Vitrification Method

A unicellular cortical section tissue (about 20 μm thick, 1×2 mm) sliced from a mulberry winter twig held with forceps was directly immersed into LN from the room temperature (cooling rate: 2×10^5 °C min^{-1}).

The ultrarapidly cooled cells (water content: 60%) remained alive when rewarmed rapidly (Sakai, 1956; 1966), indicating that very hardy cortical cells can become vitrified by ultra-rapid cooling. Recently, Fujikawa (personal communication) recomfirmed these findings and gave definite evidence for vitrification of freeze-fractured samples.

However, the ultrarapid cooling method can not be applied to less hardy cells, because such cells easily freeze intracellularly during rapid cooling. Even very hardy cells did not survive after ultra-rapid cooling when suspended in 0.03 ml water which easily induces intracellular freezing without prefreezing at −5 to −10°C (Sakai, 1966; Sakai and Yoshida, 1967). It was also demonstrated that very hardy winter twigs (10 cm long, 0.5 cm in diameter) partially prefrozen at −10°C easily vitrified upon rapid cooling in LN and developed shoots and roots when rewarmed rapidly in water at 37°C (Sakai, 1965 a,b; Sakai, 1973). Hirsh *et al.* (1985) provided strong evidence that winter hardy poplar cells can withstand freezing stress below −28°C by glass formation.

The addition of a cryoprotective solute is very effective in reducing the rate at which a solution must be cooled to avoid nucleation and the growth of ice nuclei. Very hardy mulberry cortical tissue suspended in 3 M EG solution (0.01 ml) was successfully vitrified when cooled ultrarapidly in LN (Sakai, 1958). However, a larger sample volume associated with cell suspensions (0.5 to 1.0 ml) does not allow for cooling at rates high enough to yield vitrification. A possible approach for the complete vitrification of less hardy cultured cells and meristems at practical cooling rates may involve extensive dehydration using a highly concentrated cryoprotective solution prior to rapid cooling into LN (Fahy *et al.*, 1984).

In the vitrification method, cells or meristems must be sufficiently dehydrated with a vitrification solution at about 20°C or 0°C to avoid experiencing injury prior to immersion into LN (Fig. 1, 3). We used a glycerol-

based, less toxic vitrification solution designated as PVS2 (Sakai and Kobayashi, 1990; Sakai *et al.*, 1990). For successful cryopreservation by complete vitrification it is necessary to carefully control the procedures for dehydration and cryoprotectant permeation and to prevent injury by chemical toxicity or excessive osmotic stresses during dehydration. To achieve this objective, a complicated equilibration procedure in two or three steps at about 20°C or 0°C is used (Takahashi *et al.*, 1986; Langis *et al.*, 1989; Uragami *et al.*, 1989). The one-step procedure for vitrification developed by Sakai *et al.* (1990; 1991 b) simplified the procedure and decreased the time required. This vitrification method using PVS2 was successfully applied to the cryopreservation of nucellar cells of navel orange and other three citrus plants, apical meristems of white clover (Yamada *et al.*, 1991 b), apple and pear (Niino *et al.*, 1992 b), mulberry (Niino *et al.*, 1992 c), tea plant (Kuranuki and Sakai, 1992), *Ribes* (Reed, 1992), garlic (Niwata, 1992), and multiple bud clusters of asparagus (Kohmura *et al.*, 1992) and chrysanthemum (Kohmura, 1993). Successful vitrification was also reported in the meristems of other plants: carnation (Langis *et al.*, 1990), mint (Towill, 1990), potato (Schnabel-Preikstas *et al.*, 1992 a), chrysanthemum (Schnabel-Preikstas *et al.*, 1992 b) and sweet potato (Schnabel-Preikstas *et al.*, 1991 c; Towill and Jarret, 1992).

Evidence for vitrification requires the use of physical procedures. One conventional method is to measure the latent heat released by the crystallization of ice during cooling and warming by DSC. The mulberry apical meristems treated with PVS2 were cooled to -150°C and then warmed at 10°C min^{-1}. During cooling, no freezing exotherm did occur. During subsequent warming (Fig. 8), a series of changes in the thermal behavior of the vitreous solid such as glass transition, exothermic devitrification and endothermic melting was observed (Niino *et al.*, 1992 c). These results indicate that the meristems that were sufficiently dehydrated with PVS2 became vitrified during rapid cooling.

It is particularly important that cryopreserved meristems be capable of producing plants identical with the non-treated phenotype. Haskins and Kartha (1980) demonstrated in frozen-thawed pea meristems by conventional freezing methods that the resumption of growth which usually occurred from tissues other than that of the original meristem dome led to differentiation and whole plant regeneration. Recovery of proliferated structures from surviving cells by secondary embryogenesis or adventitious buds was also reported. A callus phase prior to shoot formation is undesirable since callusing potentially increases the frequency of genetic variants. However, successfully vitrified and warmed

meristems generally produced direct shoot formation in many materials tested (Yamada, 1991 b; Niino *et al.*, 1992 a,b.; Langis *et al.*, 1990; Schnabel-Preikstas *et al.*, 1992 a,b). Thus, vitrification certainly offers a considerable advantage over the conventional freezing methods for the cryopreservation of meristems.

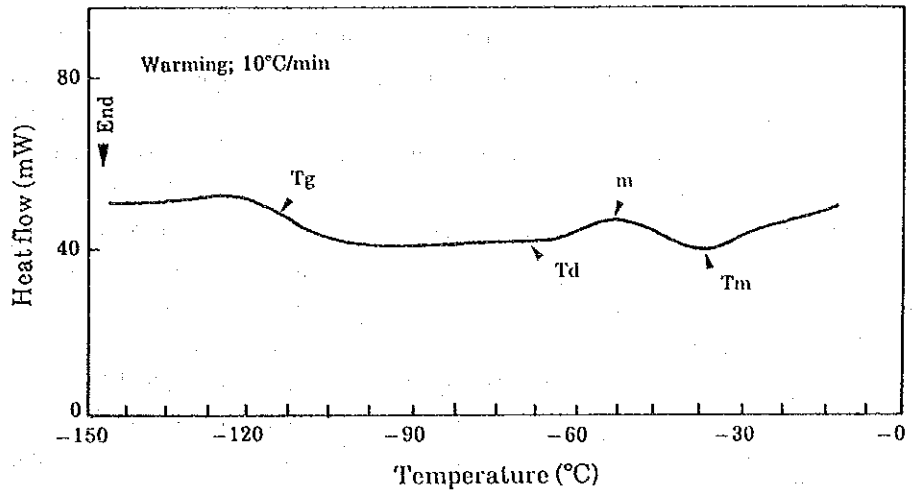

Fig. 8 DSC record of mulberry shoot tips treated with PVS2 at 25°C for 90 min (warming process).
After the surface solution was wiped off, the shoot tips were cooled at 5°C min^{-1} to −130°C and then warmed at 10°C min^{-1} to −10°C. Glass transition temperature: about −109°C (Niino *et al.*, 1992 c).

Preculture and/or cold-hardening (Reed, 1991), post-thawing handling (Chen *et al.*, 1984) and appropriate procedures with a suitable vitrification solution are essential for successful vitrification. A high level of sugar (Dereuddre *et al.*, 1988; Niino *et al.*, 1992 a,b) or sorbitol (Chen *et al.*, 1984; Yamada *et al.*, 1991 a,b) was reported to be very important for improving the survival of cryopreserved cells and meristems. However, the vitrification method did not result in high levels of survival for the majority of cultured plant cells except for a limited number of species or cultivars (Langis *et al.*, 1989; Uragami, 1991; Sakai *et al.*, 1990; Sakai, 1991 a, b). These observations suggest that direct exposure of cultured cells to a highly concentrated vitrification solution may lead to harmful effects due to osmotic stress or chemical toxicity. Thus, to improve the survival after dehydration, cultured cells were treated with various cryoprotectants before being dehydrated with PVS2 or PVS3. As shown in Table 1, the use of a mixture of 2 M glycerol and 0.4 M sucrose resulted in the

highest survival rate of vitrified asparagus cells cooled to −196°C. The use of other cryoprotectants resulted in considerably lower survival rates. Thus, recently we have developed a two-step vitrification method for cultured plant cells. Embryogenic cells were treated with a mixture of 2 M glycerol and 0.4 M sucrose for 10 min at 25°C (loading) and then dehydrated with PVS2 or PVS3 (50 w/v% glycerol plus 50 w/v% sucrose) for 5 to 20 min at 0°C or 3 to 5 min at 25°C prior to plunge into LN (Nishizawa *et al.*, unpublished). We also observed that even at 80% concentration the use of PVS3 led to high rates of survival by this technique. The harmful effects due to dehydration can be alleviated or eliminated by reducing the concentration of PVS2 or PVS3 as well as the time of exposure to the level necessary for the dehydration of the cells after which the cell suspension may become vitrified. This two-step vitrification method was successfully applied to some cultured cells. Another approach for successful vitrification of cultured plant cells is the gradual addition of PVS2. The vitrification protocol with gradual addition of PVS2 (20%, 65% and 100% PVS2 at 0°C) is currently being applied to one line of tobacco cells and has been successfully used for two lines of *Catharanthus roseus* (Van Iren, unpublished, pers. comm.). Successful results of cryopreservation by vitrification of cultured cells and meristems is presented in Table 2. It will be necessary to expand the applicability of vitrification technique to a wide range of cultured cells, meristems and embryos.

Table 1 **Effect of cryoprotective solutions on the survival of cells cooled to −196°C by vitrification**

Cryoprotective solution	Survival (%±S.E.)
No-treatment	37.1 ± 1.3
2 M glycerol + 0.4 M sucrose	85.7 ± 0.6
1.7 M glycerol + 0.4 M sucrose	81.6 ± 1.3
1.2 M glycerol + 0.4 M sucrose	68.5 ± 1.2
2 M glycerol	74.3 ± 1.3
2 M EG + 0.4 M sucrose	51.6 ± 3.7
1.7 M EG + 0.4 M sucrose	53.4 ± 1.3
1.2 M EG + 0.4 M sucrose	47.0 ± 0.2
2 M EG	0.8 ± 0.2
2 M DMSO + 0.4 M sucrose	62.5 ± 2.0
1.7 M DMSO + 0.4 M sucrose	58.8 ± 1.7
1.2 M DMSO + 0.4 M sucrose	56.9 ± 2.0
2.0 M DMSO	0.6 ± 0.3
10% DMSO + 10% sucrose + 5% glycerol	60.1 ± 0.4

Material: Embryogenic cells of asparagus; Vitrification solution: PVS3; Cells were loaded with each cryoprotective solution for 10 min at 25°C and then dehydrated with PVS3 at 0°C for 20 min prior to immersion into LN. (Nishizawa *et al.*, 1993).

Table 2 **Successful cryopreservation of cultured cells and meristems by vitrification**

Material	Vitrification solution	Authors
Brassica campestris (cultured cells)	Steponkus' sol.	Langis, Schnabel, Earle, Steponkus: 1989
Asparagus (cultured cells)	PVS1	Uragami, Sakai, Nagai, Takahashi: 1989
Citrus sinensis (Nucellar cells)	PVS2	Sakai, Kobayashi, Oiyama: 1990
Other 3 *Citrus* (Nucellar cells)	PVS2	Sakai, Kobayashi, Oiyama: 1991b
Carnation (meristems)	Steponkus' sol.	Langis, Schnabel, Earle, Steponkus: 1990
Mint (meristems)	35%EG,1M DMSO 10%PEG	Towill: 1990
White clover (meristems)	PVS2	Yamada, Sakai, Matsumura, Higuchi: 1991b
White clover (meristemoids)	PVS2	Yamada (unpublished)
Lolium perenne (meristems)	PVS2	Yamada (unpublished)
Mulberry (meristems) 13 species, cvs	PVS2	Niino, Sakai, Enomoto, Magoshi, Kato: 1992c
Pear (meristems) 8 cvs	PVS2	Niino, Sakai, Yakuwa, Nojiri: 1992b
Apple (meristems) 5 species,cvs	PVS2	Niino, Sakai, Yakuwa, Nojiri: 1992 b
Potato (meristems)	Steponkus' sol.	Schnabel-Preikstas, Earle, Steponkus:1992a
Chrysanthemum (meristems)	Steponkus' sol.	Schnabel-Preikstas, Earle, Steponkus:1992b

Table 2 Successful cryopreservation of cultured cells and meristems by vitrification (continued)

Material	Vitrification solution	Authors
Sweet potato (meristems)	Steponkus' sol.	Schnabel-preikstas, Earle, Steponkus: 1992c
Sweet potato (meristems)	PVS2	Towill, Jarret: 1992
Ribes (meristems)	PVS2	Reed : 1992
Tea plant (meristems)	PVS2	Kuranuki, Sakai: 1992
Garlic (meristems)	PVS2	Niwata: 1992
Asparagus (bud cluster)	PVS2	Kohmura, Sakai, Chokyu, Yakuwa: 1992
Asparagus (cultured cells)	PVS2 or PVS3	Nishizawa, Sakai, Amano,Matsuzawa:1993
Tobacco (cultured cells)	PVS2	Takano, Tamura: 1992
Tobacco (cultured cells)	PVS2	Van Iren (unpublished)
Catharanthus (cultured cells)	PVS2	Van Iren (unpublished)

Steponkus' solution: Langis *et al.*, 1989

6. Drying Method

Another possible approach could involve extensive dehydration by air drying (Fig. 1, 4). For example, cultured cells (Nitzsche, 1980; Kaimori, 1988), somatic embryos (Senaratna *et al.*, 1990; Shimonishi *et al.*, 1991,) lateral buds (Uragami *et al.*, 1990) and apical meristems (Niino and Sakai, 1992) from *in vitro*-grown plantlets were cryopreserved after air drying. However, the induction or modification of drought tolerance may be the main factor for successful cryopreservation in this approach. Alginate-coated shoot tips of *in vitro*-grown pear and potato plants were successfully cryopreserved following air-drying (Dereuddre *et al.*, 1990; Fabre and Dereuddre, 1991). The

encapsulation-dehydration technique is easy to handle and alleviate the dehydration process. In this method, resistance to dehydration and deep freezing was induced by preculturing encapsulated shoot tips in a medium enriched with sucrose before dehydration. In the encapsulation-dehydration technique, the sucrose molarity increased markedly during the drying process and reached or exceeded the saturation point of the sucrose solution resulting in glass transition during cooling (Dereuddre *et al.*, 1991). The encapsulation-dehydration technique appears to be a practical method for cryopreservation of meristems and embryos. Recently, we have developed a modification of the encapsulation-dehydration technique (Niino and Sakai, 1992). To induce dehydration tolerance, cold-hardened apple apical meristems were precultured before being embedded into alginate-coated beads by gradual daily transfer to media containing 0.1, 0.4, and 0.7 M sucrose. The precultured shoot tips embedded into alginate-coated beads containing 0.5 M sucrose were treated in a medium supplemented with 1.0 M sucrose for 16 hr at 5 °C. The beads were dehydrated on sterile silica gel at 25°C before being immersed into LN. This revised method was successfully applied to four apple, one mulberry and three pear species or cultivars (Niino and Sakai, 1992).

7. Conclusion

Vitrification technique does not require a controlled freezing equipment or sophisticated, expensive apparatus. The vitrification technique for cryopreservation which has been recently applied to a wide range of cultured meristems appears to be a suitable method for the cryopreservation of meristems. However, the vitrification method did not result in high rates of survival for many cultured plant cells except for a limited number of species or cultivars. Thus, we developed a two-step vitrification method for cultured cells. In this method, cells are treated with a mixture of 2 M glycerol and 0.4 M sucrose for 10 min at 25°C and then dehydrated with PVS2 or PVS3 for 3 to 5 min at 25°C or for 5 to 20 min at 0°C prior to immersion into LN. It is necessary to expand the applicability of vitrification techniques to a wide range of cultured cells and meristems. To achieve this objective, it is essential to carry out basic studies on preconditioning for enhancing the freezing or dehydration tolerance.

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Cryopreservation of Citrus

by

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CONTENTS

1. Introduction

There are only a few reports on the applications of cryopreservation techniques to citrus (Kobayashi *et al.*, 1978; Mumford and Grout, 1979; Bajaj, 1984; Marin and Duran-Vila, 1988). Marin and Duran-Vila reported that a small number of frozen embryos (3 to 5%) survived and developed into proliferating cultures that produced whole plants. This was the first report describing the regeneration of plants from cryopreserved somatic tissues of citrus. However, the survival of somatic embryos did not refer to the survival of the whole embryo, but to recovery of proliferating structures from surviving cells by secondary embryogenesis. Vardi *et al.* (1982) succeeded in regenerating whole plants from protoplasts of nucellar callus in eight citrus cultivars. Kobayashi *et al.* (1983) also obtained plants from protoplasts of 'Trovita' orange nucellar callus and succeeded in producing somatic hybrid plants of *Rutaceae* by protoplast fusion (Kobayashi *et al.*, 1988; Ohgawara *et al.*, 1985). As protoplasts derived from nucellar callus regenerate phenotypically stable plants (Kobayashi, 1987), we attempted to apply cryopreservation technique to the nucellar callus of navel orange and succeeded in obtaining a high survival rate and subsequent regeneration of whole plants. Here, we report on three different cryogenic methods used for the cryopreservation of nucellar callus of navel orange (Kobayashi *et al.*,1990; Sakai *et al.*, 1990, 1991 a, b).

2. Materials

Calli derived from nucellar tissue of navel orange were used for cryopreservation. For nucellar callus induction, ovules were excised from flowers and placed on an MT basal medium containing 0.15 M sucrose supplemented with 10 mg l^{-1} BA and 0.8% Difco Bacto agar. The medium was adjusted to pH 5.7 prior to autoclaving at 121°C for 15 min. The cultures were kept at 25°C under a 16h/day illumination with cool white fluorescent tubes (25 μE $s^{-1}m^{-2}$). Nucellar callus was induced from the ovules within 2 to 3 months, and was maintained for 2 years on the same medium. About 1 g of calli was inoculated into 40 ml of liquid MT-medium and cultured on a rotary shaker (110 rpm) under the same environmental conditions. The cells were serially transferred every 2 weeks for 2 to 3 months.

3. Viability Test and Regrowth

Viability was assessed and the cells were transferred for recovery growth immediately after thawing without washing. The viability-index of the frozen-thawed cells against controls was determined by double staining with FDA and phenosafranine (Widholm, 1972). About 200 cell colonies were observed for each treatment. Viability of the cells after various treatments was expressed as the percentage of cell survival to that of nontreated and unfrozen controls. For the regrowth, samples of about 0.5 ml suspension in cryoprotectant were dispensed onto a double layered sterilized filter paper (5 cm in diameter), placed on 20 ml MT-medium containing 5 mg l^{-1} BA and 0.8% agar in a petri dish (9 cm in diameter). After 4 to 5 hr, the filter papers with cells were transferred to other petri dishes containing the same medium (Chen *et al.*, 1984). Dishes were then sealed with Parafilm strips. They were incubated in a growth chamber at 25°C, under a 16 hr photoperiod. The weight of wet paper plus cells was measured during the growth period.

4. Slow Prefreezing Method

1) Cryoprotection

Four ml from a 6-day-old culture, containing 0.3 ml packed cell volume, was transferred to 10 ml glass tubes, and then centrifuged at 100g for 10 sec. The supernatant was discarded and the cells were resuspended with 2.5 ml of MT-medium containing 1.2 M sucrose. Glass tubes containing the cells were chilled in an ice bath. Five hundred μl of ice-cold MT-medium containing 1.2 M sucrose and 30% DMSO (w/v) was gradually added (final DMSO concentration was 5%), followed by an equilibration period of 1 hr at 0°C (Fig. 1).

2) Freezing and thawing

Five hundred μl aliquots of cell suspension, containing about 100 mg cells (fresh weight), were dispensed into cryogenic tubes (Cryotube, Nunc, Roskilde, Denmark) 1.8 ml in capacity. The tubes were placed in the freezing chamber of a programmable freezer (Cryoembryo-HP, Hoxan, Japan) precooled at -8°C. Freezing was induced by touching the tube surface with a forceps precooled into liquid nitrogen. The specimens were then cooled at desired cooling rates (0.3 to 5°C min^{-1}) to terminal freezing temperatures ranging from -25 to -50°C

followed by immersion into LN. After 1 hr storage in LN, the cryotubes were rapidly thawed in a water bath at 40°C (Fig. 1).

