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ORIGINAL ARTICLE

Development of a new vitrification solution, VSL, and its application to the cryopreservation of gentian axillary buds

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Abstract Vitrification methods are convenient for cryopreserving plant specimens, as the specimens are plunged directly into liquid nitrogen (LN) from ambient temperatures. However, tissues and species with poor survival are still not uncommon. The development of vitrification solutions with high survival that cover a range of materials is important. We attempted to develop new vitrification solutions using bromegrass cells and found that VSL, comprising 20% (w/v) glycerol, 30% (w/v) ethylene glycol, 5% (w/v) sucrose, 10% (w/v) DMSO and 10 mM $CaCl_2$, gave the highest survival following cryopreservation, as determined by fluorescein diacetate staining. However, the cryopreserved cells showed little regrowth, for unknown reasons. To check its applicability, VSL was used to cryopreserve gentian axillary buds and the performance was compared with those of conventional vitrification solutions. Excised gentian stem segments with axillary buds (shoot apices) were two-step precultured with sucrose to induce osmotic tolerance prior to cryopreservation. Gentian axillary buds cryopreserved using VSL following the appropriate preculturing approach exhibited 78% survival (determined by the regrowth capacity), which was comparable to PVS2 and PVS1 and far better than PVS3. VSL had a wider optimal incubation time (20–45 min) than PVS2 and was more suitable for cryopreserving gentian buds. The optimal duration of the first step of the preculture was 7–11 days, and preculturing with sucrose and glucose gave a much higher survival than fructose and maltose. VSL was able to vitrify during cooling to LN temperatures, as glass transition and devitrification points were detected in the warming profiles from differential scanning calorimetry. VSL and its derivative, VSL+, seem to have the potential to be good alternatives to PVS2 for the cryopreservation of some materials, as exemplified by gentian buds.

Keywords Cryopreservation · Vitrification · Gentian (*Gentiana scabra*) · In vitro conservation · Shoot apices · Thermal analysis

Abbreviations

DSC	Differential scanning calorimetry
TC	Treated control
MS medium	Murashige and Skoog medium
LN	Liquid nitrogen
PVS2	Plant vitrification solution 2
VSL	Vitrification solution L
VSL+	Vitrification solution L+
DMSO	Dimethylsulfoxide
FDA	Fluorescein diacetate

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Introduction

Cryopreservation using vitrification methods is convenient, as specimens incubated in a vitrification solution are plunged directly into liquid nitrogen (LN) from ambient

temperatures and because this approach requires fewer facilities than conventional slow prefreezing methods. Vitrification as a possible method of cryopreservation was first suggested by Luyet (1937). More recently, vitrification was demonstrated as a practical method of cryopreserving mouse embryos and human monocytes after many trials (Rall and Fahy 1985; Takahashi and Hirsh 1985) and an intracellular glassy state was observed in quench-cooled cold-hardy plant cells (Hirsh 1987). Since then, some attempts to develop vitrification solutions and protocols suitable for plant cells have been made (Langis and Steponkus 1990; Towill 1990; Uragami et al. 1989). Among them, PVS2 developed by Sakai et al. (1990) in combination with appropriate preculturing and/or pre-incubation (loading) has been most frequently used for the vitrification of plant shoot apices (Cho et al. 2002; Matsumoto et al. 1994; Turner et al. 2001; Yamada et al. 1991; Takagi et al. 1997; Sakai 2000). PVS2 was also shown to be useful in encapsulation–vitrification and droplet–vitrification protocols (Panis et al. 2005; Sakai and Engelmann 2007). Vitrification methods tend to allow the entire dome of a shoot apex to survive cryopreservation, resulting in the avoidance of callus formation and somaclonal variation during regrowth, and they are suitable for cryopreserving shoot apices (Yamada et al. 1991). However, there are still many unsuccessful cases (i.e., tissues and species) where only limited survival following vitrification has been obtained.

Problems involved in vitrification methods arise from the toxicity and high osmolarity (ca. 8 M) of the vitrification solutions, which often result in narrow optimum incubation times. Most cases of poor survival following vitrification-based cryopreservation are attributed either to insufficient osmotic tolerance of the specimen (difficulty in inducing high osmotic tolerance), toxicity of vitrification solutions, low penetration of vitrification solutions into the specimen, or insufficient dehydration of the tissues. The development of a new vitrification solution with high survival and lower toxicity, faster penetration or faster dehydration may improve such cases. Only a few papers have focused on attempts to improve vitrification solutions after PVS2 was invented (Volk et al. 2006). If there were other good vitrification solutions that exhibited high survival there would be more options for plant cryopreservation research.