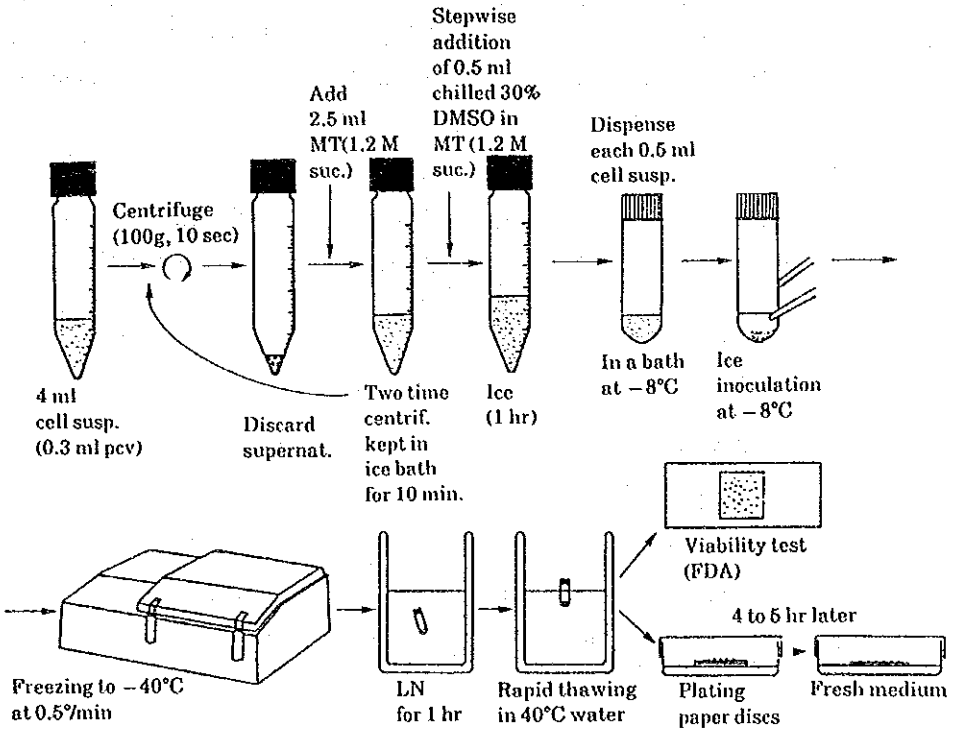

Fig. 1 Procedure for cryopreservation of nucellar cells of navel orange by the slow freezing method (Kobayashi *et al.*, 1990).

3) Results

In preliminary experiments on cryoprotection, the highest survival was obtained when the cells were treated with a solution containing 5% DMSO plus 0.7 M sorbitol, cooled at 0.5°C min^{-1} to −40°C and then stored in LN. No significant differences in cell survival were observed between 5% and 10% DMSO, in combination with 0.7 M sorbitol. Preculture in an MT-medium containing 0.5, 0.7 and 1 M sorbitol without sucrose for 16 hr did not improve the cell survival. Thus, non-precultured cells were used in the following experiments. Cryoprotection of the nucellar cells was further tested with 5% DMSO in combination with sorbitol, sucrose, glucose and glycerol, respectively. Combination of 5% DMSO with 0.7 M sucrose considerably improved the cell

survival rate (Table 1). Among sugars or polyhydric alcohols tested, cryoprotection was in the order of sucrose > sorbitol = glucose > glycerol. Therefore, the optimal sucrose concentration was tested. As shown in Table 2, the survival rate increased with increasing sucrose content, reaching a value of 73% with 1.2 M, and then rapidly decreased in a 1.5 M solution. Based on these results, 5% DMSO and 1.2 M sucrose in MT-medium were used as cryoprotectants in the following experiments.

Table 1 **Effect of cryoprotectant on the survival of nucellar cells of navel orange cooled to −196°C by slow freezing method**

Cryoprotectant	Survival (%)
Control (no additives)	0
5% DMSO + 0.7M sorbitol	30 ± 1.8
5% DMSO + 0.7M glucose	30 ± 1.6
5% DMSO + 0.7M sucrose	51 ± 1.2
5% DMSO + 0.7M glycerol	3 ± 0.8

Nucellar cells were frozen slowly to −40°C at 0.5°C min^{-1} prior to immersion into LN. Values are means of triplications ± SE (Kobayashi *et al.*, 1990).

Table 2 **Effect of sucrose concentration in combination with 5% DMSO on the survival rate of nucellar cells cooled to −196°C by slow freezing method**

Cryoprotectant	Survival (%)
5% DMSO + 0.5M sucrose	21 ± 1.4
5% DMSO + 0.7M sucrose	44 ± 1.8
5% DMSO + 1.0M sucrose	63 ± 1.6
5% DMSO + 1.2M sucrose	73 ± 1.4
5% DMSO + 1.5M sucrose	29 ± 3.0

Values are means of duplicates ± SE (Kobayashi *et al.*, 1990).

To investigate the effect of cooling rates on survival, the temperature of the cell suspensions was cooled to −40°C at various rates prior to immersion into LN and the suspensions were subsequently thawed rapidly. The optimum cooling rate was 0.5°C min^{-1} (Fig. 2). Cooling rates above and below this optimum level resulted in reduced viability. At the optimal cooling rate of 0.5°C min^{-1}, the effect of the terminal freezing temperature, prior to immersion into LN, on the survival was also examined. A terminal freezing temperature of −40°C was optimum under the experimental conditions indicated in Fig. 3.

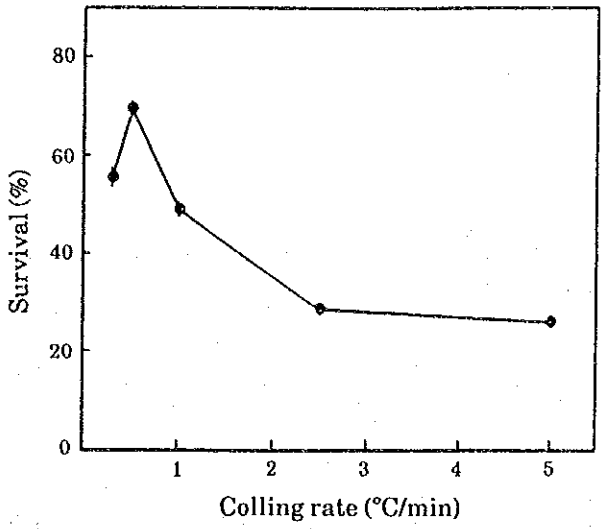

Fig. 2 Effect of cooling rate on the survival of nucellar cells cooled to −196°C by the slow freezing method.

Cell suspension was frozen in MT-medium containing 5% DMSO and 1.2 M sucrose at various cooling rates to −40°C prior to immersion into LN. Bars represent ± SE from the mean of duplicate (Kobayashi *et al.*, 1990).

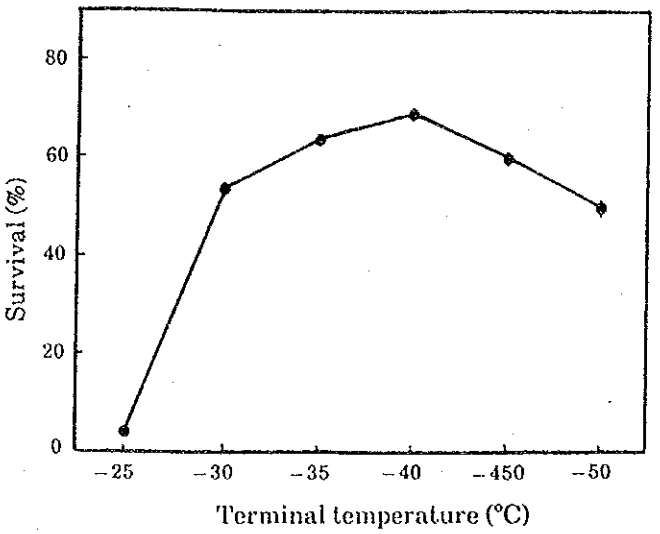

Fig. 3 Effect of terminal freezing temperature prior to immersion into LN on the survival.

Six-day-old cultures were frozen at 0.5°C min^{-1} to various temperatures prior to immersion into LN in the presence of 5% DMSO and 1.2 M sucrose and subsequently thawed rapidly. Bars represent ± SE from the means of duplicates (Kobayashi *et al.*, 1990).

Frozen-thawed cells were grown on filter paper discs over an agar medium. Recovery growth of the frozen-thawed cells is shown in Fig. 4. Control and frozen cells started to grow after a lag period of 3 days but the regrowth rate of the frozen cells was lower than that of the control cells until 12 days after plating. Rapid growth of the frozen cells was observed after 12 days of plating and followed a similar pattern to that of the control (Fig. 5).

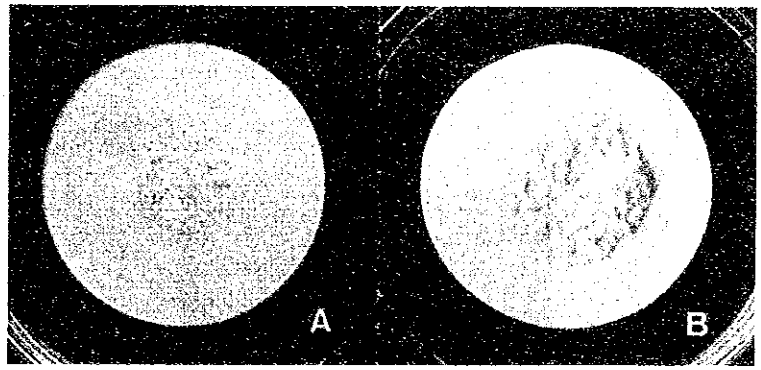

Fig. 4 Regrowth of frozen-thawed cells.
A: Cells (about 100 mg fresh weight), just after plating onto a filter paper (5 cm in diameter) over agar medium. B: Regrown cells (about 800 mg fresh weight), 30 days after plating (Kobayashi *et al.*, 1990).

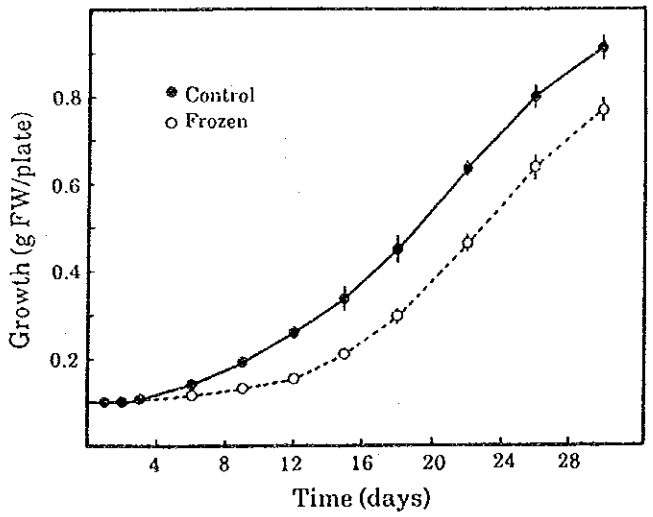

Fig. 5 Recovery growth of frozen-thawed cells.
Control: Without any treatment. Frozen: Frozen to the temperature of LN. Bars represent ± SE from the mean of duplicates (Kobayashi *et al.*, 1990).

Cells grown on filter paper were transferred to an MT-medium containing 5% galactose, 5% coconut water (Gibco, New York, USA) and 0.9% agar without sucrose. Cotyledonary embryoids were produced from the cells within 2 months of culture. When embryoids were again transferred to an MT-medium containing 2% sucrose, 500 mg l^{-1} malt extract, 0.05 mg l^{-1} NAA and 0.9% agar, they developed into whole plants after 2 to 6 months. Plants derived from frozen-thawed cells were morphologically uniform and showed the typical characteristics of navel orange (Fig. 6).

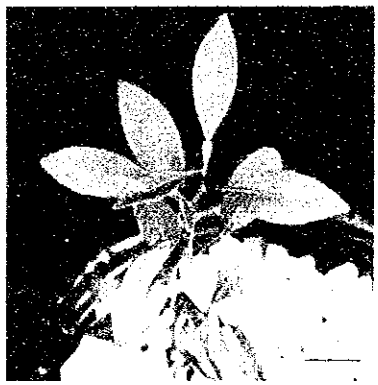

Fig. 6 Plant regenerated from the nucellar cells cooled to −196°C, about 1 month after transfer to soil in a pot.
Bar represents 1 cm (Kobayashi *et al.*, 1990).

5. Simple Freezing Method

1) Cryoprotection

As cryoprotectants, a 2- or 3-M solution of each cryoprotectant (glycerol, PG, EG and DMSO) was used in combination with 0.4 M sucrose dissolved in MT-medium. Two ml of a cell suspension from a 6-day-old culture containing 0.2 ml packed cell volume was placed in a conical centrifuge tube (15 mm diameter, 110 mm long) and allowed to settle. After the removal of the supernatant fluid, 2 ml of each cryoprotective solution prepared in an MT-medium supplemented with 0.4 M sucrose was added to 0.2 ml of packed cells at 25°C. The cell suspension was mixed, centrifuged and the supernatant fraction was discarded. The cells were resuspended with 2 ml of each cryoprotective solution and held 10 min at 25°C. Then, an aliquot of cell suspension of 0.2 or 0.4 ml was loaded into a 0.5 ml plastic straw or placed in a 1.8 ml cryotube.

The cell suspensions were frozen spontaneously by placing the straws horizontally in a freezer at −30 °C for 30 min prior to immersion into LN. The cell suspensions were supercooled to about −15°C and freezing started within 3 min after transfer to −30°C. The cooling rate after freezing was about 2°C min^{-1} between −10 and −30°C (Sakai *et al.*, 1991a). The straws placed in a freezer at −30°C for 30 min were immersed into LN. After rapid thawing in a water bath at 40°C, the cell suspension in a straw was expelled into 2 ml of a diluent solution containing 1.2 M sucrose in MT-medium at 25°C.

2) Results

Cryoprotection of the cells was compared when various cryoprotectants dissolved in MT-medium supplemented with 0.4 M sucrose were used. As shown in Table 3, the highest survival rate was obtained in the cells treated with 2 M glycerol. Among the cryoprotectants tested, the order of effectiveness was 2 M glycerol>3 M glycerol>2 M PG>2 M DMSO>3 M DMSO>2 M EG>3 M PG and 3 M EG (Table 3). Based on these results, a mixture of 2 M glycerol and 0.4 M sucrose was used in the following experiments as a cryoprotectant.

Table 3 Effect of cryoprotectant on the survival rate of cells cooled to −196°C by the simple freezing method

Cryoprotectant	Survival (%)
2 M EG	49.7 ± 1.6
3 M EG	32.3 ± 1.9
2 M PG	66.1 ± 1.4
3 M PG	35.6 ± 1.8
2 M glycerol	91.2 ± 1.4
3 M glycerol	80.4 ± 1.5
2 M DMSO	63.4 ± 1.8
3 M DMSO	52.5 ± 1.6

Cryoprotective effect on nucellar cells was compared for various cryoprotectants dissolved in MT-medium supplemented with 0.4 M sucrose at 25°C for 10 min. The cryoprotected cells were loaded into 0.5 ml straws and directly transferred to a freezer at −30°C and kept there for 30 or 60 min prior to immersion into LN. After rapid thawing, the cells were expelled into 2 ml of MT-medium containing 1.2 M sucrose (Sakai *et al.*, 1991 a).

The effect of the sucrose concentration in the diluent, after rapid thawing, on the survival was investigated. After rapid thawing, the cell suspensions were expelled into 2 ml of MT-medium containing different concentrations of sucrose and kept there for 10 min at 25°C. The highest survival rate was obtained in an MT-medium containing 1.2 M sucrose. The survival rate decreased with the decrease in the concentration of sucrose, especially below 0.4 M solution (data not shown).

The control and frozen cells resumed growth 3 days after plating, but the regrowth rate of the frozen cells was lower than that of the nonfrozen controls until 12 days and followed a similar pattern to that of the control (data not shown). Cryopreserved cells developed into whole plants.

3) Discussion

The main advantages of this procedure for cryopreservation are as follows: (1) the time required for slow freezing and the time used to equilibrate the cells in the cryoprotective solution at 0°C are significantly reduced, and (2) controlled-rate freezing equipment and ice seeding of the extracellular medium are not necessary. The temperature of −30°C corresponds to that of ordinary freezers, and frozen cells can be kept there for 30 or 60 min before their storage in LN. This method has been successfully applied to cultured cells of carrot, rice, asparagus and meristemoids of white clover. It is considered that this simple freezing method could be applied widely and may promote the adoption of cultured plant cells.

6. Vitrification Method

1) Vitrification procedure (Fig. 7)

Based on preliminary experiments with nucellar cells, a low toxicity combination that allowed the contents of a transparent straw to remain transparent without undergoing crystallization during cooling to and warming from LN was adopted as a plant vitrification solution (designated PVS2, Sakai *et al.*, 1990). This solution which can be easily supercooled below −70°C when cooled rapidly, becomes solidified into metastable glass at about −115°C. Upon subsequent slow warming in DSC, the vitrified PVS2 showed glass transition (Tg) at about −115°C, followed by exothermic devitrification

(Td) (crystallization) at about −75°C and an endothermic melting (Tm) at about −36°C (Sakai *et al.*, 1990; refer to Fig. 2 in page 7 of this report).

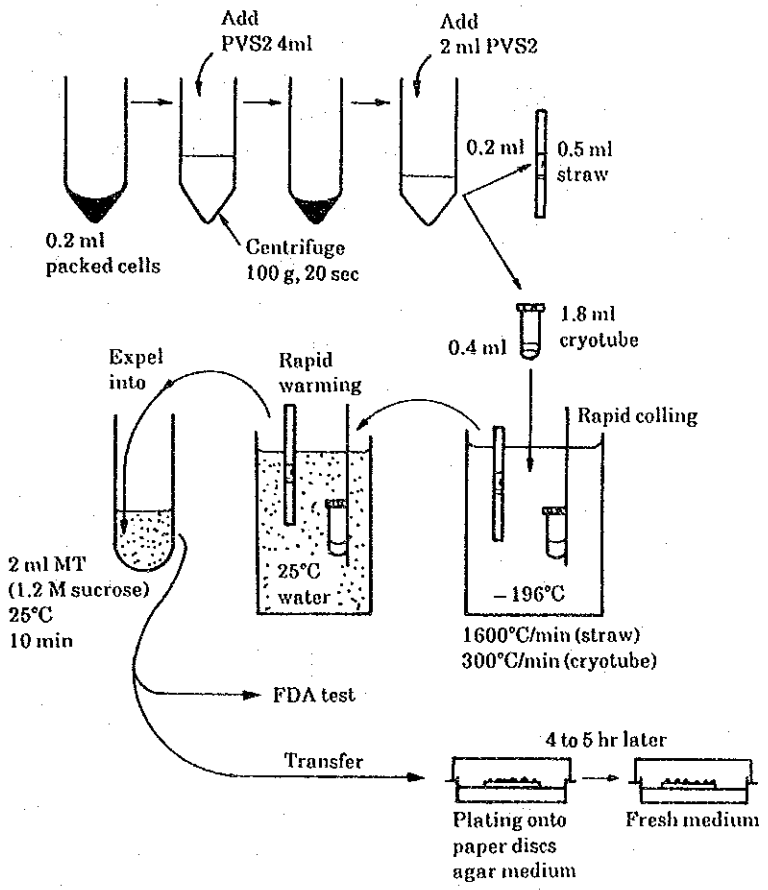

Fig. 7 Vitrification procedure for nucellar cells of navel orange (Sakai *et al.*, 1990).

Two ml of cell suspension from a 8-day-old culture containing about 0.2 ml packed cell volume was transferred to a 10 ml conical glass tube (110×15 mm) and allowed to settle. The supernatant was discarded and 4 ml of PVS2 at 25°C was added. The cell suspension was mixed and then centrifuged at 100g for 20 sec. The supernatant was discarded and 2 ml of fresh PVS2 was then added. The cells were treated with PVS2 at 25 or 0°C for various periods of time. Then, an aliquot of 0.2 or 0.4 ml cell suspension was loaded into a 0.5 ml plastic straw or placed in a 1.8 ml cryotube. The top end of each straw was sealed by a heat sealer. The straws or cryotubes were immersed into LN and kept there for at least 30 min. The mean cooling rate in the straw or the cryotube was about 1,600°C min^{-1} or about 300°C min^{-1} in the range between −30°C and −150°C, respectively. Cell suspensions in straws or cryotubes were warmed in a water bath at 25°C. The cell suspension was expelled into 2 ml of a diluting solution containing 1.2 M sucrose in MT-medium at 25°C and kept there for 10 min before the viability or growth capacity was assessed.

2) Results

To determine the optimal time of exposure to PVS2 at 25 or 0°C, the cells were treated with PVS2 for various periods of time prior to immersion into LN. Exposure to PVS2 at 25°C resulted in a time-dependent survival (Fig. 8). The highest survival was obtained for the cells treated with PVS2 for 3 min at 25°C. The vitrified cells treated with PVS2 for 1 to 5 min at 25°C still showed high rates of survival (80 to 90%), but the survival rate decreased rapidly after a longer exposure. The survival of the cells treated with PVS2 at 25°C without cooling in LN (treated control) was nearly the same as that of vitrified cells. The vitrified cells treated with PVS2 at 0°C for 3 to 20 min showed high rates of survival (75 - 70%) after cooling to −196°C.

The effect of the sucrose concentration in the diluent, after rapid warming, on the survival of vitrified cells was examined. The cell suspension was expelled into 2 ml of MT-medium containing various concentrations of sucrose and kept there for 10 min at 25°C. The highest survival rate was obtained in MT- medium containing 1.2 M sucrose (Table 4). The survival rate decreased sharply with the decrease in the sucrose concentration from 1.2 M to 0.15 M.

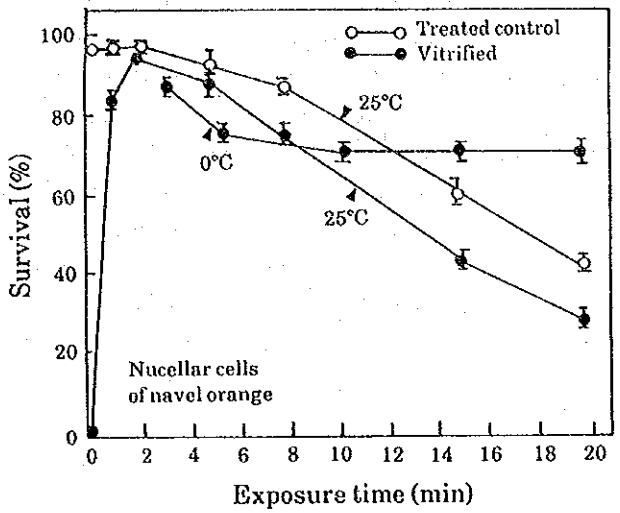

Fig. 8 Effect of exposure time to PVS2 at 25 or 0°C on the survival of vitrified cells.

Cells treated with PVS2 at 25 or 0 °C for various periods of time were placed in a 1.8 ml cryotube or loaded into a 0.5 ml plastic straw and then directly immersed into LN for 30 min. After rapid warming, the cell suspension was expelled into 2 ml of MT-medium containing 1.2 M sucrose.

Treated control: as in vitrified cells, without cooling to −196°C. The vertical bars: SE (n=2) (Sakai *et al.*, 1991 b, modified).

Table 4 Survival rate of vitrified cells unloaded with different concentrations of sucrose

Sucrose concentration (M)	Survival (%)
0.15	15.5 ± 1.74
0.40	30.7 ± 2.44
0.80	67.8 ± 2.55
1.20	89.8 ± 1.10
1.50	87.1 ± 1.17

Nucellar cells were treated with PVS2 at 25°C for 3 min. The cell suspension of 0.4 ml was placed in a 1.8 ml cryotube and immersed into LN for 30 min. After rapid warming in a water bath at 25°C, the cell suspension was expelled into MT-medium containing different concentrations of sucrose and kept there for 10 min at 25°C before viability was examined. Values are means of duplications ± SE (Sakai *et al.*, 1991 b).

The effect of the warming rate on the survival is shown in Table 5. Cells that warmed rapidly at 300°C min^{-1} or above showed a high survival rate (about 90%) regardless of the cooling rate. On the other hand, most of the vitrified cells warmed slowly at 30°C min^{-1} or below showed very low survival rates. This observation can be ascribed to the occurrence of intracellular freezing during the slow warming process. Thus, the time required for the temperature to rise from −80 to −40°C during which crystallization may occur was calculated based on the results presented in Table 5. As shown in Fig. 9, when the cells were exposed to the temperature range from −80 to −40°C within about 25 sec (rates above 100°C min^{-1}), they showed a high survival rate (about 90%), while the survival rate rapidly decreased in the cells warmed more slowly.

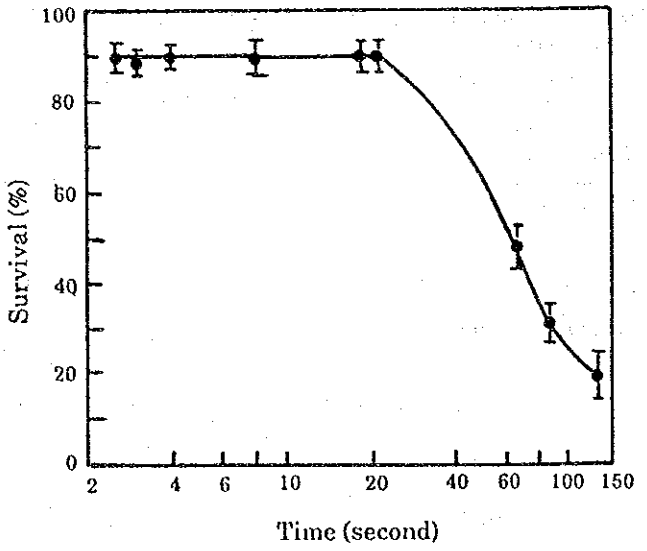

Fig. 9 Effect of warming rate on the survival of cells cooled to −196°C by vitrification.

Vitrified cells were warmed at various rates and the time required for the temperature to rise from −80 to −40°C was calculated based on the results presented in Table 5. Vertical bars: SE (n = 2) (Sakai *et al.*, 1991 b).

Table 5 Effect of warming rate on the survival rate of nucellar cells cooled to −196°C by vitrification

Containers	Cooling rate (°C/min)	Warming rate (°C/min) (method)	Survival (%)
0.5 ml straw	1,500	1,600 (25°C, water)	91.0 ± 0.8
	1,600	1,200 (0°C, water)	91.0 ± 0.8
	1,550	109 (50°C, air)	89.0 ± 0.9
	1,500	100 (25°C, air)	88.1 ± 1.2
1.8 ml cryotube	280	300 (25°C, water)	91.0 ± 1.2
	283	30 (25°C, air)	47.1 ± 1.4
	282	24 (0°C, air)	30.2 ± 1.4
	285	6 (−29°C, air)	23.9 ± 1.3

Nucellar cells treated with PVS2 at 25°C for 3 min were loaded into a 0.5 ml straw or placed in a 1.8 ml cryotube (0.4 ml cell suspension) prior to immersion into LN. Vitrified cells were warmed in a water bath at 25 or 0°C, or in the air at 25 or 0°C, respectively. Cooling rate was calculated from the time required for the temperature to drop from −30°C to −150°C. Rewarming rate was calculated from the time required for the temperature to rise from −80 to −40 °C. Values are means of duplications ± SE (Sakai et al., 19991 b).

Vitrified and warmed cells were grown on filter paper discs over agar medium (Fig. 10). Control and vitrified cells started to grow within 3 days after plating, but the regrowth rate of the vitrified cells was lower than that of controls until 12 days after plating. Thereafter the growth pattern was similar to that of the controls (data not shown). Cotyledonary embryoids were produced from the cells within 2 to 3 months of culture (Photo. 1A). When embryoids were again transferred to an MT-medium containing 2 % sucrose, 500 mg l^{-1} malt extracts, 0.05 mg l^{-1} NAA and 0.9% agar, they developed into whole plants after 2 to 3 months (Photo. 1B).

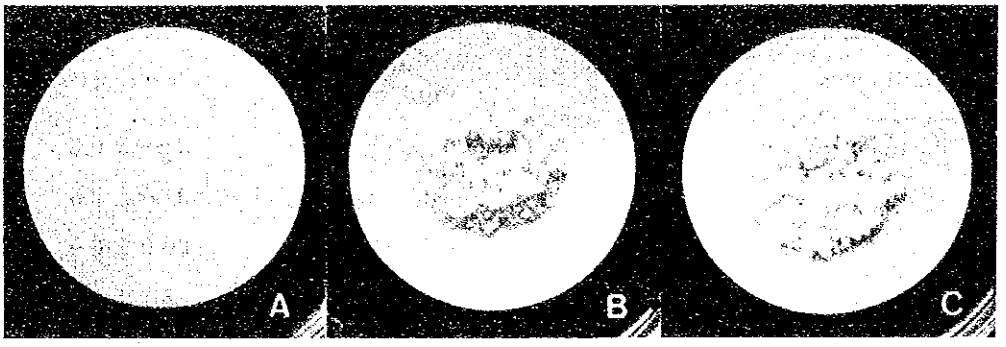

Fig. 10 Regrowth of nucellar cells cooled to −196°C by vitrification and kept for 1 hr.

The cells were treated with PVS2 for 3 min at 25°C prior to immersion into LN.

A: Cells (about 100 mg fresh weight), just after plating onto a filter paper over agar medium.

B: Control cells (1024 mg) 21 days after plating.

C: Regrown vitrified cells (800 mg) 21 days after plating (Sakai *et al.*, 1991 b).

The vitrification procedure was successfully applied to the nucellar cells of three other citrus plants. High survival rates (above 90%) were obtained in these citrus plants that were cryopreserved for 320 days (Table 6).

The survival of the nucellar cells of navel orange cooled to −196°C was compared with three different cryogenic protocols (Table 7). The highest survival rate was obtained in the vitrified cells treated with PVS2 at 25°C for 3 min.

Table 6 Survival rate of nucellar cells of four citrus plants cooled to −196°C by vitrification and stored for 320 days

Species	Survival (%)
Citrus sinensis Osb. var. *brasiliensis* Tanaka (Navel orange)	90.0 ± 1.4
Citrus paradisi Macf. (Grapefruit)	96.0 ± 0.9
Citrus sudachi Hort. ex Shirai (Sudachi)	97.0 ± 2.0
Hybrid ('Murcott' tangor)	92.0 ± 1.4

Suspension culture of nucellar cells of three species and of a hybrid of citrus plants was cryopreserved by vitrification. The cells treated with PVS2 at 25°C for 3 min were loaded into 0.5 ml straws and then immersed into LN. Values are means of duplications ± SE (Sakai *et al.*, 1991 b; modified).

Table 7 Survival rate of nucellar cells cooled to −196°C using different cryogenic protocols

Method	Survival (%)
Conventional slow prefreezing[a]	73.0 ± 1.4
Simple freezing method[b]	65.1 ± 1.6
Vitrification	90.0 ± 1.1

[a] Cooled to −40°C at 0.3°C/min in the presence of 5% DMSO and 1.2 M sucrose (Kobayashi *et al.*, 1990).

[b] Cells were treated with a mixture of 2 M glycerol and 0.4 M sucrose at 25°C for 10 min and then directly transferred to a freezer at −30°C for 1 hr prior to immersion into LN (Sakai *et al.*, 1991 a).

Treated with PVS2 at 25°C for 3 min prior to immersion into LN. Values are means of duplicates ± SE (Sakai *et al.*, 1991 b).

3) Discussion

Complete vitrification enables to avoid the potentially damaging effects of intra- and extracellular crystallization. Thus cells treated under properly controlled conditions with a suitable vitrification solution show a high rate of survival. For successful cryopreservation by vitrification it is essential to carefully control the procedures for dehydration and cryoprotectant permeation and to prevent injury by chemical toxicity or excessive osmotic stresses during dehydration. The one-step vitrification method for dehydration which we developed (Sakai *et al.*, 1990, 1991 b) enabled to simplify the procedure and increase the survival rate.

The survival of the vitrified cells depends on the fate of the glassy cytoplasm during warming. When warming was slow enough to allow sufficient time for crystallization of the cytoplasm during warming, a low survival rate was obtained. However, rapid warming did not allow sufficient time for crystallization to occur, resulting in a high survival rate. It was confirmed that in the vitrified nucellar cells, the survival rate decreased considerably in the range between −80 and −40°C during slow warming.

It is particularly important that cryopreserved cells be capable of producing plants identical with the nontreated phenotype. Vasil (1983) stated that the genetic stability found in plants regenerated from callus or tissues in many species was due to regeneration through somatic embryogenesis. In citrus also, plants regenerated from protoplasts through somatic embryogenesis showed phenotypic stability (Kobayashi, 1987). In the present study, the nucellar callus developed from cryopreserved cells by vitrification exhibited embryogenic potential identical with that of the nontreated controls, and plants regenerated from vitrified cells were morphologically uniform. Further studies are necessary to confirm the phenotype based on biochemical, cytological and morphological analyses.

7. Conclusion

The vitrification method eliminates the need for controlled slow freezing and for equilibration in a cryoprotectant. The procedure, we used for cryopreservation required only several min. The highest survival rate was obtained for the cells treated with PVS2 for 3 min at 25°C. This simple vitrification method was successfully applied to three other citrus plants. Thus,

this procedure seems to be a practical method for cryopreservation of polyembryonic citrus plants.

The nucellar callus developed from vitrified cells exibited an embryogenic potential identical with that of the nontreated controls, and plants regenerated from cryopreserved cells were morphologically uniform.

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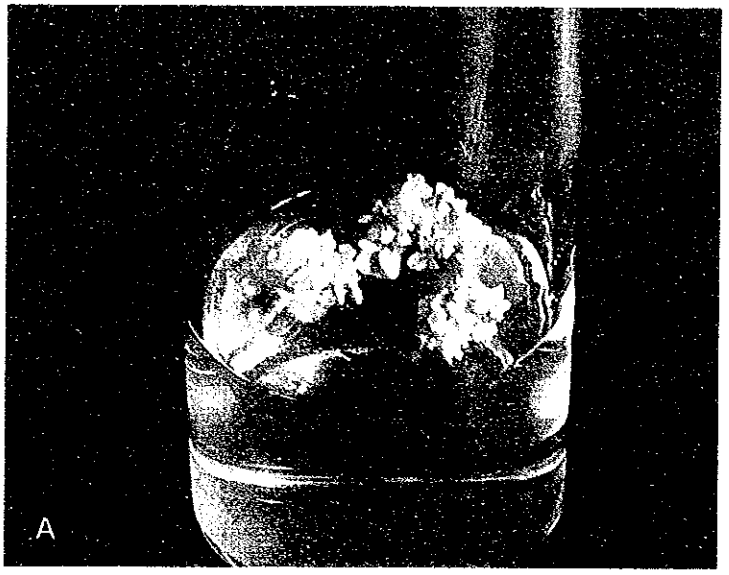

Photo. 1 Regrowth of nucellar cells cooled to −196°C by vitrification

A: Regenerated cotyledonary embryoids (×1.5), at 2 months after plating.
B: Regenerated plants, at 3 months after plating (×1.25) (Sakai *et al.*, 1991 b).

Cryopreservation of Deciduous Fruits and Mulberry Trees

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CONTENTS

1. Introduction

Most species and cultivars of woody plants are genetically heterozygotes because of their allogamous nature. The genetical integrity of these plants can be maintained only by vegetative propagation such as cutting, grafting or layering. Genetic resources of vegetatively propagated woody plants in Japan are maintained as living plants in the field in some clonal repositories. About 7,000 species or cultivars of fruit trees and more than one thousand species or cultivars of mulberry trees were preserved in Japan. These collections in the field are valuable as they provide immediate access of flowering plants to breeders through daily evaluation of characteristics. However, the preservation of woody plants in the field requires land and constant maintenance, and is costly. In addition, the susceptibility of the plants to insects, diseases and environmental stress is a major constraint on long-term preservation of these genetic resources.

2. Pollen, Seeds and Scions

The preservation of pollen and seeds of vegetatively propagated woody plants is not usually problematic. However, in the case of this germplasm, the same gene combination can not be recovered due to heterozygosity, in addition to the long periods of juvenility. Thus, these materials can only be used for base collection to preserve a gene pool, especially in the case of endangered wild species in imminent danger of loss, and for broadening the genetic diversity. Preservation of pollen and seeds has been successfully achieved for some woody plants (Stanwood, 1985; Towill, 1988; Kozaki *et al.*, 1988).

Scions or cuttings which are usually used for the propagation of fruit and mulberry tree cultivars are another form of germplasm that can be stored. Some scions of fruit trees stored for 1-5 years at -5°C to 5°C showed a growth capacity (Kozaki *et al.*, 1988).

3. *In vitro* Systems

In recent years, *in vitro* micropropagation of vegetatively propagated crops has been achieved. *In vitro*-cultured plantlets are suitable materials for germplasm preservation. Plantlets can be mass-propagated with the following advantages: limited space is necessary for their preservation; they can be cultured under disease-free conditions; and their growth can be easily controlled.

Germplasm preservation consists of short- to medium-term preservation by cryogenic methods. In the case of *in vitro* storage of plant materials, reduced or minimum growth can be obtained in woody plants such as apple, grape, kiwifruit, pear, cherry, mulberry, poplar, etc. As for apple, Lundergan and Janick (1979) reported that *in vitro* cultures had been successfully stored for up to 1 year at 1 to 4°C. Oka and Niino (1989) reported that *in vitro*- grown shoots of pear could be preserved for 2 years at 5°C under 8 hr photoperiod without requiring transfer to fresh medium. The average rate of regrowth was 80%. For hybrid poplar shoots, Son *et al.* (1991) reported that a 25% survival rate could be achieved after 5 years of storage at 4°C. Thus, the use of cold-storage system could become an alternative method for conserving genetic resources of woody plants, if suitable micropropagation systems were established.

However, the conservation of genetic resources is associated with problems, including variations during culture, the need for accession-specific media and the long time required to recover flowering plants.

4. Cryopreservation

The development of methods of germplasm preservation at cryogenic temperatures enables to save labor and space, and complements current germplasm storage methods. Cryopreservation is an important tool for long-term storage (base collection) of germplasm of vegetatively propagated plants. Several reports have indicated that shoot tips or apical meristems of some woody plants such as apple, pear, mulberry, *Rubus*, grape, etc. could be cooled successfully to −196°C. Simple and reliable cryogenic methods such as partial dehydration-prefreezing method, conventional slow prefreezing method, simple freezing method, vitrification, air-drying or alginate-encapsulation dehydration technique have been developed for fruit and mulberry meristems.

1) Cryopreservation of winter buds

Sakai (1960) first demonstrated that winter-hardy twigs survived when immersed into LN, if they were previously freeze-dehydrated to −20 or −30°C. Prefrozen willow twigs stored in LN for 1 year retained their viability and developed roots and shoots. Sakai and Nishiyama (1978) also demonstrated that cryopreserved winter buds of hardy deciduous fruit trees, such as apple, pear, gooseberry and currant, retained their ability to sprout in water after thawing,

and they demonstrated that apple buds taken from the shoots stored in LN resumed growth after grafting onto rootstocks. Yakuwa and Oka (1988) who applied these methods reported that plant regeneration could be achieved through meristem culture from mulberry winter buds cooled to −196°C. Winter buds attached to nodal shoot segments 1.0 - 1.5 cm in length were frozen to −20°C with a daily temperature decrease of 10°C before being immersed into LN. After rapid thawing, the apical meristems were aseptically excised from cryopreserved buds and cultured on MS medium. The highest frequency of shoot formation amounted to about 50 %. Tyler and Stushnoff (1988a, 1988b) also developed a technique of cryopreservation of dormant buds of apple, using 2.5 cm pieces of dormant twigs, each containing one bud which was dehydrated at −4°C to 20% to 30% water content. These dehydrated winter buds were prefrozen at the rate of 2°C/hr to −16°C and then 10°C/hr to −30°C, after which the samples were kept for 24 hr at −30°C before immersion into LN. After slow thawing overnight at 0 to 5°C and rehydration in moist peat moss, the winter buds were grafted onto dwarf rootstocks. Average recovery rate of frozen apple winter buds was about 70%.

Cryopreservation of dormant winter buds offers several advantages: (1) clonal integrity is maintained; (2) it is a simple and reliable method which requires limited space; (3) it requires no expensive and sophisticated cooling apparatus; (4) prolonged storage is theoretically possible; (5) *in vitro* clonal propagation from cryopreserved materials is easy; (6) possible toxic effects of chemical cryoprotectants which are indispensable in conventional methods can be avoided. Two protocols of cryopreservation using mulberry winter buds are presented as follows.

(1) Slow prefreezing method of winter buds

Mulberry shoots were collected in winter, when the buds were still in a state of 'quiescence'. The axillary buds were removed from the stems with about 5 mm of vascular tissue attached to the buds. Buds in polyethylene bags were placed in stainless steel freezing canisters and kept at 0°C for one day before freezing. Buds were cooled at the rate of 5°C/day from 0°C to −20°C and kept at −20°C for 1 day prior to immersion into LN. After storage in LN, the buds were rapidly thawed in a water bath at 37°C.

Buds were sterilized with 70% ethanol for 30 sec followed by SHC solution (effective chlorine concentration: 0.5%) for 15 to 30 min. After rinsing in sterilized water, shoot tips consisting of the meristem and five to eight leaf primordia were dissected. The shoot tips were then cultured on MS medium containing 2% fructose, 0.8% agar supplemented with 1 mg/l BA. The medium was adjusted to pH 6.0 before autoclaving at 120°C for 15 min (Oka, 1985). Shoot tips were placed on 25 ml of medium in 100 ml glass flasks. The culture was maintained at 25°C under a 16 hr photoperiod (Fig. 1).

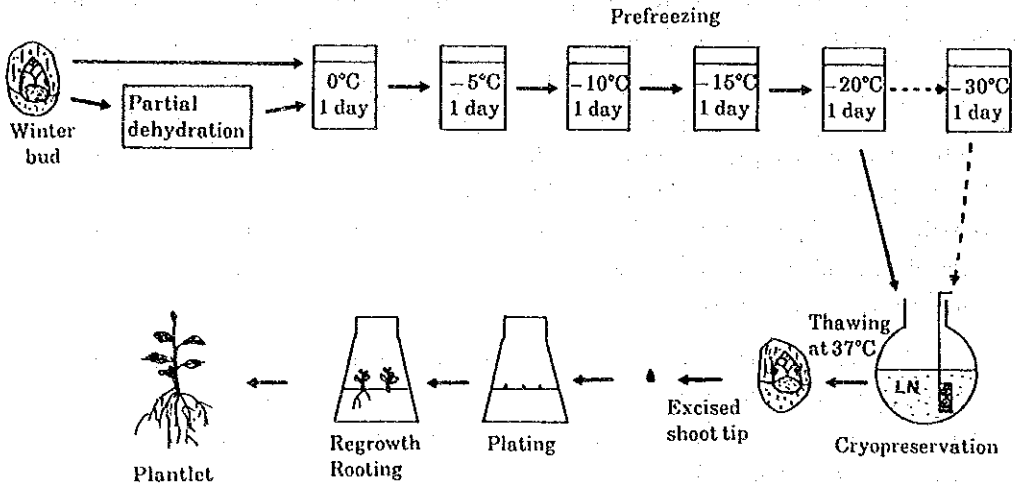

Fig. 1 Schematic protocol of slow prefreezing method of winter buds.