The objective of the first part of this study was to develop an alternative vitrification solution with a high survival and a potentially wide applicability to the cryopreservation of plant-cultured materials and genetic resources. To find good vitrification solution candidates and optimize the conditions, which require a time-consuming trial and error approach in order to match narrow optimal windows, we used homogeneous and fast-growing bromegrass suspension cells. The objectives of the second part of this paper were (1) to determine whether the newly developed vitrification solution, VSL, can provide high survival (regrowth capacity) in the cryopreservation of gentian axillary buds and to compare this with the performances of other conventionally used vitrification solutions, (2) to optimize the conditions that enable the high survival of gentian buds cryopreserved using VSL, and (3) to examine the physicochemical properties of VSL with differential scanning calorimetry (DSC). Gentian axillary buds were used to achieve the latter aim, as there is accumulated knowledge on the cryopreservation of this material (Suzuki et al. 1998, 2005, 2006).

Materials and methods

Development of vitrification solutions using bromegrass suspension cells

A nonembryogenic suspension culture of smooth bromegrass (*Bromus inermis* Leyss cv. Manchar) was used. It was maintained as previously described with a slight modification (Ishikawa et al. 1990, 1996). Briefly, cultures were initiated biweekly by transferring 3 mL of cells to 50 mL of fresh Erickson's (ER) medium (pH 5.8, 0.5 mg L^{-1} 2,4-D) and incubated on a rotary shaker (80 rpm) at 25 °C. It is known that bromegrass cells of the late log or early stationary phase (8–12 days after subculture) are most tolerant to cryopreservation and abiotic stresses (Ishikawa et al. 2006). Ten-day-old cells of a flask were harvested by filtering through a nylon mesh of 80 μm and washed with 250 mL sterilized distilled water. The cells were transferred onto sterilized filter paper in a Petri dish and processed for cryopreservation using vitrification procedures.

Cells (0.2–0.3 g) were dispensed in conical centrifuge tubes (10 mL, graduated glass with screw caps) and treated with 0.3 mL of different vitrification solutions (Table 1) for various durations (30–120 s) at room temperature (25 °C). Then the tubes were immediately submerged in LN and stored there for >1 h. Following rapid thawing in 40 °C water, the cells were diluted by 2.7 mL of chilled 1.2 M sucrose solution unless otherwise noted. The tubes were centrifuged at 3,000 rpm for 5 min at 0 °C and the washing solution was decanted quickly. To the pellet of cells 2 mL of 1.2 M sucrose was added and the sample was recentrifuged as described above. After removing the washing solution, 2 mL of liquid ER medium were added dropwise with gentle shaking and processed for viability assays. Cells treated with vitrification solutions but not submerged in LN were also washed and diluted in the same manner. All operations were done on ice.

Table 1 Composition of various vitrification solution candidates (% w/v)

Vitrification solutions	Glycerol	Sucrose	Ethylene glycol	DMSO
A	20	10	10	0
B	20	10	0	10
C	20	0	10	10
D	0	10	20	10
E	20	10	10	10
F	30	10	10	10
G	20	10	20	10
H (PVS2)	30	15	15	15
I	20	5	25	10
J	25	5	20	10
K (PVS1)	30	5	15	15
L (VSL)	20	5	30	10
M	20	5	25	5
N	20	5	20	5
O	20	10	10	5
L+ (VSL+)	20	15	30	10

These solutions also contained 10 mM $CaCl_2$, except for H and K

The viability of the bromegrass cells was determined by fluorescein diacetate (FDA) staining following Ishikawa et al. (1995). About 300 randomly selected cell clumps were observed to score the percentage of cells showing green fluorescence in each cell clump (0–100%). The scores of these ~300 clumps were averaged to represent the viability of one treatment. To confirm that the surviving cells could regrow, the cells treated with vitrification solutions and those exposed to LN were incubated on semi-solid (0.8% agar) ER medium at 25 °C. Each set of experiments was repeated three times. The data were shown as the mean ± SD.

Cryopreservation of axillary buds of in vitro gentian plants using VSL

The stem segments of gentian (*Gentiana scabra* Bunge var. *buergeri* Maxim.) with axillary buds were propagated as previously described (Suzuki et al. 1998, 2005, 2006). Briefly, they were cultured on MS (Murashige and Skoog 1962) medium containing half-strength inorganic salts and 15 g L^{-1} sucrose (hereafter referred to as 1/2 MS medium) solidified with 8 g L^{-1} agar with the pH adjusted to 5.8. The segments were incubated at 25 °C under a 16 h-photoperiod (50 μmol s^{-1} m^{-2}) and subcultured on the same medium at an interval of 60 days by transplanting the excised nodal segments with axillary buds.

To induce dehydration tolerance in gentian buds, two-step preculturing with sucrose was employed according to the method detailed previously (Suzuki et al. 1998, 2005, 2006). After removing leaves and apical buds, about 2–3 mm long nodal stem segments with axillary buds were excised from the 60-day-old in vitro-grown plantlets and immediately used for preculturing. The axillary buds contained lateral vegetative shoot apices that were not actively growing due to the apical dominance. These excised entire nodal stem segments with axillary buds are hereafter referred to as "axillary buds" or simply "buds." In the first preculture step, the buds were precultured on full-strength MS agar medium containing 0.1 M sucrose (2.3-fold the sucrose concentration in the subculture medium) for 11 days at 25 °C under a 16 h photoperiod (50 μmol s^{-1} m^{-2}) unless otherwise specified. In the subsequent step, the buds were consecutively cultured on full-strength MS agar media containing 0.4 M sucrose, then 0.7 M sucrose for one day each at 25 °C under the same light conditions. These precultured axillary buds (2–3 mm long nodal segments with axillary buds) were used for vitrification-based cryopreservation. In some experiments to see the effects of other sugars, the sucrose in the preculture media was replaced by glucose, fructose and maltose.

Precultured axillary buds (ten or more for each treatment) were transferred to each plastic cryotube where they were directly incubated in 1 mL of a vitrification solution for 5–60 min at 25 °C. During the incubation, the vitrification solutions in the cryotubes were replaced by fresh solutions once or twice. The solution volume was reduced to 0.6 mL before immersion in LN. The vitrification solutions used were PVS1 (30% w/v glycerol, 15% w/v ethylene glycol, 5% w/v sucrose, 15% w/v DMSO) (Uragami et al. 1989), PVS2 (30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v sucrose, 15% w/v DMSO) (Sakai et al. 1990), PVS3 (50% w/v glycerol, 50% w/v sucrose) (Nishizawa et al. 1993) and VSL (20% w/v glycerol, 30% w/v ethylene glycol, 5% w/v sucrose, 10% w/v DMSO, 10 mM $CaCl_2$).

Following incubation in vitrification solutions for designated periods, the cryotubes containing 0.6 mL of solution and ten or more buds were plunged into LN and stored there for at least 1 h. Then they were rewarmed by shaking them in a water bath at 37 °C for about 1–2 min. After immediately decanting the vitrification solutions from the cryotubes, the buds were rinsed twice with 1.5 mL of 1.2 M sucrose solution (5 min each). The buds subjected to vitrification solutions alone were rinsed with 1.2 M sucrose in the same manner.

The rinsed buds (exposed to vitrification solutions alone or then exposed to LN) were placed on full-strength MS medium containing 30 g L^{-1} sucrose solidified with 0.8% (w/v) agar (pH adjusted to 5.8) and recultured under a 16 h photoperiod (50 μmol s^{-1} m^{-2}) at 25 °C. After seven days, the surviving buds resumed growth, which was recognized visually. Then the buds were transferred to fresh medium

(same composition and conditions as before) to see if they grew into normal plants. The survival (regrowth capacity) of the axillary buds was defined as the percentage of buds that produced normal shoots and roots after 20 days of reculturing in vitro.

Experiments were replicated at least three times (ten or more buds for each treatment in a replicate). The data were shown as the mean ± SD. Statistical analysis (Tukey's test) was performed where necessary.

Differential scanning calorimetry (DSC)

Samples (5–10 mg) of vitrification solutions were encapsulated in hermetically sealed aluminum pans and loaded onto a differential scanning calorimeter (Perkin-Elmer Pyris 1, Waltham, MA, USA) at 25 °C, cooled at an average rate of 100 °C min^{-1} to −175 °C (180 °C min^{-1} to −140 °C) and held there for 2.5 min unless specified. Then the samples were scanned at a warming rate (10 °C min^{-1}) to characterize various phase transitions. Temperature and peak area calibration was performed using indium, water and cyclohexane.

We also made an additional vitrification solution, L+: 20% (w/v) glycerol, 30% (w/v) ethylene glycol, 15% (w/v) sucrose, 10% (w/v) DMSO and 10 mM $CaCl_2$. This had an increased concentration of sucrose, 15% (w/v). This was created in an analogy to PVS2 (Sakai et al. 1990), modified from PVS1 (Uragami et al. 1989).

Results and discussion

Attempts to develop new vitrification solutions using bromegrass suspension cells

We attempted to develop new vitrification solutions with high survival using homogeneous and fast-growing bromegrass suspension cells and fluorescein diacetate (FDA) staining for viability assays (Tables 1, 2).

The best vitrification solution found through the survey using FDA staining was named VSL, and was composed of 20% (w/v) glycerol, 30% (w/v) ethylene glycol, 5% (w/v) sucrose, 10% (w/v) DMSO and 10 mM $CaCl_2$ (Table 2). This was followed by solutions I and K (equivalent to PVS1). The optimum survival of bromegrass cells following exposure to liquid nitrogen (LN) was obtained when treated with VSL for 30 s at 25 °C (Table 3). As the period of incubation increased, the viability of cells declined sharply. Bromegrass cells cryopreserved using VSL were stained well with FDA immediately after the freeze–thaw, but gave poor regrowth (less than 5%) after seven days of reculturing (even PVS2-treated cells) for unknown reasons. FDA stainability indicates the intactness of the plasma membranes of the cells (Widholm 1972). This implies that the integrity of plasma membranes was retained just after the cryopreservation procedures, but that the cells lost the ability to regrow for unknown reasons.