Shoot formation of excised shoot tips from mulberry winter buds cooled to −196°C with prefreezing to −20°C and with temperature decreases of 5 or 10°C daily prior to immersion in LN, is shown in Table 1. Prefreezing with a daily temperature decrease of 5°C resulted in higher shoot formation compared with 10°C/day decrease (Niino *et al.*, 1992b). Yakuwa and Oka (1988) observed that the prefreezing temperature required for survival from cryopreservation depends on the thawing rate. Favorable conditions for a high rate of shoot formation were obtained either by prefreezing at −10 or −20°C followed by rapid thawing at 37°C or prefreezing at −20 or −30°C followed by slow thawing at 0°C. Tyler and Stushnoff (1988) also reported that prefreezing at −30°C for 24hr and slow thawing at 2°C for 24hr resulted in the highest survival rate of cryopreserved buds of apple cultivars. When the prefreezing temperature decreased to −40°C, the survival rate decreased. Recently, it has been observed

that partial dehydration at 25°C prior to prefreezing to −20°C improved the recovery rate of mulberry shoot tips cooled to −196°C. The optimal water content of dehydrated buds was about 38.5% and the highest rate of shoot formation amounted to about 70% (Fig. 2). This improved procedure enables to use the dormant buds collected from late autumn to early spring for cryopreservation (Niino *et al.*, 1991b). These prefreezing methods were successfully applied to winter buds of other deciduous woody trees, such as pear, blueberry, raspberry (Photo. 1), cherry and apricot (Oka *et al.*, 1991; Niino *et al.*, 1990; Stushnoff, 1991).

Table 1 Shoot formation rate of excised shoot tips from mulberry winter buds cooled to −196°C at different cooling rates

Cultivar	Shoot formation (% ± S.E.)	
	10°C/day	5°C/day
Akagiichibei	21 ± 10	46 ± 15
Aobanezumi	23 ± 8	80 ± 10
Fusounishiki	30 ± 0	53 ± 7
Magozaemon	34 ± 5	73 ± 5
Akaunetaoshi	40 ± 7	46 ± 10
Honyamato	55 ± 15	69 ± 7
Fushimagari	48 ± 8	68 ± 3
Kenmochi	55 ± 10	75 ± 10

Mulberry winter buds were cooled to −20°C with 5 or 10°C decrease of the temperature per day prior to immersion into LN, and then thawed rapidly in a water bath at 37°C. After sterilization, the shoot tips were excised and plated on MS medium. Shoot formation of cryopreserved shoot tips was defined as the percentage of shoot tips inducing normal shoots 40 days after plating. Approximately 20 buds were tested for each of the two replications. Difference between 10°C/ day and 5°C/day treatment was significant at P=0.01 (t-test). (Niino *et al.*, 1992b).

It is important to determine whether cryopreserved shoot tips are capable of maintaining their viability during prolonged storage and of producing plants identical with the non-treated phenotype. Shoot formation of excised shoot tips from winter mulberry buds stored at various temperatures for different periods of time are shown in Table 2 (Niino *et al.*, unpublished). The rate of shoot formation of the shoot tips stored in LN for 3.5 years were 75%. The rate of shoot development of shoot tips cryopreserved at −135°C for less than 3.5 years did not decrease compared with those stored in LN.

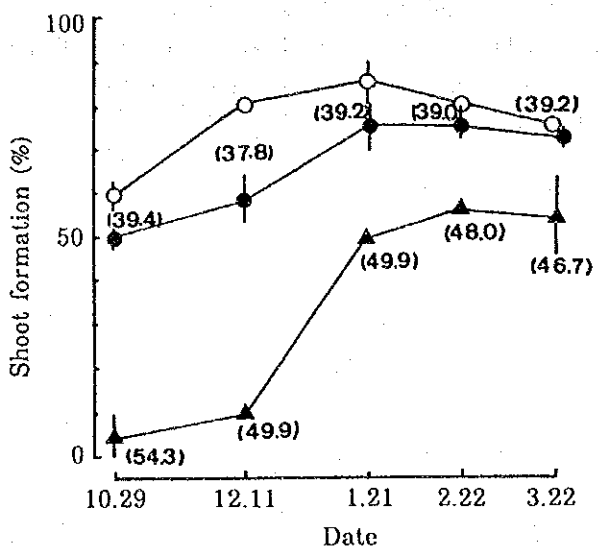

Fig. 2 Effects of sampling date and partial dehydration on shoot formation of winter buds after immersion into LN.

Numerals in parentheses indicate the water content of the buds before or after partial dehydration for 2 to 3 hr at 25°C. Winter buds were prefrozen to −20°C with a temperature decrease of 5°C/day prior to immersion into LN. Vertical bars: S.E. (Niino *et al.*, 1991b).

○—○ : Unfrozen control; Buds were partially dehydrated at 25°C for 2 to 3 hr.
●—● : Cooled to −196°C after partial dehydration.
▲—▲ : Cooled to −196°C without partial dehydration.

Table 2 **Shoot formation of excised shoot tips from mulberry winter buds stored at various temperatures for different periods of time**

Duration	Shoot formation (% ± S.E.)			
	−40°C	−70°C	−135°C	−196°C
1 month	0 ± 0	2.5 ± 2.5	72.5 ± 2.5	75.0 ± 10.0
6 months	0 ± 0	12.5 ± 2.5	75.0 ± 5.0	72.5 ± 2.5
1 year	–	12.5 ± 2.5	77.5 ± 2.5	70.0 ± 0
3.5 years	–	–	75.0 ± 5.0	75.0 ± 5.0

Material: *Morus bombycis* Koiz., cv. Kenmochi. Mulberry winter buds were slowly frozen to −30°C at the rate of 10°C/day prior to storage at various temperatures. Winter buds were thawed rapidly in a water bath at 37°C. After sterilization, the shoot tips were excised and plated on MS medium. Shoot formation of cryopreserved shoot tips was defined as the percentage of shoot tips inducing normal shoots 40 days after plating. Approximately 20 buds were treated for each set of duplications. (Niino *et al.*, unpublished).

(2) Cryopreservation of excised shoot tips from winter buds

Shoot tips excised from winter buds of apple survived after immersion into LN following slow freezing below −10°C without cryoprotectants (Katano *et al.*, 1983). Pear shoot tips excised from dormant buds also survived after immersion into LN following prefreezing below −40°C, regardless of the rewarming procedure applied (Moriguchi *et al.*, 1985). Suzuki *et al.* (1988a) demonstrated that excised shoot tips from dormant buds of Japanese pear were successfully cryopreserved. Excised shoot tips were dipped into a cryoprotectant solution containing 8% DMSO and 3% sucrose at 0°C for 2 hr. The shoot tips were slowly (0.5 °C /min.) frozen to −40°C and then immersed into LN. After rapid thawing in a water bath at 37°C, the shoot tips were plated on MS medium. The rate of shoot formation of the cryopreserved shoot tips was about 50%. However, almost all the shoots remained in the rosette form. This slow freezing method was applied to shoot tips from winter buds of cherry and honeysuckle (Suzuki *et al.*, 1988b).

Niino *et al.* (1992b) reported that dehydrated shoot tips excised from mulberry winter buds could be cryopreserved without any cryoprotectant. Excised shoot tips were placed on a sterilized filter paper in a parafilm-sealed Petri dish containing dry silica gel at 25°C. After dehydration for various periods

of time, the shoot tips were put in 2 ml cryotubes and immersed into LN. The shoot tips were then rewarmed by placing the cryotubes in a water bath at 37°C and were plated on MS medium. Shoot formation of dried shoot tips without or with exposure to LN at various water contents is shown in Fig. 3. The shoot formation rate increased markedly with decreasing water content from 30 to 20% and reached a maximum value (about 80%) at water content of about 19% (fresh weight base).

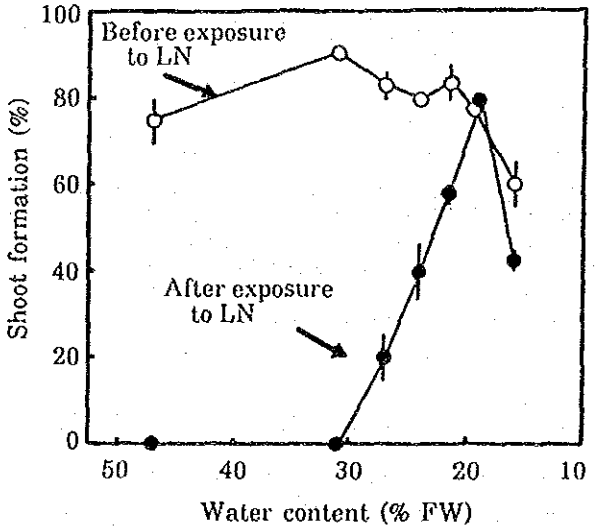

Fig. 3 Shoot formation rate of excised shoot tips from winter buds of mulberry trees without or with exposure to LN for various water contents.

Dehydrated shoot tips were placed in a cryotube and then immersed into LN. After 1 day, the cryotube was rapidly transferred into a water bath at 37°C. Shoot formation (%): percentage of shoot tips inducing normal shoots 40 days after plating. Approximately 20 shoot tips were tested for each of the 2 replications. Cultivar: Kenmochi. (Niino *et al.*, 1992b).

2) Cryopreservation of *in vitro*-grown shoot tips

Cryopreservation of plant meristems and cells has become an important method for the long term preservation of germplasm due to the limited area and maintenance required. *In vitro*-cultured plantlets are suitable for germplasm preservation. Plantlets are maintained under disease-free conditions and are mass propagated, the growth rate being easily controlled. For successful cryopreservation, it is essential to dehydrate cells sufficiently in order to avoid

the lethal effect of intracellular freezing during subsequent rapid cooling into LN. The cryogenic strategies for cultured cells and meristems are divided into four categories depending on the conditions of dehydration: freeze dehydration, partial osmotic and freeze dehydration, vitrification and air-drying method.

(1) Conventional slow freezing method

Many factors are involved in the successful cryopreservation of *in vitro*-grown shoot tips. The survival rate significantly increased by the adequate combination of cooling rates and prefreezing temperatures; kinds of cryoprotectant; preconditioning such as cold-hardening and/or preculture and post-thawing handling of shoot tips. Reed (1990) clearly demonstrated that cold-hardening and slow cooling significantly improved the recovery rates of shoot tips of all four pear cultivars tested. In her experiment, the pear plantlets were cold-hardened for 1 week at 22°C days for 8 hrs and −1°C for 16 hrs. Samples were frozen in a cryoprotectant (a mixture of each 10% of PG, glucose and DMSO in water) at the rate of 0.1°C/min to −40°C and then immersed into LN. Cryotubes were thawed for 1 min at 40°C. Regrowth rate of shoot tips ranged from 55% to 95% (Reed, 1990). By similar methods, *in vitro*-grown shoot tips of several woody plants were successfully cryopreserved in LN, including apple (Katano *et al.*, 1984), *Rubus* accessions (Reed and Lagerstedt, 1984), *Vaccinium* species (Reed, 1989) and mulberry (Niino, 1990; Niino and Oka, 1990). However, this conventional slow freezing method requires a complicated procedure with controlled freezing equipment. Therefore, a simpler procedure should be developed.

(2) Partial osmotic and freeze dehydration (Simple freezing)

Osmotic dehydration using a 2 or 3 M solution of cryoprotectant at room temperature may be an effective method for avoiding lethal intracellular freezing during cooling to −30°C or −40°C, making it possible to simplify the conventional cryoprotective and freezing procedures (Sakai *et al.*, 1991b). Niino *et al.* (1991a) reported that *in vitro*-grown shoot tips of the pear cultivar 'Beurré d'Amanlis' could be preserved in LN by a simple freezing method. In their experiments, the hardened shoot tips were dipped into a solution containing 0.4 M sorbitol and 1.25 M glycerol at 5°C for 18 hr. The shoot tips were placed in 2.0 ml cryotubes with 0.6 ml of fresh solution. The cryotubes were placed in a

freezer at −30°C and kept for 1 hr prior to immersion into LN. Cryotubes were thawed in water at 37°C. The cryoprotectant was drained from the cryotubes and replaced with 1.25 M glycerol. Shoot tips were transferred onto sterilized filter paper discs and plated on medium for regrowth (Fig. 4). The average rate of shoot formation was about 70%. This simple freezing method was successfully applied to eight pear cultivars (Table 3).

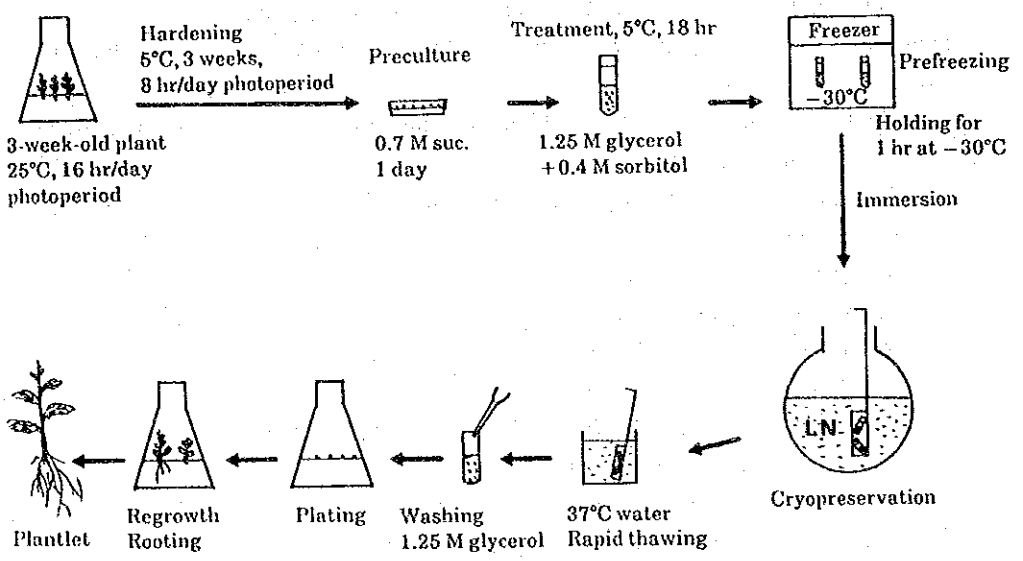

Fig. 4 Schematic protocol of simple freezing method.

Table 3 Shoot formation rate of cryopreserved *in vitro*-grown shoot tips of eight pear cultivars by simple freezing method

Cultivar	Survival (% ± S.E.)	Shoot formation (% ± S.E.)
Pyrus communis		
cv. Joséphine de Malines	55 ± 7	33 ± 5
cv. Idaho	63 ± 5	48 ± 3
cv. Beurré Jean Van Geert	80 ± 6	60 ± 4
cv. Duchesse d'Angouleme	73 ± 6	65 ± 7
cv. Beurré Superfin	60 ± 4	38 ± 3
Pyrus pyrifolia		
cv. Senryo	85 ± 5	70 ± 4
cv. Yoshino	83 ± 5	50 ± 4
cv. Hokkaiwase	80 ± 5	60 ± 5

Hardened shoot tips were cryoprotected with a mixture of 1.25 M glycerol and 0.4 M sorbitol at 5°C for 18 hrs. The shoot tips were placed in a 2.0 ml cryotube with 0.6 ml of a cryoprotective solution. They were then placed in a freezer at −30°C for 1 hr prior to immersion into LN. (Niino *et al.*, 1991a)

(3) Vitrification method

Vitrification refers to the physical process by which a concentrated aqueous cryoprotective solution solidifies into metastable glass at sufficiently low temperatures without the occurrence of crystallization. Complete vitrification eliminates the damaging effects of intra- and extra-cellular crystallization, hence the high rate of meristem survival. Successful vitrification requires careful control of the highly concentrated vitrification solution to prevent injury associated with toxicity or excess osmotic stress during dehydration, in order for the cells to be dehydrated to become vitrified cells upon rapid cooling into LN. The vitrification procedure eliminates the need for a controlled slow freezing apparatus and enables the meristems to be cryopreserved by direct immersion into LN (Sakai *et al.*, 1990; Yamada *et al.*, 1991).

Niino *et al.* (1992c) developed a successful vitrification method of shoot from tissue-cultured plantlets of apple and pear. Three-week old plantlets were cold-hardened 5°C for 3 weeks under 8 hr photoperiod. The cold-hardened shoot tips were precultured at 5°C for 1 day on MS medium supplemented with 0.7 M sucrose under 8 hr photoperiod. Following the preculture, ten shoot tips were transferred to 1.0 ml of the PVS2 vitrification solution (Sakai and Kobayashi, 1990; Sakai *et al.*, 1990; Sakai *et al.*, 1991a) in 2 ml plastic cryotubes at 25°C for various periods of time. During the PVS2 treatment, PVS2 was replaced twice. Shoot tips were suspended finally in 0.6 ml of the fresh PVS2 solution immediately before immersion and kept in LN. After rapid warming in a water bath at 25°C, PVS2 was drained from the cryotubes and replaced twice with liquid MS medium containing 1.2 M sucrose. After the surface solution was blotted, shoot tips were transferred onto 0.8% agar-MS medium and grown under standard conditions (Fig. 5).

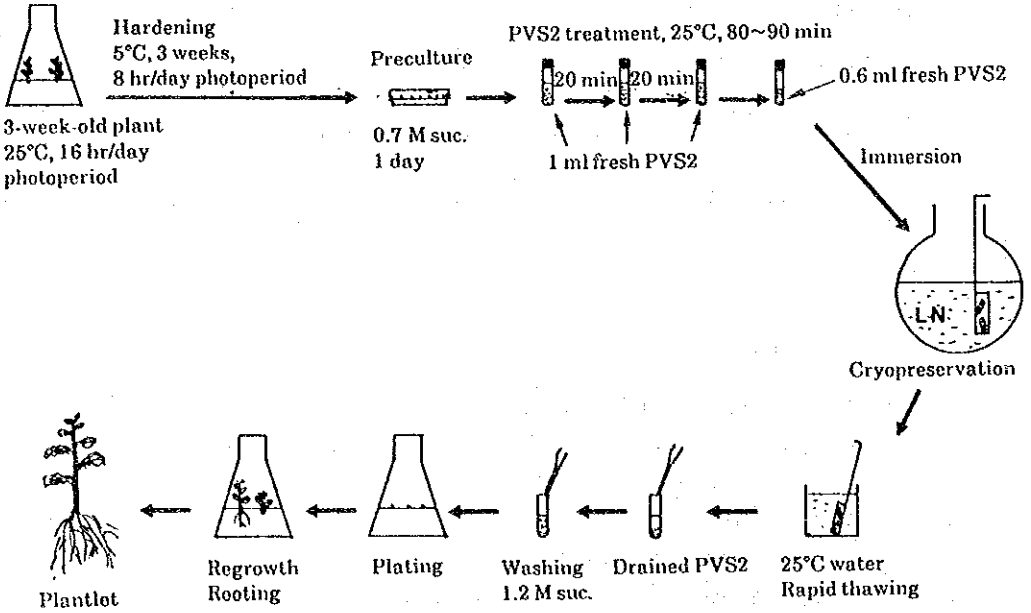

Fig. 5 Schematic protocol of vitrification.

The shoot formation rate of the vitrified apple shoot tips increased gradually with the duration of exposure to PVS2 and reached a maximum value after 80 min of exposure (Fig. 6). Shoot formation rates by the same vitrification procedure was compared among five apple species or cultivars and eight pear

cultivars. High rates of shoot formation were observed in apples and pears (Table 4) (Niino *et al.*, 1992c). High rates of shoot formation were also obtained in 12 mulberry cultivars belonging to 7 species except for one cultivar by cooling to −196°C using the same vitrification system (Niino *et al.*, 1992a). Even less- or non-hardy mulberry cultivars in Southeast Asia showed a moderate or high shoot formation.

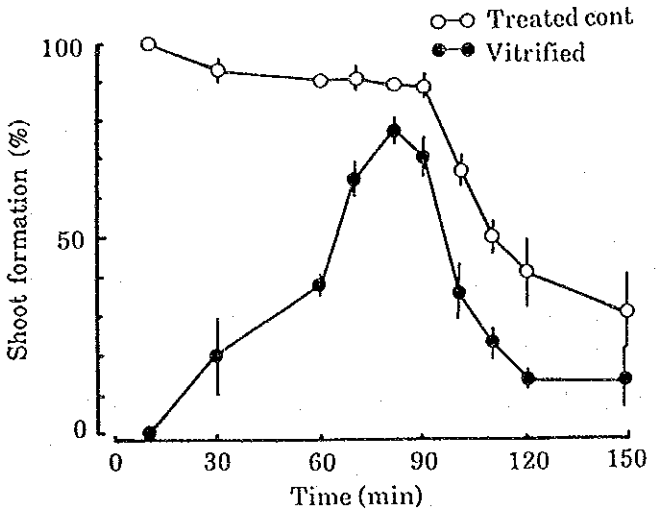

Fig. 6 Effect of exposure time to PVS2 at 25°C on the shoot formation of apple shoot tips cooled to − 196°C after vitrification.