In general, vitrification protocols are suitable for cryopreservation of shoot apices rather than suspension cells (Ishikawa et al. 1996, 2006). PVS2 (the most frequently used vitrification solution for shoot apices) was originally developed using callus of navel orange, where the initial growth of cryopreserved callus was low compared to untreated control (Fig. 2 in Sakai et al. 1990). This allows us to speculate that VSL may give high survival (regrowth capacity) if used for the cryopreservation of shoot apices and prompted us to cryopreserve gentian axillary buds using VSL.

$CaCl_2$ were added to the vitrification solutions in expectation that it may help to stabilize the integrity of the membranes, as seen in the case of protoplast isolation (Constabel 1982) and freeze–thaw survival of plant cells (Sugawara and Sakai 1975; Terumoto 1959).

Application of the VSL to the cryopreservation of gentian axillary buds: comparison with other known vitrification solutions

The cryopreservation of gentian axillary buds using two-step preculture and desiccation methods (with or without encapsulation) has been established in our laboratory (Suzuki et al. 1998, 2005, 2006). Since this material and system are well characterized, we attempted to compare the performances of the vitrification solutions PVS1, PVS2, PVS3 and VSL in cryopreserving gentian axillary buds.

Gentian axillary buds that had been precultured in a two-step manner (0.1 M sucrose containing medium for 11 days, followed by serial incubation on 0.4 and 0.7 M sucrose containing medium for one day each) were directly incubated in the vitrification solutions for 10, 20 and 45 min at 25 °C and immersed in LN (Fig. 1). Among the vitrification solutions tested, PVS2 (45 min), VSL (20 and 45 min) and PVS1 (45 min) gave comparable survival (70–80% determined by regrowth capacity). PVS3, which consisted of glycerol and sucrose, gave the lowest survival rates for all of the incubation periods tested.

The performances of the vitrification solutions PVS2 and VSL were studied in more detail (Fig. 2). Incubation of two-step precultured gentian buds in VSL did not greatly affect the viability (regrowth capacity) for up to 60 min (treated control). When the buds thus incubated were immersed in LN, 73–78% survival (regrowth capacity) was attained with the buds incubated in VSL for 20–45 min (Fig. 2b). The incubation of precultured gentian buds in PVS2 for 45 min decreased the viability by 10% (treated control), while the highest survival following LN exposure

Table 2 Effect of different vitrification solutions on the survival of bromegrass suspension cells before and after cryopreservation, as determined by FDA staining

Vitrification solutions	Duration of incubation (s)	Survival by FDA staining (%)	
		Treated control	LN
A	120	97.0 ± 1.4	7.5 ± 1.6
B	120	98.9 ± 0.7	6.5 ± 1.0
C	120	98.8 ± 0.6	9.3 ± 1.5
D	120	91.8 ± 2.1	1.6 ± 0.5
E	120	89.6 ± 1.7	16.9 ± 2.1
F	60	91.8 ± 2.1	23.1 ± 2.3
G	30	91.4 ± 1.5	32.5 ± 3.0
H (PVS2)	30	60.9 ± 2.9	32.4 ± 2.8
I	30	91.2 ± 1.4	57.8 ± 1.9
J	30	91.8 ± 1.6	30.0 ± 2.4
K (PVS1)	30	88.9 ± 1.7	55.4 ± 2.8
L (VSL)	30	86.9 ± 1.4	79.7 ± 1.8
M	30	89.4 ± 1.5	49.7 ± 1.7
N	30	94.4 ± 1.9	37.5 ± 1.9
O	30	95.6 ± 2.1	9.2 ± 2.2

Data were the mean ± SD. Each vitrification solution gave the best survival after exposure to LN for the incubation period shown

Table 3 Effect of incubation time with VSL on the survival of bromegrass suspension cells before and after cryopreservation, as determined by FDA staining

Time (s)	Survival by FDA staining (%)	
	Treated control	LN
15	89.2 ± 1.4	28.7 ± 2.9
30	86.0 ± 1.4	78.4 ± 2.1
60	84.0 ± 1.6	61.0 ± 3.0
90	80.1 ± 1.2	57.5 ± 2.3
120	59.4 ± 2.7	42.4 ± 3.0
300	8.7 ± 1.1	1.6 ± 0.3
600	3.9 ± 0.6	0.7 ± 0.2

Data were the mean ± SD

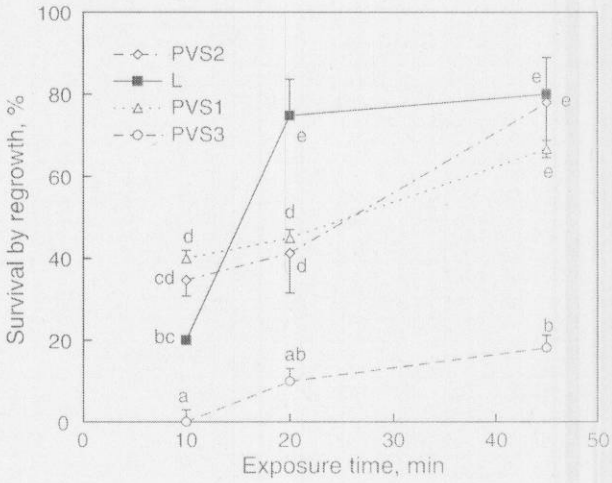

Fig. 1 Effect of different vitrification solutions on the survival of gentian axillary buds after exposure to LN. Excised gentian buds were precultured on MS medium containing 0.1 M sucrose for 11 days (step 1), and then with medium containing 0.4 and 0.7 M sucrose for one day each (step 2) at 25 °C. The buds were subsequently incubated in vitrification solutions at 25 °C for designated periods prior to immersion in LN. The two-step preculture procedure, vitrification procedures, thawing and reculturing are detailed in "Materials and methods." The survival (regrowth capacity) of the axillary buds was defined as the percentage of buds producing normal shoots and roots after 20 days in vitro. The data are the mean ± SD of triplicates (ten buds or more for each replicate). Different letters besides the symbols indicate significant differences ($P < 0.05$) by Tukey's test

(78%) was achieved with an incubation period of 45 min (Fig. 2a). Longer incubation periods with PVS2 resulted in decreased survival both in the treated control and the LN-exposed gentian buds. A similar PVS2 incubation period dependency (survival 20% at 20 min, 76% at 30–40 min and 50–20% at 50–60 min) was also obtained by Tanaka et al. (2004). The results showed that the best survivals obtained (78%) were similar for both PVS2 and VSL.

The higher survival at an incubation period of 20 min suggests that VSL became effective in vitrifying the shoot apices of gentian buds faster than PVS2, probably due to its faster penetration and/or faster dehydration of tissues comprising the apices. The survival of buds treated with VSL only (treated control) indicates that VSL is less toxic for gentian buds than PVS2. These properties most probably allowed VSL to have a wider optimal incubation period than PVS2.

Axillary buds that survived cryopreservation using VSL developed normal shoots without callus formation (Fig. 3). Similar results were obtained with PVS2 (data not shown). This allows somaclonal variation to be avoided and is useful for conserving vegetatively propagated gentians.

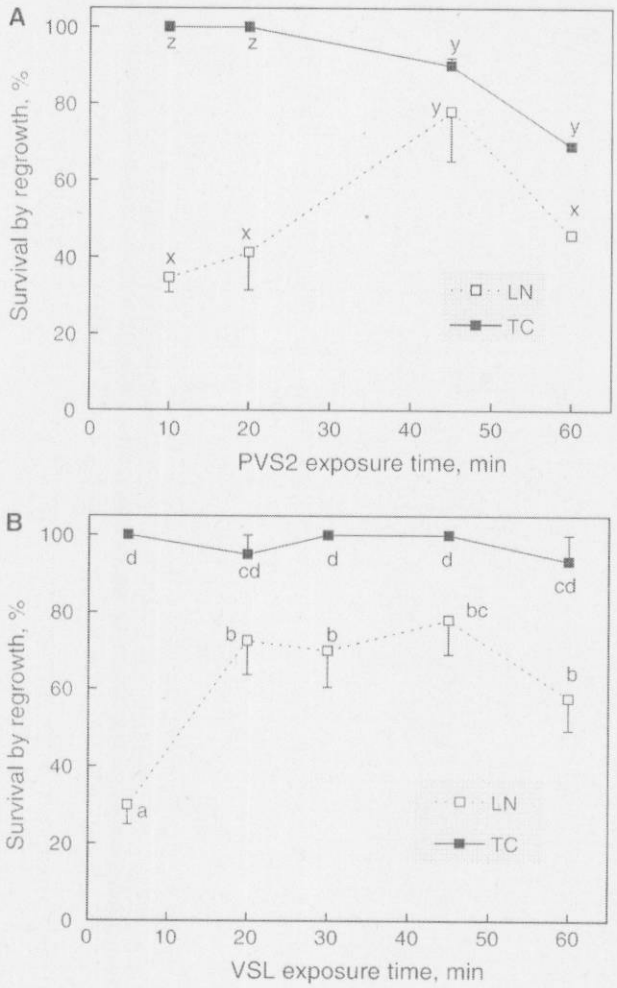

Fig. 2 Effect of duration of incubation in **a** PVS2 and **b** VSL on the survival (regrowth capacity) of gentian buds before (*TC*) and after (*LN*) immersion in LN. Excised axillary buds of gentian were two-step precultured and used for vitrification procedures as described in Fig. 1. The data are the mean ± SD of triplicates (ten buds or more for each replicate). Different letters besides the symbols indicate significant differences ($P < 0.05$) by Tukey's test