Material: *Malus domestica* cv. Fuji. Cold-hardened, precultured shoot tips were treated with PVS2 for different periods of time at 25°C and then directly immersed into LN for 1 day. Treated control: same as vitrified meristems, without cooling to −196°C. Cold-hardening was carried out for 3 weeks at 5°C (8hr photoperiod). Shoot tips excised from cold-hardened *in vitro* plantlets were precultured on agar MS medium supplemented with 0.7 M sucrose for 1 day at 5°C. Approximately 20 shoot tips were tested for each of the two replications. (Niino *et al.*, 1992c).

Table 4 Shoot formation of *in vitro*-grown shoot tips of apple and pear cooled to −196°C by vitrification

Cultivar or species	Shoot formation (% ± S.E.)
Malus domestica	
cv. Golden Delicious	55.0 ± 5.0
cv. hybrid 423-1	77.5 ± 7.5
Malus paradisiaca	
M. 9	45.0 ± 5.0
M. 26	67.5 ± 2.5
Malus prunifolia	70.0 ± 5.0
Pyrus communis	
cv. Fondante Thirriot	57.5 ± 7.5
cv. Early Seckel	61.1 ± 11.1
cv. Beurré Jean Van Geert	67.0 ± 3.0
cv. Doyenné du Comice	50.0 ± 15.0
cv. Beurré d'Amanlis	72.5 ± 2.5
Pyrus pyrifolia	
cv. Senryo	60.0 ± 0
cv. Yoshino	40.0 ± 0
cv. Hokkaiwase	70.0 ± 0

Cold hardening: 3 weeks at 5°C (8 hr photoperiod); Preculturing: at 5 °C for 1day on MS agar medium supplemented with 0.7 M sucrose. Hardened, precultured shoot tips were treated with PVS2 for 80 min (apple) or 90 min (pear) at 25°C prior to immersion into LN. Shoot formation : percentage of shoot tips inducing shoots 40 days after plating. Approximately 20 shoot tips were tested for each of the two replications. (Niino *et al.*, 1992c)

When vitrification was successful, the shoot tips resumed growth within 3 to 5 days after reculture, and started to develop shoots within two or three weeks without intermediary callus formation. Photo. 2 shows the formation of shoots from the vitrified apical shoot tips after one month of culture. No morphological abnormalities were observed in the plants developed from

cryopreserved shoot tips. This method appears to be suitable for the cryopreservation of shoot tips from *in vitro*-grown plantlets of woody plants.

(4) Air-drying

Air-drying is a simple and reliable method for *in vitro*-grown shoot tips in the same way as vitrification. In air-drying method, the induction or modification of dehydration tolerance is essential for the successful cryopreservation (Uragami *et al.*, 1990; Nitzsche, 1980; Senaratna *et al.*, 1990; Anandarajah and McKersie, 1990). Cold-hardened shoot tips of *in vitro*-grown apple shoots were precultured at 5°C on agar MS medium supplemented with 0.7 M sucrose for one day, and dehydrated to various water contents. Dehydrated shoot tips were placed in cryotubes and then cooled to −196°C. The cryotubes were rapidly transferred into a water bath at 25°C and then the shoot tips were plated on MS medium. The rates of shoot formation of the dried apple shoot tips without or with exposure to LN for various water contents are shown in Fig. 7. The shoot formation rate increased from 0% to about 50% in response to the decrease in water content from 45% to 22%. Subsequently, the shoot formation rapidly decreased due to desiccation injury.

Alginate-encapsulation dehydration technique is another drying method. This method is easy to handle and simplifies the dehydration process. The alginate-coated shoot tips from winter hardy mulberry buds were placed in a medium supplemented with 1.0 M sucrose for 16 hr at 5°C and then dehydrated up to about 22-25% prior to immersion into LN. Shoot formation rate was approximately 70% after warming (Niino *et al.*, 1992b).

Alginate-coated shoot tips of *in vitro*-grown pear, grape and apple were successfully cryopreserved following air-drying (Dereuddre *et al.*, 1990; Plessis *et al.*, 1991; Niino and Sakai, 1992). The protocol of cryopreservation of *in vitro*-grown apple shoot tips coated with alginate gel is shown in Fig. 8. Three-week old plantlets were cold-hardened at 5°C for 3 weeks under 8 hr/day photoperiod. Progressive preculture was performed at 5°C by daily transfer of the shoot tips onto MS media supplemented with 0.1, 0.4 or 0.7 M sucrose under 8 hr/day photoperiod. Alginate gel beads containing shoot tips were prepared according to the technique of Bapat *et al.* (1987) and Dereuddre *et al.* (1990) with some modifications. Shoot tips were suspended in a calcium-free culture medium supplemented with 3%(W/V) Na-alginate solution and 0.5M sucrose. The mixture was placed in 50 ml of culture medium containing 100 mM calcium

chloride and 0.5 M sucrose using a dispenser with a sterile tip and kept for 30 min at 25 °C. Beads about 5 mm in diameter containing one shoot tip were placed in MS medium supplemented with 1.0 M sucrose and kept for 16 hr at 5 °C. After treatment with 1.0 M sucrose, the surface solution was wiped off and the beads were subjected to dehydration in Petri dishes containing 50 g dry silica gel kept at 25 °C. Dried beads were put in 2-ml cryotubes and immersed into LN. Samples were warmed by placing the cryotubes in a water bath at 25°C. Regrowth of the alginate-coated shoot tips was achieved by transfer onto the appropriate MS medium under standard culture conditions (Fig. 8).

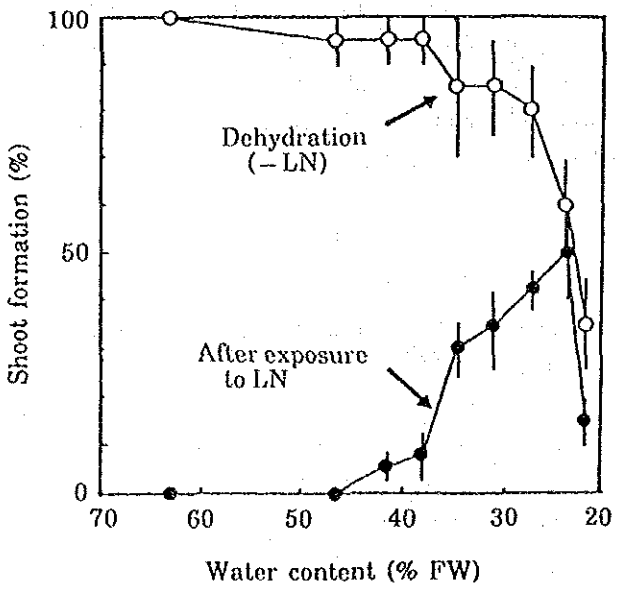

Fig. 7 Shoot formation of *in vitro*-grown apple shoot tips for various water contents without or with exposure to LN.

Material: *M. domestica* cv. Fuji. Cold-hardening: 3 weeks at 5°C; Preculture: at 5°C on agar MS medium supplemented with 0.7 M sucrose for 1 day at 5°C. Dehydrated shoot tips were placed in a cryotube and then transferred on agar MS medium without (−LN) or with (LN) cooling to −196°C. After 1 day, the cryotube was rapidly transferred into a water bath at 25°C. Shoot formation rate: percentage of shoot tips inducing normal shoots 30 days after plating. Approximately 20 shoot tips were tested for each of the two replications. (Niino and Sakai, 1992).

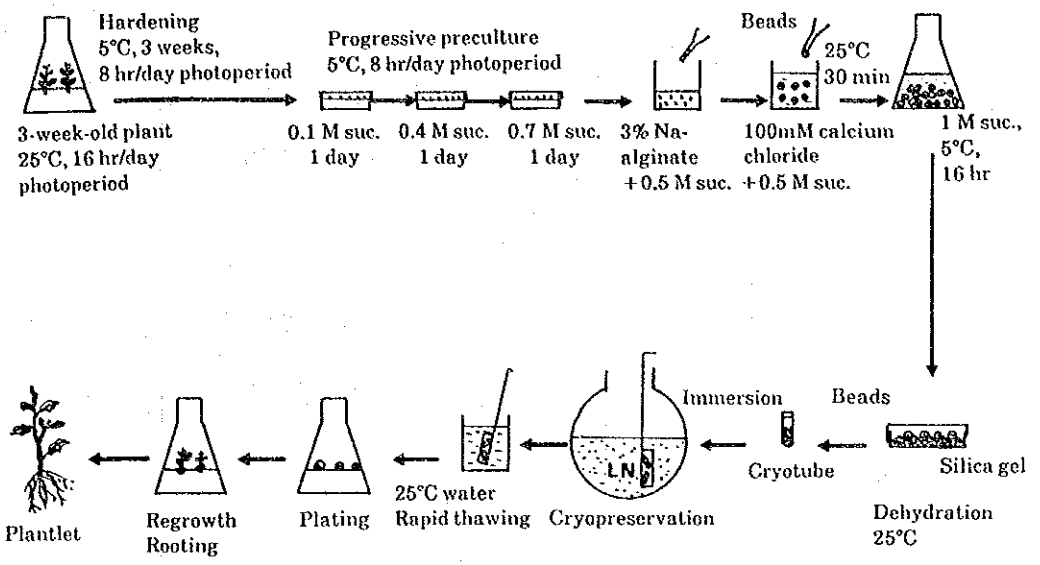

Fig. 8 Schematic protocol of alginate-encapsulation dehydration technique.

Shoot formation rate of dehydrated *in vitro*-grown apple shoot tips coated with alginate gel without or with cooling at −196°C is shown in Fig. 9. The maximum rate of shoot formation was obtained at a 33% water content. The alginate-coated shoot tips with a 37% water content yielded a very low rate of shoots (15%) when directly immersed into LN, while prefreezing at −20°C or −30°C following dehydration induced a shoot formation rate of about 70% (Table 5). On the other hand, the alginate-coated shoot tips with a 33% water content which were prefrozen at −20°C or −30°C for 1 day showed a little better shoot formation rate of 77 to 83% compared with the 73% of non-prefreezing. This modified encapsulation-dehydration technique was successfully applied to shoot tips from *in vitro*-grown plants of three apple, one mulberry and three pear cultivars (Niino and Sakai, 1992). Dried alginate-coated shoot tips stored at −135°C for more than 5 months showed a slight or no decrease in the rate of shoot development compared with those stored in LN (Table 6). Photo. 3 shows the development of alginate-coated shoot tips and plantlets from the shoot tips cryopreserved after dehydration. No morphological abnormalities were observed in the plants developed from cryopreserved shoot tips. The results presented here confirmed that this technique is suitable for cryopreservation of meristems.

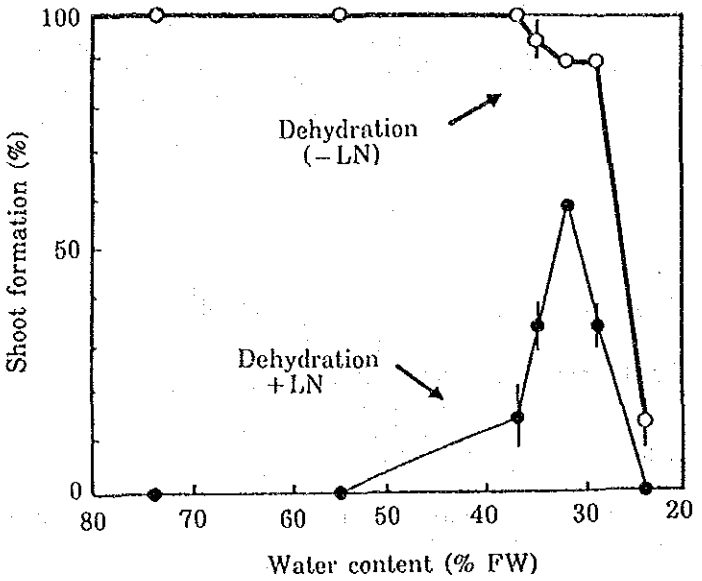

Fig. 9 **Shoot formation rate of dehydrated *in vitro*-grown apple shoot tips coated with alginate gel, without or with immersion into LN.**

Material: *M. domestica* cv. Fuji. Cold-hardening: 3 weeks at 5°C; Preculture: on agar MS medium supplemented with 0.7 M sucrose for 1 day at 5°C. Cold-hardened, precultured shoot tips in alginate-gel beads supplemented with 0.5 M sucrose were placed in MS medium supplemented with 1.0 M sucrose at 5°C for 16hr. Dehydrated beads were placed in a cryotube and transferred on agar MS medium without (−LN) or with (LN) cooling to −196°C. After 1 day, the cryotube was rapidly transferred into a water bath at 25°C. Shoot formation rate: percentage of shoot tips inducing normal shoots 40 days after plating. Approximately 20 shoot tips were tested for each of the two replications. (Niino and Sakai, 1992).

Table 5 **Effect of prefreezing at −20°C or −30°C for 16 hr after partial dehydration on shoot formation of *in vitro*-grown apple shoot tips coated with alginate gel and cooled to −196°C**

Temperature for prefreezing (°C)	Water content of beads (%)	Shoot formation (% ± S.E.)
−20	37	61 ± 4
−30	37	72 ± 6
Non-freezing[a]	37	15 ± 5
−20	33	77 ± 4
−30	33	83 ± 4
Non-freezing[a]	33	73 ± 4

Material: *Malus domestica*, cv. Fuji. Cold-hardened: 3 weeks at 5°C. Progressive preculturing: on MS agar media with daily increased concectration (0.1 M, 0.4 M and 0.7 M). Cold-hardened, precultured shoot tips were encapsulated in alginate-gel beads containing 0.5 M sucrose and then treated with 1.0 M sucrose at 5°C for 16 hr. These beads were subjected to dehydration for 6 or 7 hr (water content; about 37 or 33%) at 25°C prior to immersion into LN. Shoot formation: percentage of shoot tips inducing normal shoots 40 days after plating. Approximately 10 shoot tips were tested for each of the 4 replications.
[a] Cooled to −196°C directly from room temperature. (Niino and Sakai, 1992).

Table 6 **Shoot formation of dehydrated alginate-coated apple shoot tips stored at various temperatures**

Temperature and duration	Shoot formation (% ± S.E.)
−196°C, 3 days	73 ± 3
−196°C, 5 months	73 ± 3
−135°C, 3 days	77 ± 3
−135°C, 5 months	77 ± 3
−70°C, 3 days	3 ± 3
−70°C, 5 months	7 ± 3

Material: *Malus domestica*, cv. Fuji. Cold-hardened: 3 weeks at 5°C. Progressive preculture: on MS agar media with daily increased concentration (0.1 M, 0.4 M and 0.7 M). Cold-hardened, precultured shoot tips were encasulated in alginate gel beads containing 0.5 M sucrose and then placed in MS medium containing 1.0 M sucrose at 5°C for 16 hr. The encapsulated shoot tips dehydrated for 7 hr (water content; about 33%) were exposed to different temperatures. Shoot formation: percentage of shoot tips inducing normal shoots 40 days after plating. Approximately 10 shoot tips were tested for each of the 3 replications. (Niino and Sakai, 1992).

5. Conclusion

Protocol of cryopreservation of two materials, shoot tips of winter buds and *in vitro*-grown plantlets was outlined. Cryopreservation of winter vegetative buds was found to be suitable for the preservation of gene sources of woody plants. Slow freezing method following partial dehydration of winter buds and air-drying or alginate-coated dehydration technique may be applied for base collection.

Recently, the author has observed that the mulberry winter buds cryopreserved in LN or −135°C (deep freezer) for 3.5 years were able to regenerate plantlets after plating on culture medium. No morphological abnormalities were observed in the mulberry trees developed from cryopreserved shoot tips. Chromosome analysis showed that all the regenerated plants examined were diploid with 28 chromosomes.

In vitro-culture plants are suitable for germplasm preservation. The vitrification procedure, simple freezing method and the encapsulation-dehydration technique with *in vitro*-grown shoot tips may eliminate the need for controlled slow freezing devices. Meristems can be cryopreserved by direct

immersion into LN or with pre-freezing. These three cryogenic methods appear to be suitable for routine cryopreservation of *in vitro*-grown shoot tips of woody plants.

In our laboratory, approximately about 470 apple cultivars, 410 pear species and cultivars and about 420 cultivars of mulberry are maintained in the field. Concurrently, winter buds of these accessions have been cryopreserved in a deep freezer at −135°C for more than 3.5 years. About 300 pear species or cultivars and about 200 mulberry cultivars have been stored as *in vitro*-grown plantlets in test tube at 5°C for more than 2 years. Cryopreservation of *in vitro*-grown shoot tips of mulberry was initiated 3 years ago. In addition, basic studies have been carried out on long-term viability, genetic stability, improvement of procedures and basic cell survival mechanisms, at biochemical and biophysical levels. Further technical improvements may contribute to a wider applicability of cryogenic techniques to various species.

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Photo. 1 Plantlets developed from shoot tips excised from raspberry winter buds immersed into LN. (20 days after reculture). (Niino *et al.*, 1990).

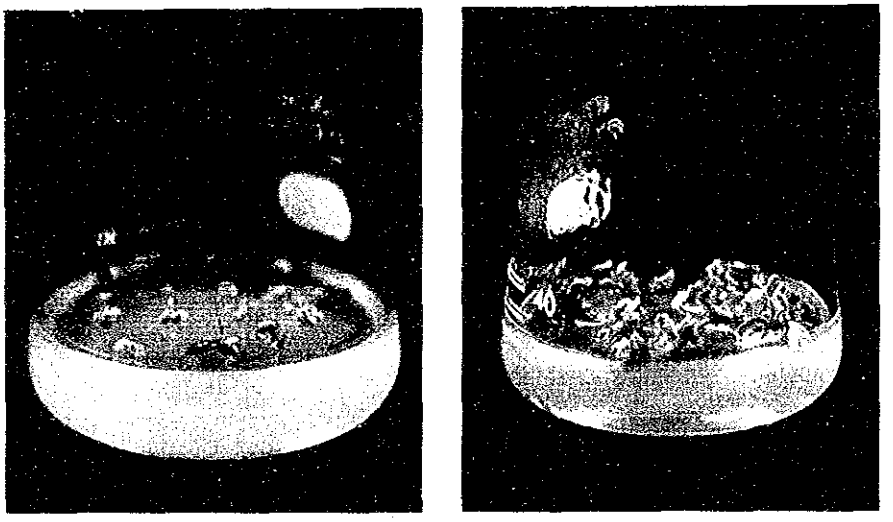

Photo. 2 Plantlets developed from *in vitro*-grown apple shoot tips cooled to −196°C by vitrification, 10 days (left) and 25 days (right) after reculture. Material: *Malus domestica* cv. Fuji.

Photo. 3 Plantlets regenerated from cryopreserved *in vitro*-grown apple shoot tips for 30 days after plating. Material: *M. domestica* cv. Fuji.
Upper: Left; Dehydrated apple shoot tips embedded in alginate-coated beads after thawing; Right: 5-10 days after plating. Lower: 30 days after plating. (Niino and Sakai, 1992).

Cryopreservation of Forage Crops

by

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CONTENTS

1. Introduction

Many important forage crops are outcrossing species and their seeds are highly heterozygous. Although seeds are the means of commercial propagation and distribution, plant breeders and researchers must maintain the genotypes vegetatively, particularly the basic plants of cultivars that are used as parent materials for seed production. Until now, the genotypes have usually been maintained as potted plants in the nursery or green house. A great deal of time and labour is required for the maintenance of these plants, and moreover, some loss of genotypic lines may occur due to accidents or diseases. Shoot meristems are suitable materials for the preservation of virus-free genetic resources which are capable of genetically stable mass proliferation.

Recently, plant cell culture has been increasingly used to obtain genetically modified cells and plants. Thus, the preservation of cultured cells with unique attributes such as high regenerative ability is assuming a greater importance.

Cryopreservation may be a useful method for long-term storage of genetic resources because of the minimum space and maintenance. The long-term storage of frozen shoot meristems and plant cells in LN results in an almost complete cessation of biochemical activities, hence reducing risk of changes in the physiological and genetic characters of the preserved lines (Kartha, 1985; Sakai, 1985; Withers, 1985).

Successful cryopreservation of shoot meristems and plant cells was achieved using the conventional slow freezing method in many species of plants (Kartha, 1985; Sakai, 1985; Withers, 1985). However, this cryogenic method requires a controlled freezing equipment and a complicated cryoprotective treatment for 1 hr or more at 0°C. Thus, the development of simpler procedures is essential. In recent years, a simple vitrification procedure (Sakai *et al.*, 1990; Sakai *et al.*, 1991a) and simple freezing method (Sakai *et al.*, 1991b) have been developed.

There are only a few reports on the application of cryopreservation technique to forage crops (Finkel *et al.*, 1985). We carried out experiments to develop methods for the cryopreservation of apical meristems and meristematic callus of a forage legume, white clover (*Trifolium repens* L.) (Yamada *et al.*, 1991a, b). In this paper, the author describes a successful method of cryopreservation of apical meristems and meristematic calli of white clover

utilizing three different cryogenic protocols: the conventional slow freezing method, simple vitrification method and simple freezing method.