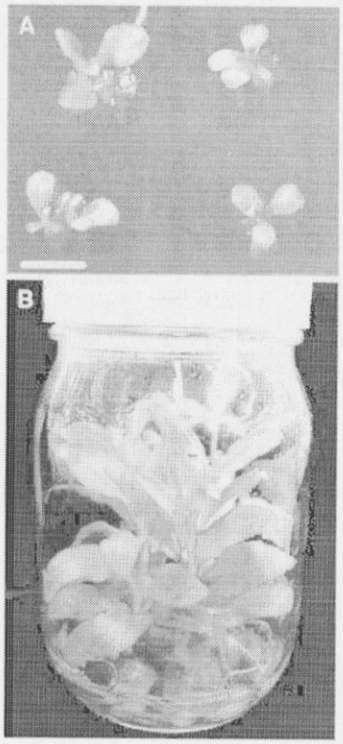

Fig. 3 Regrowth of gentian axillary buds cryopreserved using the two-step preculture and VSL after **a** 10 days and **b** 90 days of reculturing in vitro. The *bar* indicates 5 mm

Further optimization of the vitrification-based cryopreservation of gentian buds: length of the first preculture step

VSL had an osmolarity of ca. 8 M, like other vitrification solutions, and induction of osmotic tolerance prior to cryopreservation was crucial. In gentian buds, desiccation or osmotic tolerance can be induced by two-step preculturing with sucrose or by prolonged cold-hardening treatment (Suzuki et al. 1998; Tanaka et al. 2004). To make the protocol shorter, two-step preculturing was selected. The first step of this preculturing gives mild osmotic stress and we determined the optimal period of this step (Fig. 4). The results show that preculturing buds with MS medium containing 0.1 M sucrose for 7–11 days gave high survival of the buds exposed to LN. This was similar to previously reported cases where the two-step preculturing was applied to the desiccation-based cryopreservation of gentian buds with and without encapsulation (Suzuki et al. 1998, 2005, 2006).

Effect of sugars contained in the preculture medium on the survival of gentian axillary buds incubated with VSL and exposed to LN

To determine which sugar species is effective for two-step preculturing, gentian buds were precultured on MS medium containing four sugars (0.1 M for 11 days followed by 0.4 and 0.7 M for one day each) and tested for vitrification with VSL. The results showed that preculturing with sucrose and glucose gave 80 and 70% survivals, respectively, following exposure to LN, while fructose and maltose gave 15 and 0% survivals, respectively (Fig. 5). In our previous study where the effects of various sugars during identical two-step preculturing were compared in terms of inducing desiccation tolerance (water content 10% on f. wt. basis) in gentian buds, glucose and sucrose gave the highest survivals (>90%) and maltose and fructose gave 50–60% survivals (Suzuki et al. 1998). Preculturing

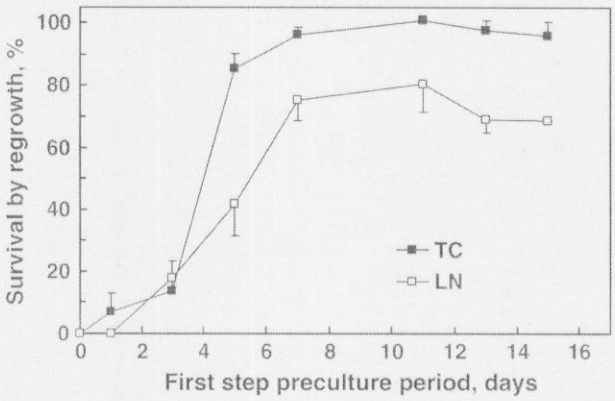

Fig. 4 Effect of the length of the first preculture step using 0.1 M sucrose on the survival (regrowth capacity) of gentian buds exposed to VSL (*TC*) and subsequently to LN (*LN*). Following the first step of preculturing for the designated period, the buds were precultured on 0.4 and 0.7 M sucrose for one day each at 25 °C before being incubated in VSL for 45 min at 25 °C and immersed in LN. The data are the mean ± SD of triplicates (ten buds or more for each replicate)

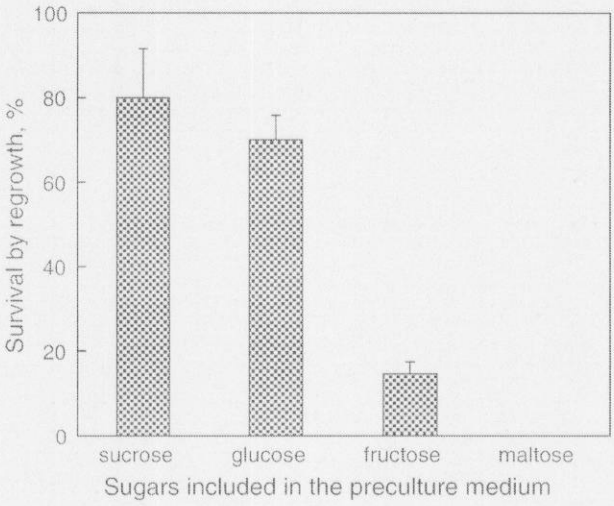

Fig. 5 Effects of four different sugars (*sucrose, maltose, glucose* and *fructose*) included in the preculture medium on the survival (regrowth capacity) of gentian buds cryopreserved using VSL. The buds were two-step precultured on MS medium containing the designated sugar (0.1 M for 11 days in the first step and 0.4 and 0.7 M for one day each in the second step) before being incubated in VSL for 45 min at 25 °C and submerged in LN. The data are the mean ± SD of triplicates (ten buds or more for each replicate)

of gentian buds with sucrose and glucose is an effective way to increase survival in cryopreservation using both desiccation and vitrification methods. In contrast, preculturing with fructose and maltose gave much lower survivals in the vitrification method than the desiccation method. These results imply that sugars in the preculture medium do not work merely as an osmoticum.

Previous studies have revealed that the first preculture step (2.3-fold increase in sucrose concentration from normal subculture medium) involves a transient ABA increase and results in ABA-mediated cellular changes, while in the second preculture step the sugar in the medium was loaded into bud cells (Suzuki et al. 2006). The differences in the performances of the sugars may arise from the ability of each sugar to vitrify per se in the desiccated state or upon rapid cooling in LN, and/or the ability of each sugar to be incorporated into the cells in the second step. It is also possible that the results may reflect the abilities of the sugars to elicit signals for inducing dehydration tolerance in the first step (Suzuki et al. 1998, 2006).

Thermal behavior of VSL and VSL+

VSL and VSL+ vitrified upon rapid cooling to LN temperatures, as indicated by the DSC heating profiles, where glass transition (T_g) and devitrification (T_d) occurred before the melting (T_m) of the samples (Fig. 6). These heating profiles of VSL and VSL+ were similar to those of PVS1 and PVS2, respectively. PVS2 and VSL+ had much smaller devitrification exotherms and melting endotherms due to the increased concentrations of sucrose. T_g (onset), T_d (onset) and T_m of the vitrification solutions are summarized in Table 4. VSL+ and PVS2 exhibited T_g and T_d at higher temperatures and T_m at lower temperatures than VSL and PVS1, respectively. Cooling rates of between −100 and −10 °C min^{-1} did not greatly affect the heating profiles (T_g, T_d, T_m) (figure not shown), which indicates that the samples can be vitrified at these cooling rates. The cooling profiles of these solutions had only a single shift (T_g) in the heat flow when cooled at rates of between −10 and −100 °C min^{-1} (figure not shown). The onset of this shift (T_g) for VSL occurred at −118 °C in the cooling profile. These data allow us to conclude that VSL and VSL+ are capable of being vitrified upon rapid cooling to LN.

PVS2 is rich in glycerol (30% w/v). This makes the solution fairly viscous (the viscosity of 100% glycerol at 20 °C is 1,499 mPa s), and the resulting decreased mobility of molecules is considered to contribute to the high vitrifying ability of the solution (Chang and Baust 1991). The high viscosity of PVS2 may also result in a slower penetration and/or slower dehydration of the tissues (Fig. 2). VSL, on the other hand, is rich in ethylene glycol (30% w/v), and contains less DMSO, glycerol and sucrose compared to PVS2. The viscosity of 100% ethylene glycol at 20 °C is 23.5 mPa s. This makes the VSL less viscous than PVS2, most probably resulting in faster penetration and/or faster dehydration of the tissues (Fig. 2) while maintaining its vitrification capabilities (Fig. 6). The toxicity of the solution depends on the materials, since the lower content of DMSO and the higher content of ethylene glycol may cancel each other.