2. Cryopreservation of Apical Meristems

1) Conventional Slow Freezing Method

The conventional slow freezing method has been generally applied using a programmed freezer to about −40°C in the presence of suitable cryoprotectants containing 5 - 10% DMSO and 5 - 15% sugar or sugar alcohol (Kartha, 1985; Sakai, 1985; Withers, 1985). In white clover, high rates of survival and plant regeneration were obtained when apical meristems were frozen to −40°C at a rate of 0.3°C/min in the presence of 10% DMSO and 10% glucose, then plunged into LN and thawed in a water bath at 40°C for 1 min (Yamada *et al.*, 1991a). Fig. 1 shows the procedure of conventional slow freezing method developed for white clover.

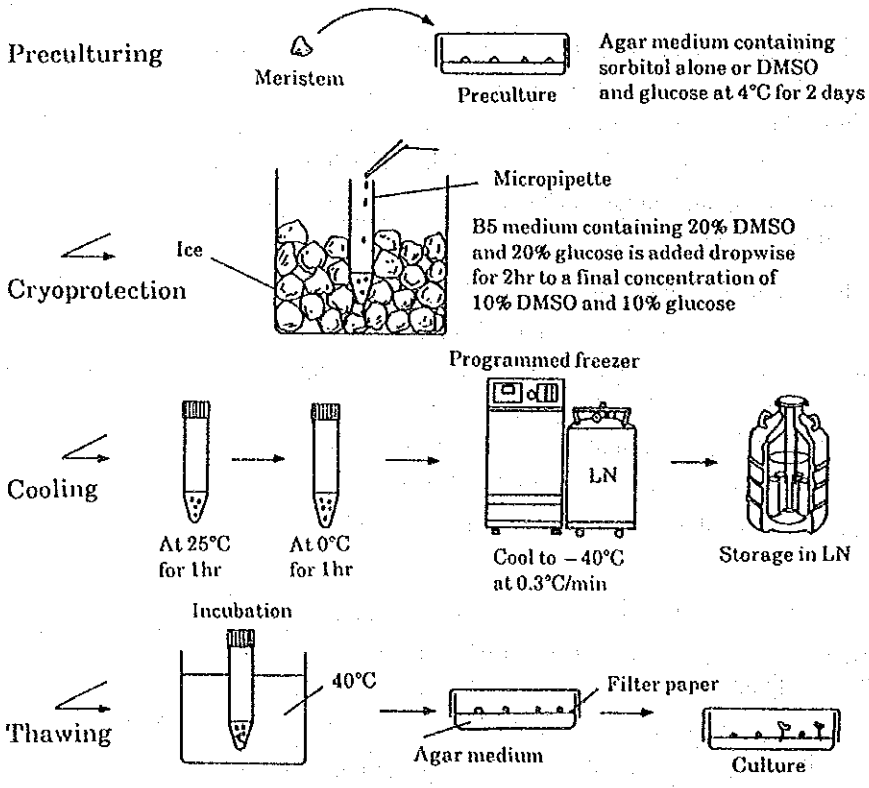

Fig. 1 Procedure of conventional slow freezing method for apical meristems of white clover.

The survival rates of the meristems frozen in LN under optimal conditions exceeded 80% in all the cultivars tested (Table 1). Survival rates were not correlated with the field cold hardiness of the cultivars tested.

Many factors are involved in the successful cryopreservation of shoot meristems. In white clover, the major factors are preculture and post-thawing handling. Preculture on a solidified agar B5 medium supplemented with DMSO and glucose or with sorbitol alone at 4°C for 2 days under continuous light resulted in a significant increase in the survival rate of shoot meristems cooled in LN. After rapid thawing in 40°C water, the regrowth of the LN stored meristems was achieved by transfer without washing onto filter paper discs over agar-solidified B5 medium. In addition to triggering injury by rapid deplasmolysis, post-thawing washing may damage the cells due to the removal of the solutes lost by the specimens (Withers and King, 1979). The filter paper regrowth technique may have enabled the cryoprotectants to leak into agar slowly, resulting in the considerable enhancement of normal shoot formation. This technique not only simplified the post-thawing handling, but also enabled to avoid unnecessary washing damage.

Table 1 Survival of apical meristems of different cultivars of white clover frozen in LN by conventional slow freezing method

Cultivar	Cold hardiness[a]	Survival (% ± S.E.)
Kitaooha	8	96 ± 4
Kent Wild	8	86 ± 4
Nora	8	78 ± 11
Grasslands Huia	6	92 ± 2
Haifa	2	82 ± 4
Nolin's Improved	2	83 ± 6

Survival was defined as the percentage of apical meristems resuming normal growth 1 month after plating.

[a] The degree of cold hardiness in the field was rated from 1 (tender) to 9 (hardiest).

Some of samples were stored in LN for 5 and 10 months. Storage in LN for different periods of time did not significantly different depending on the duration of the storage in LN, suggesting that long-term storage of white clover germplasm using cryogenic techniques may be possible.

2) Simple Cryogenic Procedures

(1) Simple Vitrification Method

Vitrification refers to the physical process by which a concentrated aqueous solution solidifies into metastable glass (glassy solid) at sufficiently low temperatures without the occurrence of crystallization. At sufficiently low temperatures, a highly concentrated cryoprotective solution becomes so viscous that it solidifies into metastable glass at an adequate cooling rate. The vitrification procedure does not require a controlled slow freezing equipment and allow cells and meristems to be cryopreserved by direct transfer to LN. Recently, Sakai *et al.* (1990, 1991a) succeeded in developing a method for the cryopreservation of nucellar cells of navel orange by the use of a simple vitrification procedure (one step method). The author and co-workers also succeeded in developing a simple vitrification method for the cryopreservation of apical meristems of white clover with subsequently normal shoot development (Yamada *et al.*, 1991b).

Fig. 2 shows the procedure of the simple vitrification method developed for white clover. Apical meristems were precultured on a solidified agar-B5 medium containing 1.2 M sorbitol in a Petri dish at 4°C under continuous illumination for 2 days. Precultured meristems were sufficiently dehydrated with a highly concentrated cryoprotective mixture (designated as PVS2) prior to immersion into LN. PVS2 consists of 30% (w/v) glycerol, 15% (w/v) EG and 15% (w/v) DMSO in B5 medium containing 0.4M sucrose (pH 5.8) (Sakai *et al.*, 1990; Sakai *et al.*, 1991a). Upon warming at 10°C/min from −196°C in a DSC, the vitrified PVS2 showed a 'glass transition' at about −115°C, a devitrification (crystallization) at about −75°C and a melting point at about −36°C (Sakai *et al.*, 1990). Precultured meristems were placed in a 1.8-ml cryotube and then 1 ml of PVS2 was added and mixed. After PVS2 was removed with a Pasteur pipette, 1 ml of fresh PVS2 was added, replaced twice and the materials at 25°C or 0°C for different periods of time. The meristems treated with PVS2 were directly plunged into LN. The cryotubes in which meristems were finally suspended in 0.5 ml of PVS2 were plunged into LN. The cooling rate was about

280°C/min. After rapid warming in water at 25°C (warming rate: about 280°C/min), PVS2 was drained from the cryotubes and replaced twice with liquid B5 medium containing 1.2 M sucrose. Then the meristems were transferred onto filter paper discs over agar-B5 medium containing 0.5 mg/l BA in a Petri dish. After one day, the meristems were transferred onto a fresh paper disc over same agar medium.

The supply of a high level of sugar (Dereuddre *et al.*, 1988) or sorbitol (Chen *et al.*, 1984) during the preculture was reported to be essential for improving the survival of cryopreserved cells and meristems. In white clover, preculture on B5 medium supplement with 1.2 M sorbitol for 2 days at 4°C led to the highest survival rate of apical meristems cooled in LN by vitrification (Table 2). It was suggested that the high osmolarity created by sucrose may have induced the synthesis of ABA. However, little is known about possible beneficial events that may occur in the cells and meristems during the preculture.

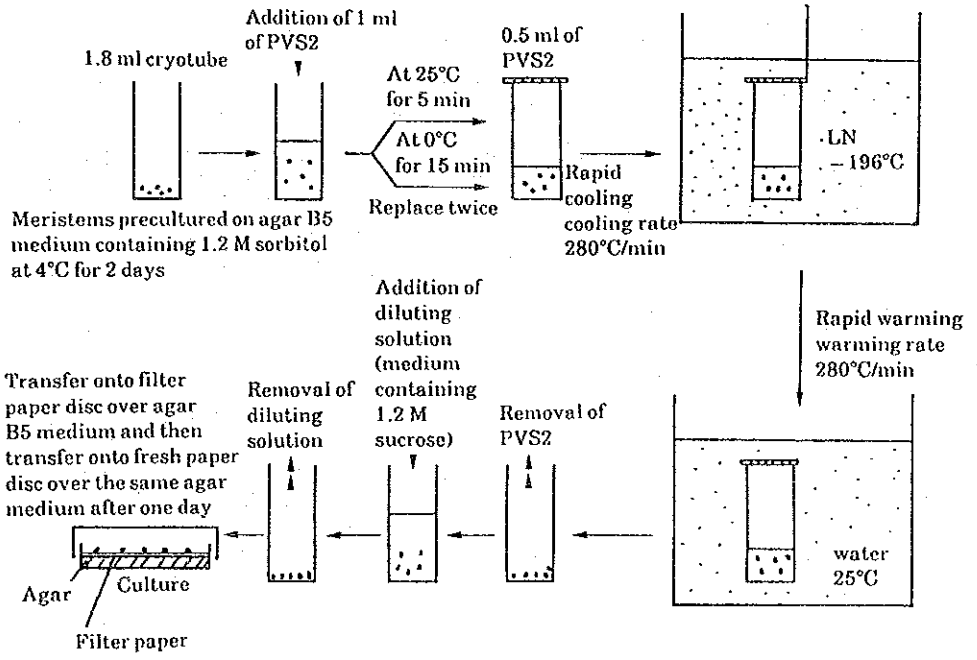

Fig. 2 Procedure of simple vitrification method for apical meristems of white clover.

Table 2 **Effect of preculture on the survival of white clover apical meristems cooled to −196°C by vitrification**

Preculturing condition	Survival (% ± S.E.)
Control	9 ± 5
Only B5 midium	32 ± 4
5% DMSO + 5% glucose	69 ± 3
0.6 M sorbitol	42 ± 2
0.8 M sorbitol	43 ± 6
1.0 M sorbitol	74 ± 4
1.2 M sorbitol	85 ± 4
LSD ($P = 0.05$)	10

Survival was defind as the percentage of vitrified-warmed meristems producing shoot growth 3 weeks after planting.

To determine the optimal time of exposure to PVS2 at 25°C or 0°C, the meristems were treated with PVS2 for different periods of time prior to immersion into LN. Exposure to PVS2 resulted in time-dependent survival (Fig. 3). The highest survival was obtained with meristems treated with PVS2 for 5 min at 25°C or 15 min at 0°C, respectively. The meristems treated with PVS2 for 3 - 7 min at 25°C or up to 25 min at 0°C without cooling in LN (treated control) maintained high levels of survival rates (80 - 90%) which however decreased with increasing exposure.

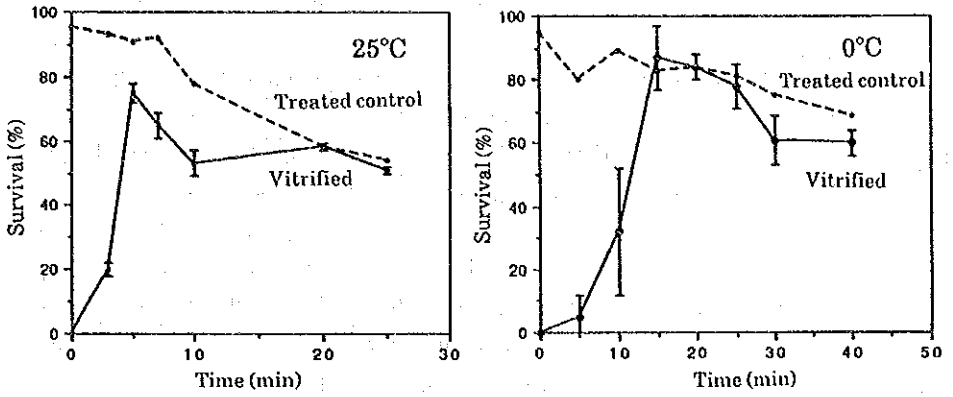

Fig. 3 Effect of exposure time to PVS2 at 25°C or 0°C on the survival of meristems cooled to −196°C by vitrification.
Bar represents standard error. Treated control: as in vitrified meristems, without cooling to −196°C.

The survival rate of *T. repens* following vitrification was compared with that of two other species (for 5 min at 25°C or for 15 min at 0°C). Red clover (*T. pratense*), a hardy species and Egyptian clover (*T. alexandrinum*), a non-cold hardy species showed a moderate survival rate (about 50%) (Table 3).

Table 3 Survival of apical meristems from three *Trifolium* species cooled to −196°C by vitrification

Species and cultivar	Hardiness	Survival (%±S.E.)	
		0°C, 15 min	25°C, 5 min
Trifolium repens Kitaooha	Very hardy	83 ± 4	80 ± 12
T. pratense Sapporo	Hardy	55 ± 13	53 ± 4
T. alexandrinum Camel	Non hardy	48 ± 4	50 ± 4

Survival was defined as the percentage of vitrified-warmed meristems producing shoot growth 3 weeks after plating.

Successfully vitrified and warmed apical meristems retained green continuously after reculture, resumed growth in about 3 days after reculture, and developed shoots within 10 days without intermediary callus formation. Fluorescence microscopic examination of longitudinal sections through the meristematic dome of vitrified meristems after 4 days of reculture revealed that in most of the meristems, the dome appeared to be viable based on FDA staining (Photo. 1). Photo. 2 shows the proliferation of the shoots developed from a vitrified and warmed apical meristem after three weeks of culture. Almost all the shoot formed roots on hormone free B5 medium (Photo. 3) and were successfully transferred to pots. No morphological abnormalities were observed in the plants developed from cryopreserved meristems.

(2) Simple Freezing Method

Sakai *et al.* (1991b) developed a simple freezing method for the cryopreservation of cells of navel orange. In this method, cells were partially dehydrated with 2 or 3 M glycerol and 0.4 M sucrose at 25°C for 10 min and then additionally freeze-dehydrated by direct transfer to a freezer at −30°C prior to immersion into LN.

Fig. 4 shows the procedure of a simple freezing method developed for white clover. After preculture on solidified B5 agar medium containing 1.2 M sorbitol at 4°C for 2 days, the meristems were placed in 1.8 ml cryotubes and a cryoprotective solution (B5 medium containing 3 M glycerol plus 0.4 M sucrose) was added and mixed. The cryoprotective solution was replaced twice and kept at 25°C for 10 min. The meristems were finally suspended in 0.5 ml of the cryoprotective solution in the cryotubes. Samples were frozen by direct transfer to a freezer at −30°C for 60 min prior to immersion into LN. Samples were frozen spontaneously after supercooling to about −18°C and then cooled to −30°C at about 1°C/min (Sakai *et al.*, 1991b). After storage in LN, the samples were thawed rapidly in a water bath at 40°C. After removal of the cryoprotective solution, the meristems were expelled into liquid B5 medium supplemented with 1.2 M sucrose. Then the meristems were transferred onto filter paper discs over agar medium as same in the case of the simple vitrification method.

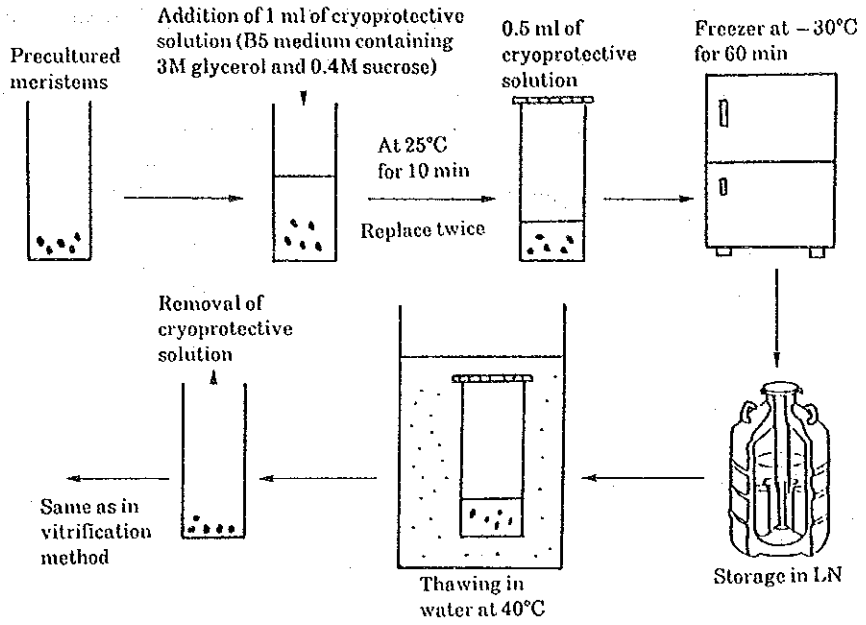

Fig. 4 Procedure of the simple freezing method for apical meristems of white clover.

The survival rates of the apical meristems of white clover (cv. Kitaooha) were compared among three cryogenic protocols: the conventional slow freezing method, the simple freezing method and the simple vitrification method (Table 4). The survival rates obtained in the three cryogenic protocols were not significantly different. The simple freezing method was successfully applied to the apical meristems of white clover.

Based on Dr. Sakai's personal communication, this simple freezing method produced a high rate of survival for rice and tobacco cells without requiring preculturing. This method may be applicable to wide range of plant materials, in particular, less resistant cells. The main advantages of this simple freezing method are that (1) the time required for slow freezing and time used to equilibrate cells in cryoprotective solutions at 0°C are significantly reduced, and (2) controlled-rate freezing equipment and ice seeding of the extracellular medium are not necessary (Sakai *et al.*, 1991b).

Table 4 Survival of apical meristems of white clover cooled to −196°C by three different cryogenic protocols

Cryogenic protocol	Survival (%±S.E.)
Conventional slow freezing method[a]	86 ± 8
Simple freezing method[b]	84 ± 2
Vitrification (15 min at 0°C)[c]	83 ± 4
Vitrification (5 min at 25°C)[c]	80 ± 12
LSD ($P=0.05$)	21

Survival was defined as the percentage of cryopreserved meristems producing shoot growth 3 weeks after planting.

a Frozen at 0.3°C/min to −40°C in the presence of 10% DMSO plus 10% glucose.

b Meristems treated with a mixture of 3 M glycerol and 0.4 M sucrose for 10 min at 25°C were frozen in a freezer at −30°C for 60 min before being immersed into LN.

c Followed by immersion into LN.

3. Cryopreservation of Meristematic Callus

The ability to regenerate whole plants is a matter of vital importance for *in vitro* tissue culture, and the application of molecular and somatic genetics to crop improvement. One of the features of white clover regeneration from callus and cells has been the genotype specificity. Efficient regeneration from callus or cell culture is limited to a few genotypes. Yamada (1989) selected a genotype of white clover which forms numerous dense, globular, green meristematic cell masses (meristemoids) and develops directly into shoots spontaneously forming roots on hormone-free agar medium. The preservation of cultured cells with unique attributes such as high regenerative capacity is of great importance.

A callus line of white clover forming numerous meristemoids was used in the experiments. This callus line was selected from a seedling of the Swedish cultivar 'Undrom' and has maintained high levels of plant regeneration during long-term subculture (Yamada, 1989). About 5 g of meristematic calli from a 10 to 15-day-old culture were precultured in liquid B5 medium supplemented with 0.6 M sorbitol at 25°C for 16 hr. Recovery growth (the increase in fresh weight after 30 days of culture) of the meristematic calli of white clover cooled to −196°C was compared among three cryogenic protocols (Table 5). In the

conventional slow freezing method, the calli were frozen at a cooling rate of 0.3°C/min to −40°C in the presence of 10% DMSO and 10% glucose prior to immersion into LN. In the simple freezing method, calli were treated with B5 medium containing 3 M glycerol and 0.4 M sucrose at 25°C for 10 min and then frozen by direct transfer to a freezer at −30°C for 60 min prior to immersion into LN. In the simple vitrification method, the calli were treated at 25°C for 7 min or 0°C for 20 min with a highly concentrated vitrification solution (PVS2) followed by direct immersion into LN. In the calli cryopreserved by the simple freezing method or the vitrification method, the recovery growth was much more rapid than that of the calli cryopreserved by the conventional slow freezing methods. Meristematic calli cryopreserved by three different cryogenic protocols developed numerous shoots. Plants derived from cryopreserved meristematic calli were morphologically uniform and had the characteristics true to white clover. Thus these simpler cryogenic methods appear to be suitable for the routine cryopreservation of meristematic calli of white clover.

Table 5 Recovery growth of meristematic calli of white colver coooled to −196°C by three different cryogenic protocols

Cryogenic protocol	Recovery growth rate (%±S.E.)	Tiine required for procedure (min or hr)
Conventional slow freezing method[a]	11.5 ± 2.1	7 hr
Simple freezing method[b]	32.0 ± 11.3	70 min
Vitrification (7 min at 25°C)[c]	16.5 ± 2.1	7 min
Vitrification (20 min at 0°C)[c]	26.0 ± 3.5	20 min

Meristematic calli were precultured in liquid B5 midium containing 0.6 M sorbitol for 16 hr at 25°C.
Recovery growth rate was the increase in fresh weight after 30 days culture.

a Prefrozen at −40°C at 0.3°C/min in the presence of 10% DMSO plus 10% glucose before being immersed into LN.

b Meristematic callus treated with 3 M glycerol and 0.4 M sucrose for 10 min at 25°C was spontaneously frozen by direct transfer to a freezer at −30°C for 1 hr prior to immersion into LN.

c Treated with PVS2 at 25°C or 0°C prior to immersion into LN.

4. Conclusion

In white clover, cryopreservation of apical meristems and meristematic calli has been developed using the conventional slow freezing method, simple vitrification method and simple freezing method. Further studies are necessary to identify the regenerated phenotypes of the cryopreserved meristems and calli by cytological, biochemical and morphological analyses.

Another simple cryogenic protocol could involve extensive dehydration by air drying. The improvement of drought tolerance may be the key for successful cryopreservation for this approach. Further studies are necessary to improve the drought tolerance of the meristems and meristematic calli of white clover.

Cryogenic protocols such as the simple vitrification method and simple freezing method seem to be suitable for routine preservation of genetic resources in the vegetative phase. It is anticipated that the simple cryogenic methods described here will be applied to other species of plants. Recently, the author has succeeded in applying the simple vitrification method to the shoot meristems of forage grass, perennial ryegrass (*Lolium perenne* L.).

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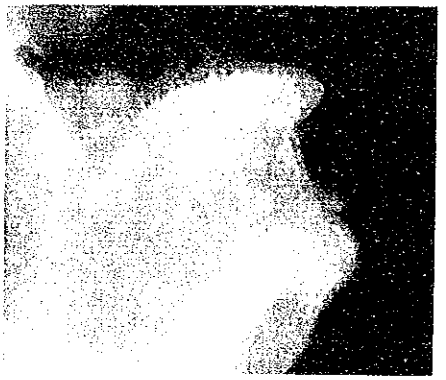

Photo. 1 Longitudinal section through the meristematic dome of vitrified meristem after 4 days of reculture stained with fluorescein diacetate.

Photo. 2 Proliferation of shoots from an apical meristem cryopreserved by vitrification for 3 weeks after plating.

Photo. 3 A plantlet developed from an apical meristem cooled to − 196°C by vitrification.

Cryopreservation of Cultured Cells and Organs of Vegetables

by

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CONTENTS

1. Introduction

Tissue culture has played an important role in the propagation and breeding of vegetable crops. The relatively high price fetched by vegetable products makes them targets for research into the practical use of tissue culture. Tissue culture has been utilized for producing virus-free plants, for vegetative propagation, and for genetic engineering. In addition, genetically altered cell lines or cell lines with special characteristics, such as embryogenic potential,virus-free properties, or high production of secondary metabolites, can be obtained by using tissue culture.

The widespread use of tissue culture requires the development of new preservation techniques for *in vitro* cultured materials. Although numerous new cell lines and plantlets have been produced by tissue culture, they all had to be maintained by periodic subculture. Other disadvantages associated with this method of maintenance, include high cost, the risk of contamination and genetic or phenotypic modification during subculturing. The use of tissue culture allows vegetatively propagated plants, for which methods of germplasm preservation other than maintenance in the field were not available, to be preserved *in vitro*. Recently, although a growing number of reports has been presented concerning the cryopreservation of *in vitro* cultured materials, there are few studies on their genetic stability after long-term preservation in LN.

Successful cryopreservation of a number of species that are commonly utilized as vegetables has been reported (Table 1). The current studies describe various methods of cryopreservation for a wide range of materials, from protoplasts to embryos.

The cryopreservation protocols reported to date can be classified into 4 groups according to the cell dehydration procedures used prior to immersion in LN (Sakai, 1991) as follows: (1) slow-freezing method, (2) vitrification method, (3) air-drying method, and (4) a combination of the above methods. The results of cryopreservation by the application of these methods using cultured cells and organs of asparagus and oilseed rape are described below.

Table 1 Summary of cryopreservation protocols for cultured cells and organs of vegetables

Species	Material	Pretreatment and cryoprotectants	Freezing and storage	Rewarming and dilution	Viability
Asparagus officinalis (asparagus) (Kumu *et al.* 1983)	Shoot tips (spear, 1.5 - 2.0 mm)	4% DMSO + 3% glucose 2 day preculture Frozen in 11% DMSO	0.5°C/min to −40°C LN storage	in 40°C water	100% survival
Asparagus officinalis (asparagus) (Uragami *et al.* 1989)	Cultured cells	12% EG + 0.5 M sorbitol 22°C, 10 min Dropwise addition of PVS1 0°C, 10 min PVS1 0°C, 10 min	Direct immersion in LN	in 22°C water 1.2 M sucrose	65% survival (FDA staining)
Asparagus officinalis (asparagus) (Uragami *et al.* 1989)	Somatic embryos (globular)	12% EG + 0.5 M sorbitol 22°C, 10 min Dropwise addition of PVS1 0°C, 10 min PVS1 0°C, 10 min	Direct immersion in LN	in 22°C water	48% survival (FDA staining)
Asparagus officinalis (asparagus) (Uragami *et al.* 1990)	One node segments (*in vitro*, 5 mm)	0.7 M sucrose, 2 day preculture Air-dried	Direct immersion in LN	in air, room temperature	63% shoot formation
Asparagus officinalis (asparagus) (Nishizawa *et al.* 1992)	Cultured cells (embryogenic, 4 day-old)	2 M glycerol + 0.4 M sucrose 25°C, 10 min	Frozen at -30°C, 1 h, then in LN	in 40°C water 1.2 M sucrose	80% survival (FDA staining)
Asparagus officinalis (asparagus) (Kohmura *et al.* 1992)	Multiple bud clusters (3-5 mm)	PVS2 0°C, 120 min	Direct immersion in LN	in 38°C water 1.2 M sucrose	90% shoot formation

Table 1 Summary of cryopreservation protocols for cultured cells and organs of vegetables (Continued)

Species	Material	Pretreatment and cryoprotectants	Freezing and storage	Rewarming and dilution	Viability
Brassica napus (oilseed rape) (Uragami *et al.* in press)	Microspore embryos (torpedo)	0.75 M sucrose, 1 day preculture, encapsulated Desiccated in laminar air-flow	Direct immersion in LN	in air, room temperature	83% bipolar development
Brassica oleracea (brussels sprouts) (Harada *et al.* 1985)	Shoot tips (1-2 mm)	Collected in winter (min.temperature −3.5°C), 3% glucose + 1.5M DMSO 20°C, 2h	0.5°C/min to −40°C LN storage	in 40°C water	93% survival
Brassica campestris (Langis *et al.* 1989)	Cell suspension	1.5 M EG + 3% sucrose 0°C, 90 min, then 7M EG + 0.88M sorbitol 0°C, 3 min	Direct immersion in LN	in 20°C water 1.5 M sorbitol	40% survival (TTC reduction)
Cichorium intybus (chicory) (Demeulemeester *et al.* 1992)	Shoot tips (2 mm)	2 days on nutrient medium Frozen in 15% DMSO	0.5°C/min to −40°C, LN storage	in 40°C water	83% regrowth
Cucumis melo (melon) (Shimonishi *et al.* 1991)	Somatic embryos (3 mm)	10 mg/l ABA, 3 day preculture Dessicated at 50 - 65% RH	Direct immersion in LN	in 40°C water	65% greening, rooting or shooting
Daucus carota (carrot) (Nag and Street 1975)	Cell suspension	Frozen in 5% DMSO	1 - 2°C/min to −100°C LN storage	in 37°C water	65% survival (FDA staining)
Daucus carota (carrot) (Nitzsche 1980)	Callus	10 mg/l ABA + 50 g/l sucrose, 16 day preculture Desiccated in laminar air-flow	at −80°C, 1 year storage	in air(?)	100, 90, 55 % regrowth with 3 cultivars

Table 1 Summary of cryopreservation protocols for cultured cells and organs of vegetables (Continued)

Species	Material	Pretreatment and cryoprotectants	Freezing and storage	Rewarming and dilution	Viability
Daucus carota (carrot) (Takeuchi *et al.* 1980)	Protoplasts	Frozen in 10%(v/v) DMSO + 20%(w/v) glucose	1.0 - 2.0°C/min to −35°C, LN storage	in 30 − 40°C water, 0.4M mannitol	40% survival (TTC reduction)
Daucus carota (carrot) (Dereuddre *et al.* 1991)	Somatic embryos (torpedo)	0.3 M sucrose, 18 h preculture, encapsulated Desiccated in laminar air-flow	Direct immersion in LN	in air, room temperature	71% germination
Daucus carota (carrot) (Lecouteux *et al.* 1991)	Somatic embryos (heart-torpedo)	0.4 M sucrose, 24 h preculture	Frozen at −20°C, 24 h, then in LN	in 40°C water	80% bipolar development
Fragaria × *ananassa* (strawberry) (Sakai *et al.* 1978)	Meristems (runner, 0.5 - 1.0 mm)	4°C, 3 day hardening Frozen in 10 - 16% DMSO + 3% sucrose	Prefrozen at −20°C to −30°C, LN storage	in 40°C water	60 - 80% shoot regeneration
Fragaria × *ananassa* (strawberry) (Kartha *et al.* 1980)	Meristems (*in vitro*, 0.4 - 0.5 mm)	5% DMSO, 2 day preculture Frozen in 5% DMSO	0.84°C/min to −40°C LN storage	in 37°C water	95% plant regeneration
Lycopersicon esculenta (tomato) (Grout *et al.* 1978)	Meristems	Frozen in 10-15% DMSO	22-55°C/min LN vapor freezing	in 40°C water	30% callus and plants
Pisum sativum (pea) (Kartha *et al.* 1979)	Meristems (0.4 - 0.5 mm)	5% DMSO, 2 day preculture Frozen in 5% DMSO	0.6°C/min to −40°C LN storage	in 37°C water	60% plant regeneration

2. Slow-freezing

The slow-freezing method, also referred to as the conventional method, consists of cryoprotection using 5 - 15% DMSO followed by slow freezing (0.5 - 2°C/min) to about −40°C, immersion in LN, and rapid thawing in water at about 40°C. This cryopreservation protocol, especially for cell suspensions, requires comparatively expensive facilities for rate-controlled cooling.

The first report on the cryopreservation of asparagus shoot-tips *in vivo* utilized this slow-freezing method (Kumu *et al.*, 1983).

1) Materials and Methods

Plant materials

Small unbranched lateral shoot tips (1.5 to 2.0 mm in length) were aseptically excised from asparagus spears (cv. Mary Washington 500), which had sprouted from 5 year-old plants in a greenhouse (Fig. 1).

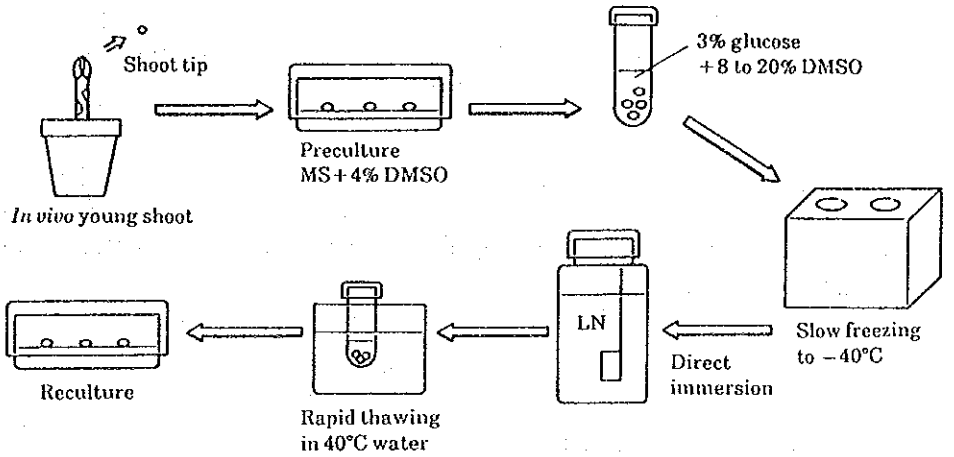

Fig. 1 Slow-freezing procedure.

Preculture

Shoot tips were cultured on solidified MS medium, containing 3% glucose and 4% (v/v) DMSO, for 2 days at 25°C, under illumination for 16hr .

Cooling

After the preculture, the shoot tips were immersed in a 0.25 ml solution containing 3% glucose and 4, 8, 12, 16, or 20% DMSO in a spitz tube. After 2-hr treatment at 3°C, the spitz tubes were plunged into an ethanol bath at 0°C, and then cooled to −10°C in 10 min. To induce freezing in the solution and the sample tissues, the outer surface of the tube wall that came into contact with the solution was cooled with dry ice for a few seconds. The frozen samples were successively cooled to −40°C at a rate of 0.5°C/min, and were maintained at that temperature for 10 min. These tubes were then quickly immersed in LN.

Thawing and reculture

The samples in the tubes were stored in LN for 30 min, then thawed rapidly in water at 40°C (rewarming rate: 500°C/min). After thawing, the shoot tips were immersed in MS liquid medium for 30 min to remove DMSO, and recultured on MS solid medium supplemented with 2% sucrose, 5×10^{-7} M IBA, and 10^{-7} M BA. They were cultured at 25°C under illumination for 16 hr.

2) Results

In the presence of 8 to 20% of DMSO in the freezing solution, the survival rates of the asparagus shoot tips ranged from 95 to 100%. Some of the shoot tips which survived developed into plantlets.

3. Vitrification

When cells are sufficiently dehydrated, the intracellular solutes become concentrated enough to undergo vitrification (formation of an amorphous solid) during cooling, and can sustain subsequent rewarming without being damaged by significant recrystallization, in the range of ordinary cooling and rewarming rates. Rall and Fahy (1985) successfully cryopreserved mouse embryos by

dehydrating the embryos with a highly concentrated cryoprotective solution to allow vitrification during rapid cooling in LN.

Sakai *et al.* (1991) reported an improved method for vitrification of nucellar cells of navel orange (*Citrus sinensis* Osb.) using PVS2, which contained 30% (w/v) glycerol, 15% (w/v) DMSO, and 15% (w/v) EG in MT medium supplemented with 0.4 M sucrose. The average rate of survival was 90%.

This improved procedure for vitrification was applied to embryogenic callus cultures of asparagus using PVS1, which contained 22% glycerol, 13% PG, 13% EG, and 6% DMSO in 0.5 M sucrose MS medium, as a vitrification solution (Uragami, 1991).

1) Materials and Methods

Plant materials

Embryogenic callus cultures were initiated from single cells of the asparagus line 'KBF×3-9'. The calli were maintained on solidified MS medium containing 5×10^{-6} M 2, 4-D in Petri dishes (9 cm in diameter) and were subcultured to the fresh medium every 4 weeks. The plates were incubated at 25°C in the dark.

PVS1 treatment

A mass of about 0.1 g (FW) of 2 week-old calli was pretreated with 5 ml of PVS1 in a 10 ml Pyrex tube at 25°C (Fig. 2). The calli were placed in a tube, and PVS1 was gently added to them. After gentle stirring with a Pasteur pipette, the supernatant was removed thoroughly by centrifugation at 100×g for 2 min. Fresh PVS1 was then added to the packed calli. In order to avoid the dilution of PVS1, the calli were washed twice with fresh PVS1. Calli suspended in 30 μl of PVS1 were then loaded into the central part of 0.5 ml plastic straws and the top end of each straw was sealed using a heat sealer.

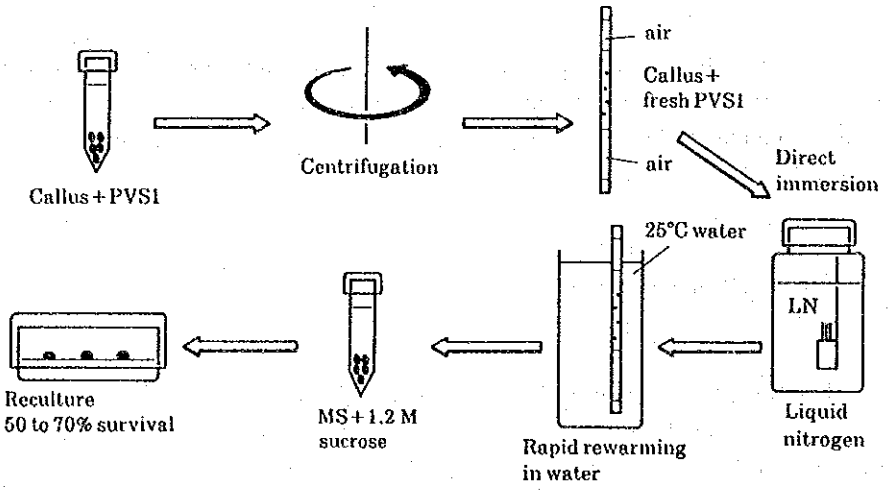

Fig. 2 Vitrification procedure.

Cooling and rewarming

The straws were then directly immersed in LN. After storage in LN, the straws were immediately transferred from LN to water to avoid the detrimental recrystallization caused by slow rewarming. The cells in the straws were then rewarmed rapidly in a water bath at 25°C. After both ends of each straw were cut, the callus suspension was expelled into a diluting solution containing 1.2 M sucrose in MS medium. The suspended cells were placed in the diluting solution just after it reached room temperature to avoid harmful effects due to the toxicity of PVS1 at high temperatures. The supernatant of the diluted callus suspension was discarded, and the remaining calli were placed and recultured on solidified MS medium without phytohormones.

The viability of the cells after dilution was determined by FDA vital staining (Widholm, 1972).

2) Results

Effect of PVS1 treatment

In order to determine the optimum time of exposure to PVS1 at 25°C, the asparagus calli were treated with PVS1 for different periods of time prior to immersion in LN. Exposure to PVS1 produced time-dependent injury (Fig. 3). The maximum survival rates were obtained for the calli treated for 2 to 5 min. The survival rates of the calli treated for 2 to 3 min without immersion in LN were 10 to 20% higher than those of vitrified calli. The survival rates remained high when the PVS1 treatment lasted for less than 5 min, while treatment for more than 7 min seriously damaged the cells due to the toxicity of the cryoprotectants and excessive osmotic stress. Treatment applied for 4 min with PVS1 seemed optimal for the vitrification of the asparagus calli.

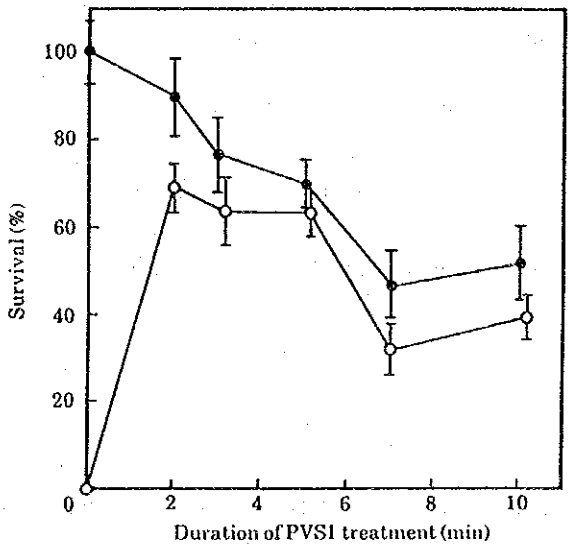

Fig. 3 Effect of duration of PVS1 treatment at 25°C on the survival of embryogenic callus.

The callus suspension was treated for various periods of time with PVS1 and then loaded into plastic straws. The straws were then immersed directly in LN. After rapid warming, the callus suspension was expelled into 2 ml of MS medium containing 1.2 M sucrose. ○: vitrified callus; ●: callus treated with PVS1 but not cooled to −196°C.

Effect of holding temperature after removal from LN

In the present study, the vitrified calli were kept at temperatures ranging from −115 to −45°C for 5 min, at which intracellular crystallization may occur and damage the calli. Survival rates decreased significantly when the calli were maintained at temperatures higher than −65°C. At those temperatures, the callus suspensions in the straws became whitish due to crystallization during the 5 min-treatment, indicating that the crystallization proceeded at temperatures higher than −70°C. These findings are consistent with the devitrification temperature (−76.2°C) observed in the DSC record of PVS1 (Uragami *et al.*, 1989).

Effect of warming rate

To further investigate the detrimental effect on the callus of cooling to −196°C by vitrification due to crystallization during slow warming, vitrified calli were rewarmed at varying rates in the temperature range between −80 and −40°C after removal from LN. The calli, which were warmed rapidly at 100°C/min or above, showed high survival rates ranging from 50 to 70%. However, the vitrified calli subjected to slow warming at 60°C/min or below showed a very low survival rate of approximately 10%. These results presumably reflect the effects of intracellular crystallization taking place during slow warming.

Effect of storage in LN for various periods of time

Storage in LN for various periods of time did not result in a significant decrease of the survival rate. The calli that were preserved in LN for 6 months retained a 61.5% viability. The vitrified calli stored in LN for 8 weeks resumed growth after reculture for 3 days, suggesting that long-term storage of the asparagus calli may be possible by using this method.

Regeneration of plantlets through embryogenesis

The vitrified calli on the regrowth medium resumed growth within about 3 days (Fig. 4). Numerous embryos were developed from embryogenic calli cooled to −196°C within 2 weeks after reculture. Torpedo-shaped embryos were then transferred to fresh medium and developed into plantlets.