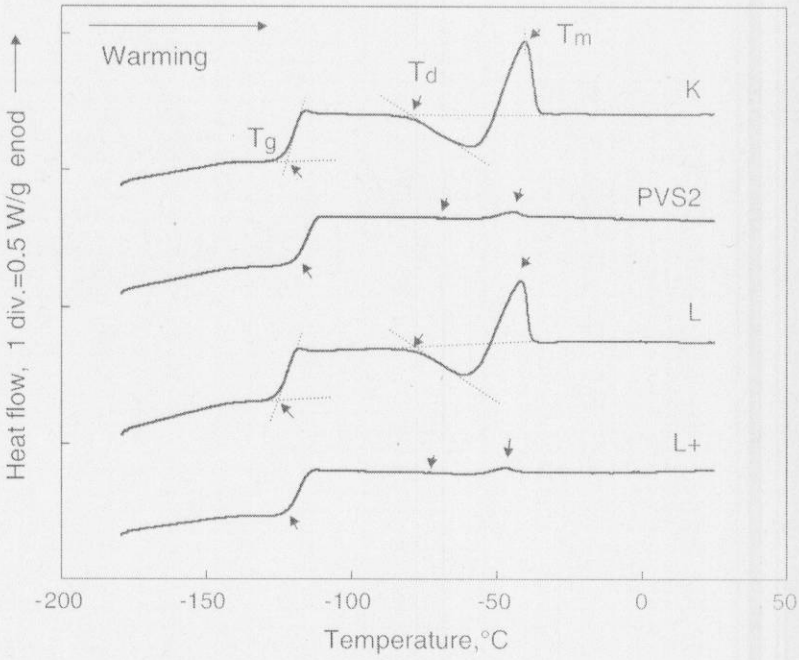

Fig. 6 DSC profiles showing the heating processes of the vitrification solutions *K* (PVS1), *PVS2*, *L* (VSL) and *L+* (VSL+). The samples (5–10 mg) were cooled at 100 °C min^{-1} from 25 to −175 °C (180 °C min^{-1} to −140 °C) and held there for 2.5 min before being warmed at 10 °C min^{-1} from −175 to 25 °C. T_g, glass transition temperature; T_m, melting temperature; T_d, devitrification temperature

Table 4 Summary of the thermal behaviors of the vitrification solutions, determined with differential scanning calorimetry (DSC)

Vitrification solutions	T_g (onset) °C	T_d (onset) °C	T_m °C
VSL	−125	−84	−41
VSL+	−121	−82 to −74[a]	−47
PVS1	−122	−80	−41
PVS2	−119	−83 to −75[a]	−44

The methods are described in the "Materials and methods" section and the legend for Fig. 6

[a] The values depend upon extrapolation

Application of VSL and VSL+ to the cryopreservation of other materials

VSL and VSL+ were applied to the vitrification-based cryopreservation of garlic shoot apices (without pretreatment). However, VSL and VSL+ gave lower survivals (regrowth) than PVS2 (Dr E. Niwata, personal communication). More recently, VSL+ was applied for the cryopreservation of recalcitrant tropical plant seeds by Dr Y. L. Hor (UPM, Malaysia), who learned the techniques in our laboratory. He attempted the vitrification of zygotic embryos of recalcitrant and semi-recalcitrant seeds, including comparisons of various vitrification cocktails (VSL, PVS2, PVS3, etc.). In the case of embryonic axes of lime (*Citrus madurensis*), 75 and 65% survivals (regrowth capacity) following cryopreservation were obtained using PVS2 and VSL+, respectively (Cho et al. 2002). In contrast, for cryopreserving embryos of jackfruit (*Artocarpus heterophyllus*) and rambutan (*Nephelium lappaceum*), VSL+ gave the best survival (regrowth) over PVS2 (Dr Y. L. Hor, personal communications). For others, such as rubber (*Hevea brasiliensis*), it was not as good as PVS2 (Sam and Hor 1999). In these cases, viability after vitrification in LN is generally in the 30–50% range, as they are recalcitrant species characterized by high water contents and sensitivity to desiccation, and it is generally difficult to obtain high survival for them. Hopefully, VSL or VSL+ will be applied to the cryopreservation of various species and compared with other vitrification solutions.

In summary, candidates for good vitrification solutions were surveyed using bromegrass suspension cells. The best vitrification solution found through the survey using FDA staining, named VSL, was rich in ethylene glycol, and had the ability to vitrify during cooling to LN, as shown by DSC. Bromegrass cells cryopreserved following direct treatment with VSL were stained well with FDA but did not regrow. However, when it was applied to gentian axillary buds following an appropriate two-step preculture, VSL gave comparable survival to PVS2 with a wider range of optimal incubation times. VSL seems more suited to the cryopreservation of gentian buds. Conditions for inducing osmotic tolerance were also optimized: the highest survival was obtained with axillary buds that had been precultured on medium containing 0.1 M sucrose for 7–11 days (first step), followed by a second step preculturing on medium containing 0.4 and 0.7 M sucrose for one day each. For the sugar used for two-step preculturing, sucrose and glucose gave much higher survivals than fructose and maltose. Application of VSL and VSL+ to the cryopreservation of

garlic shoot apices and zygotic embryos of four tropical recalcitrant seeds revealed that in three cases VSL or VSL+ was more toxic than PVS2, while in the other two cases VSL+ gave better survival than PVS2. Optimal conditions, including the appropriate vitrification solution for successful cryopreservation, vary depending upon the materials. VSL or VSL+ may perform better in materials that need faster penetration of the vitrification solution and/or faster dehydration of the tissues, such as gentian buds. VSL or VSL+ seem to have the potential to become good alternatives to PVS2 in such materials.

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