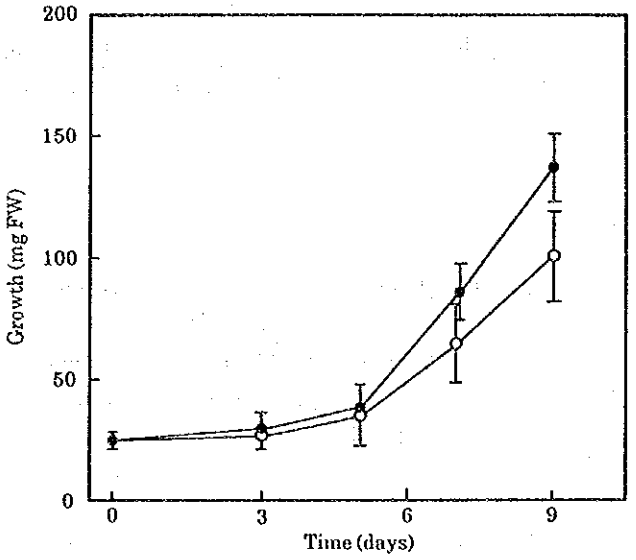

Fig. 4 Regrowth of vitrified callus stored in LN for 8 weeks.

A vitrified callus suspension in plastic straws was rewarmed rapidly and diluted with MS liquid medium containing 1.2 M sucrose. The dilution medium was then discarded and the callus was placed and cultured on a solidified MS medium without phytohormones. ○: vitrified callus; ●: untreated control.

4. Air-drying

Nitzsche (1980) demonstrated that carrot calli precultured on Gamborg medium supplemented with ABA and high concentrations of sucrose could tolerate desiccation and regenerated into plants after storage at −80°C for a few days. Withers (1979) also reported the successful cryopreservation of dried plantlets in carrot. Thus, desiccation prior to direct immersion in LN seems to be an effective method for the cryopreservation of plant materials. Recently, storage of *in vitro* cultured plant materials in LN, or at higher temperatures after considerable desiccation in the air, has been reported in carrot calli (Kaimori, 1988), alfalfa embryos (Senatatna *et al.*, 1989), and melon embryos (Shimonishi, 1991). These materials were dehydrated after becoming tolerant to dehydration by preculture with ABA and/or high concentrations of sugar.

These dehydration methods generally involve rapid cooling by immersion of the materials in LN directly from the room temperature, followed by slow rewarming in the air. These methods do not require the use of a special

equipment to control the cooling rate as in case of the slow-freezing method. In addition, medium-term preservation at higher temperatures such as −20°C is possible when the materials are precultured with less toxic substances, such as sucrose, although there is a potential risk that recrystallization and crystal growth may occur during preservation.

1) Direct Dehydration

Single node segments, which consist of a fragment of stem bearing an axillary bud, were commonly used for the micropropagation of asparagus (Desjardins *et al.*, 1987), potato (Hussey and Stacy, 1981), and other species. The axillary buds from asparagus plantlets grown *in vitro* were cooled to −196°C after air drying (Uragami *et al.*, 1990).

(1) Materials and Methods

Plant materials

Asparagus female clone '873-32' (selected at the Hokkaido National Agricultural Experiment Station, Japan, from line '873' from the University of California, U.S.A.) was maintained *in vitro* as described by Yang and Clore (1973). The *in vitro* plantlets developed from buds were transferred to fresh MS medium solidified with 0.25% Gelrite supplemented with 5.4×10^{-7} M NAA and 4.4×10^{-7} M BA every 4 to 6 weeks after shoot harvesting. They were cultured at 25°C under continuous cool white fluorescent light ($37\mu Em^{-2}s^{-1}$). The plantlets with fully-developed crowns and roots were considered to be a superior material. A microscope was used to dissect the segments with uniform buds.

Preculture

Stem segments were excised from the middle part of the shoots of 6 to 8 week-old plantlets that were approximately 10 cm in height (Fig. 5). The diameter of the shoots was 0.5 - 0.8 mm. The excised shoots were cut into smaller segments approximately 5 mm in length, and bearing one bud in the center. The rank of the single node segments in the middle part of a stem did not affect the growth rate or the appearance of the new shoot that developed from the axillary buds. The segments were precultured on solidified MS medium supplemented with 0.7 M sucrose at 25°C in the light for 2 days.

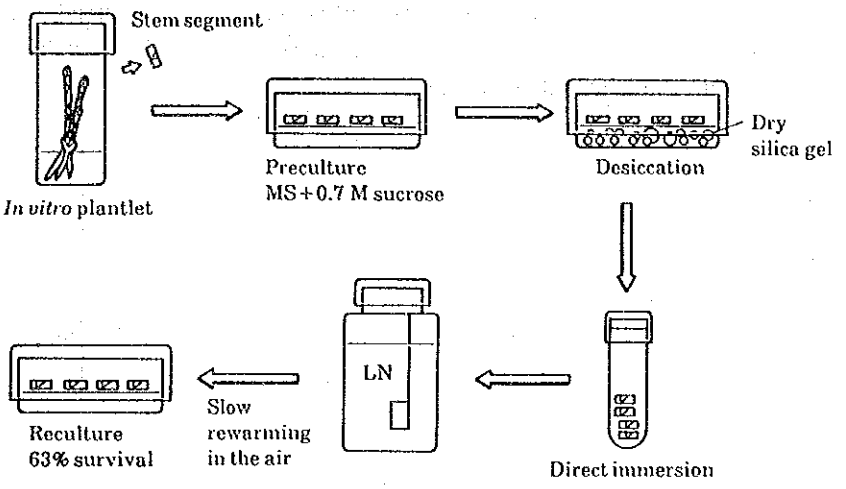

Fig. 5 Air-drying procedure of asparagus stem segments.

Desiccation

The pretreated segments were collected on two sheets of 500-μm nylon mesh in a small Petri dish (3 cm in diameter) and were then subjected to dehydration in a Petri dish (6 cm in diameter) containing 15 g dry silica gel. The Petri dishes were sealed with Parafilm and were maintained at 25°C. The segments inside the Petri dish were then desiccated to lower water contents.

Cooling and rewarming

About 15 segments were placed in 1-ml plastic tubes which were then sealed and cooled rapidly by immersion in LN (cooling rate: 720°C/min). Samples were stored in LN for 60 min, then rewarmed by placing the tubes in the air at room temperature (rewarming rate: 120°C/min), and were recultured on MS medium solidified with 0.25% Gelrite supplemented with 5.4×10^{-7} M NAA and 4.4×10^{-7} M BA.

(2) Results

Time course of dehydration

The time course of the dehydration of the asparagus stem segments was determined. Fig. 6 shows the time course of dehydration of precultured and non-precultured single node segments. The precultured segments were dehydrated more slowly than the non-precultured segments.

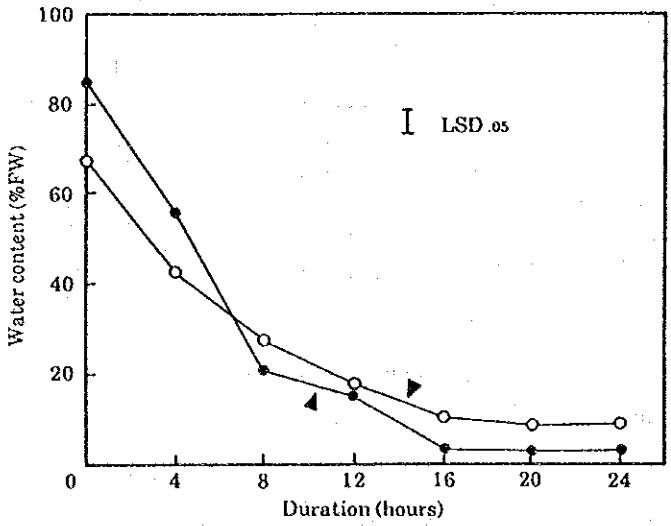

Fig. 6 Time course of dehydration of single node segments.

The segments were precultured on a solidified MS medium containing 0.7 M sucrose for 2 days in the light at 25°C. ●: non-precultured; ○: precultured; Arrows indicate LD_{50}.

Effect of preculture

As shown in Table 2, the maximum survival rate of non-precultured buds immersed in LN following dehydration was only about 7%, while the precultured buds showed a survival rate of 63%, accounting for 71% of the survival rate of the untreated control, since only 89% of the buds of the untreated control developed shoots (Fig. 7). Thus, the preculture for 2 days at 25°C on a culture medium containing 0.7 M sucrose was effective in increasing the survival rate of the buds after immersion in LN. During the preculture at a high sucrose concentration for 2 days, the water content decreased by about 16.4% (from 84.7 to 68.3%) and the dry matter content increased approximately 2.7 times.

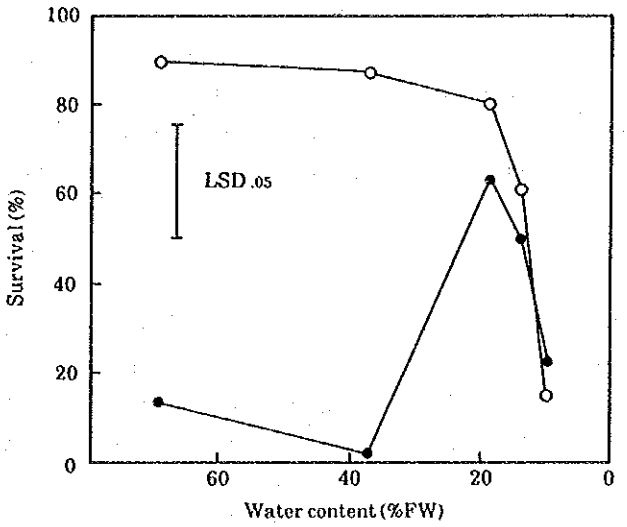

Fig. 7 **Survival of precultured axillary buds with and without exposure to LN at various water contents.**

○: without, ●: with exposure to LN.

Table 2 **Effect of pretreatment on the survival of axillary buds cooled to −196°C and changes in water content and dry matter during preculture**

	Non-precultured	Precultured
Maximum survival (%)	6.7	63.2
Water content (%)	84.7	68.3
Dry matter (mg/segment)	1.0 ± 0.34	2.7 ± 0.49

Preculture: precultured on an MS medium supplemented with 0.7 M sucrose for 2 days at 25°C in light.

Effect of water content

Fig. 7 shows that the threshold water content, below which the axillary buds remained alive after immersion in LN, ranged from approximately 20 to 17% (FW) (LD_{50}). However, the survival rate decreased rapidly at a water content below about 13% (FW), presumably due to desiccation injury, since the same trend was observed in the segments that were not exposed to LN.

Plant regeneration

Viable shoot primordia resumed growth on the regrowth medium within one week and developed shoots directly without any callus formation. Approximately 70% of the cryopreserved buds regenerated into plantlets with multiple shoots and roots. Some axillary buds, that sustained desiccation injury, resumed growth within 2 or 3 weeks after reculture. However, even those buds developed shoots without intermediate callus formation.

Chromosome observation showed that all of the 20 regenerated plants examined were diploid with 20 chromosomes.

2) Encapsulation-dehydration

Recently, there have been reports of successful cryopreservation of cultured shoot tips of pear (Dereuddre *et al.*, 1990) and *Solanum* (Fabre and Dereuddre, 1990), and of somatic embryos of carrot (Dereuddre *et al.*, 1991) through advanced dehydration after encapsulation with Ca-alginate beads. Embryos derived from the microspores of oilseed rape have recently been cryopreserved using this method (Uragami *et al.*, in press).

(1) Materials and Methods

Plant materials

Haploid embryos were induced from microspores of oilseed rape (*Brassica napus* L. cv. Topas). The microspores were collected from buds and were cultured on NLN medium without hormones in the dark at 30°C for 14 days. The resulting embryos were 1 - 3 mm long at a stage between the heart and early cotyledonary stages. The culture conditions hereafter were 23 ± 1°C with a photoperiod of 16 h and a light intensity of $30 \mu Em^{-2}s^{-1}$.

Encapsulation and preculture

The embryos were suspended in NLN medium, lacking calcium and containing 3% sodium alginate and 0.38 M sucrose (Fig. 8). Alginate beads were produced by dropping them into another NLN medium supplemented with 100 mM calcium nitrate, using Pipetman 5000 (Gilson Co. Ltd., France). The diameter of the beads was approximately 4 mm. Alginate beads with 1 to 3 embryos were collected and precultured on NLN medium, containing 0.75 M sucrose, for 1 day.

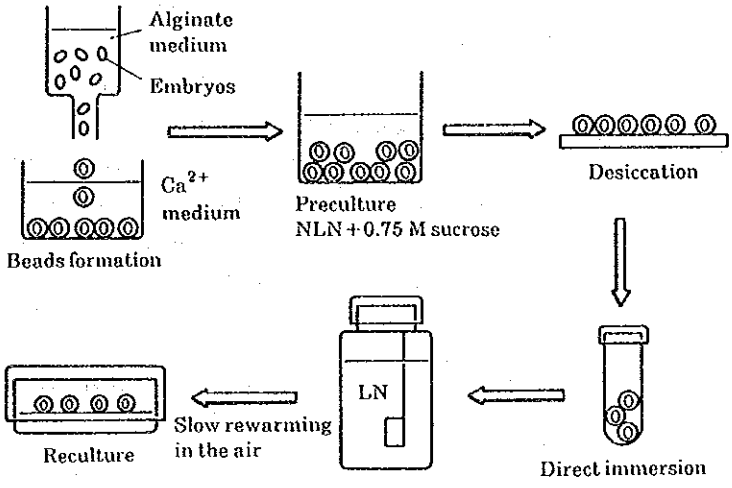

Fig. 8 Encapsulation-dehydration procedure.

Dehydration

After the preculture, the encapsulated embryos were dehydrated under sterile air-flow for 3.5 h. The final water content of the beads was 18 - 20%. The beads were then placed in 2-ml plastic tubes and directly immersed in LN, where they were stored for at least 60 min. The beads were slowly rewarmed by placing the tubes under sterile air-flow at room temperature for 10 min. The rewarmed beads were then placed on solidified NLN medium and were recultured.

Determination of survival and plantlet regeneration

After 1 week of reculture on solidified NLN medium, the surviving embryos were counted and then subcultured on B_5 medium without 2,4-D and with 0.1 mg/l GA_3 for 1 week at 4°C in the light, and then again for 3 weeks at 25°C in the light. The plants with roots and true leaves or fully elongated cotyledons were considered to be regenerated plantlets. The ploidy of the regenerated plantlets after cryopreservation was evaluated by counting the number of chloroplasts in the stomatal guard cells (Lucas *et al.*, 1991).

(2) Results

Effect of preculture medium

The concentration of sucrose in the preculture medium affected considerably the survival rate of the cryopreserved embryos (Table 3). Without preculture using a sucrose concentration above 0.38 M, no survival was observed. There was no appreciable difference in the survival of the embryos precultured in sucrose at concentration between 0.75 M and 1.50 M. Therefore, the 0.75 M sucrose NLN medium was used as the preculture medium.

Table 3 **Effect of sucrose concentration of the preculture medium on the survival of cryopreserved embryos**

Sucrose content (M)	Survival (%)
0.38	0
0.75	97 ± 3
1.50	91 ± 4

The embryos were encapsulated with Ca-alginate beads and were precultured on each preculture medium for 1 day before dehydration and cooling. Each result represents the average and the standard deviation of 3 experiments. Twenty-five embryos were used for each experiment.

Effect of alginate coating

The duration of the alginate coating after dehydration and LN treatment affected the survival rate of the rapeseed embryos and the regeneration rate of the plantlets (Table 4). When extracted from beads 1 day after LN treatment, the survival rate of the embryos was similar to that of the controls, while the plantlet regeneration rate was slightly lower than that of the controls. However, when the embryos were kept inside the alginate coating for 7 days, the survival rate was lower than that of the controls, and the plantlet regeneration rate decreased considerably. Penetrometric hardness of the alginate cubes of the NLN medium containing 0.38 M sucrose (650±40 g) was significantly higher than that of the medium containing 0.09 M sucrose (480±50 g), the concentration used in previous reports on encapsulation-dehydration. In the preliminary experiment, the NLN medium containing 0.09 M sucrose was used to make beads, but the survival rate after dehydration and LN treatment was very low (data not shown).

Table 4 Effect of duration of alginate encapsulation on plantlet regeneration

Treatment	Duration of alginate coating after treatment	Survival (%)	Plantlet regenation (%)
Control	—	100	94 ± 1
Dehydration	1 day	99 ± 1	95 ± 1
	7 days	78 ± 28	67 ± 17
LN	1 day	98 ± 1	83 ± 5
	7 days	84 ± 8	55 ± 8

Alginate-coated embryos were either dehydrated or both dehydrated and then treated with LN. The embryos were extracted from beads 1 day or 7 days after each treatment. Each result represents the average and the standard deviation of 2 experiments. Fifty embryos were used for each experiment.

Ploidy of the regenerated plantlets

The ploidy of the regenerated plantlets with or without cryopreservation was evaluated by counting the number of chloroplasts in the stomatal guard

cells (Table 5). The percentages of diploidized plantlets regenerated from the control and the LN-treated embryos were not significantly different.

Table 5 Ploidy of the plantlets regenerated from cryopreserved embryos

Treatment	Number of plantlets	
	n	2n
Control	47	10 (18)*
LN	41	9 (18)

* Parenthesis indicates percentage of total.

5. Conclusion

Plant materials must be placed under specific physiological conditions in order to achieve a good survival rate following storage in LN. Hardening of whole plants *in vivo* (Harada *et al.*, 1985) or *in vitro* (Dereuddre *et al.*, 1990) reportedly affects the survival after cryopreservation. Preculture and pretreatment are also important factors affecting the survival rate. While DMSO, ABA, and sugars are reported to be effective, little is known about the actual function of these cryoprotective agents. Recently, Gazeau *et al.* (1992) reported that carrot and *Catharanthus* cells were successfully cryopreserved after treatment with cytochalasin D (actin-filaments fragmentation agent).

There has been a growing number of studies on the effects of the cooling and rewarming procedures on the survival of plant cultured materials. Future studies will focus on the analysis of the pre- and post-conditioning at both the cellular and molecular levels that will ensure that the cell survives cryopreservation, and also on a more precise and long-term analysis of genetic stability after cryopreservation.

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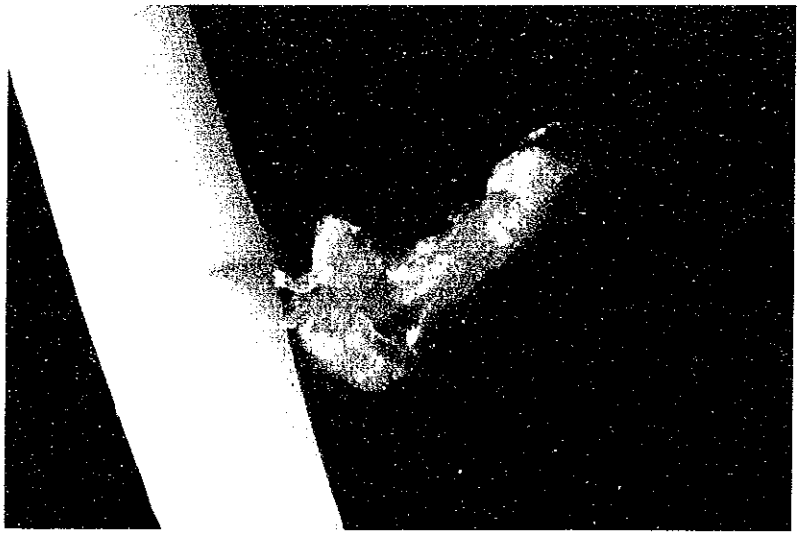

Photo. 1 Direct shoot formation of asparagus after cryopreservation by air-drying method. Three weeks after reculture.

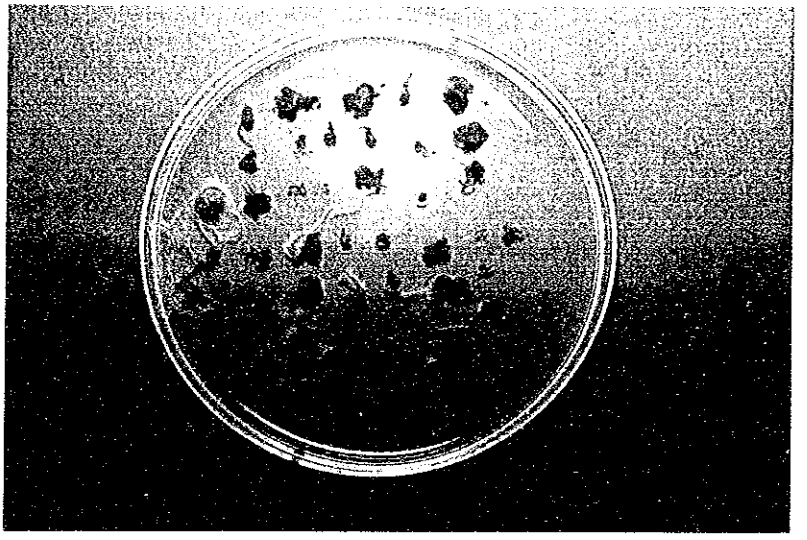

Photo. 2 Development of cotyledons and roots from microspore derived embryos of oilseed rape after cryopreservation by encapsulation-dehydration method.
One week after reculture.