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Cryopreservation of Alkaloid-Producing Cell Cultures of Periwinkle (*Catharanthus roseus*)[1]

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TONY H. H. CHEN, KUTTY K. KARTHA*, NICHOLAS L. LEUNG, WOLFGANG G. W. KURZ, KENNETH B. CHATSON, AND FRIEDRICH CONSTABEL
Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan S7N 0W9 Canada

ABSTRACT

A procedure for cryogenic storage of alkaloid producing cell lines of periwinkle, *Catharanthus roseus* (L.) G. Don., has been developed. The procedure differs from established cryopreservation protocols in several aspects. Specifically, 4-day-old suspension subcultures of three cell lines were precultured in nutrient media supplemented with 1 molar sorbitol for 6 to 20 hours. The cells were then incubated in nutrient media with 1 molar sorbitol plus 5% DMSO in an ice bath for 1 hour and, thereafter, were frozen in this solution at a cooling rate of 0.5°C per minute to −40°C prior to immersion in liquid nitrogen (LN). After rapid thawing in a 40°C water bath, the regrowth of LN stored cells was achieved by transferring them without washing onto filter paper discs over nutrient media solidified with agar for a period of 4 to 5 hours. The filter paper discs with the cells were then transferred to fresh media of the same composition for regrowth. The viability immediately after thawing as evaluated by the 2,3,5-triphenyl tetrazolium chloride method was about 60% of controls. Suspension cultures established from LN stored cells retained the capability for alkaloid synthesis and accumulation.

Plant cells cultured *in vitro* are potential sources of useful chemicals such as alkaloids (1, 5, 16, 23, 31). The production of alkaloids by *in vitro* cultures can vary among cell lines and in individual lines be fairly stable (2, 4); in other cell lines, alkaloid content and spectra have been found subject to gradual change over years of subculture (6). Instead of repeated selection to maintain a high alkaloid level, cryopreservation would appear as an alternative to overcome stability problems (14, 31).

Storage in LN[2] followed by recovery of viable plant cells have been reported for many species (7, 10, 12, 14, 21, 22, 28, 30) and cells of certain species retained the biosynthetic capacity for biotin (25), anthocyanin (8), and cardenolides (7). However, whether such a cryogenic technique could be applied to the preservation of cell cultures with capability for alkaloid synthesis and accumulation has not been determined and thus served as the primary objective of the present study.

Cell lines of periwinkle, *Catharanthus roseus*, designated nos. 200, 615, and 91601 accumulated various indole alkaloids including catharanthine (18). The preservation of the alkaloid-synthesizing capability of these cell lines is not only important for an ongoing study of the biosynthesis of iboga type alkaloids, but also for industrial exploitation of *Catharanthus* cell cultures.

A procedure for prolonged cryogenic storage of periwinkle cell line no. 916, which did not produce alkaloids in detectable amounts, has been reported (14). We employed the procedure established for no. 916 (14) as well as other published protocols for the freeze-preservation of cultured plant cells (10, 14, 21, 22, 25, 26, 27, 28, 29); but none of them was found suitable for any of the alkaloid-producing periwinkle cell lines.

In the present paper, we report a method for the successful cryopreservation of alkaloid-producing cell lines of periwinkle. It differs in several respects from previously established protocols.

MATERIALS AND METHODS

Plant Material. Alkaloid-producing periwinkle (*Catharanthus roseus* L., G. Don.) cell lines designated PRL nos. 200, 615, and 91601 were used in this study. These cell lines were initiated from callus of anther walls and filaments of periwinkle as previously reported (18), and were maintained on solid and in liquid media. Subcultures of cell line no. 200 were grown in liquid B5 medium (11) supplemented with 1 mg/l 2,4-D, 1 g/l casein hydrolyzate, 200 mg/l ribose, 200 mg/l glucose, and 200 mg/l arginine. Subcultures of nos. 615 and 91601 were grown in liquid B5 medium supplemented with 1 mg/l 2,4-D. All cell suspensions were subcultured as 50-ml batches in 250-ml Delong flasks on gyratory shakers (130 rpm) under a continuous light of about 100 wm^{-2} at 24 ± 1°C.

Pretreatment. Unless specified otherwise, all cultures were precultured in nutrient media containing 1 M sorbitol before preparation for freezing. For preculturing, 30 ml of a 4-d-old culture, containing about 10 ml packed cell volume, was transferred to a 50-ml Erlenmeyer flask, and 10 ml of 4 M sorbitol was added gradually to the final sorbitol concentration of 1 M. This material was maintained on a gyratory shaker at 130 rpm for 17 to 20 h. At the end of the preculture period, the flask containing the cells was chilled in an ice bath. DMSO was added to a final concentration of 5% (v/v) by gradually adding culture medium containing 1 M sorbitol and 30% DMSO (v/v) over a period of 15 min followed by an equilibration period of 1 h.

Freezing and thawing. One-ml aliquots of cell suspension were dispensed into cryogenic glass ampoules (1.2 ml capacity, Wheaton) and flame-sealed. The sealed ampoules were arranged in the freezing chamber (CRYOMED 972 Freezer) precooled to 0°C. The specimens were cooled at desired cooling rates (0.3–5°C/min) with a programmable CRYOMED 1000 controller to terminal freezing temperatures ranging from −20°C to −45°C followed by immersion in liquid nitrogen. After 1 h storage in LN, the ampoules were rapidly thawed in a 40°C water bath.

Viability Test and Regrowth. The thawed cell suspensions were immediately used for assessing the viability and for regrowth, without washing away the cryoprotectants. The rate of

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[2] Abbreviations: LN, liquid nitrogen; TTC, 2,3,5-triphenyl tetrazolium chloride.

viability of frozen-thawed cells was compared with the controls by means of TTC reduction assay (24). The viability of cells after various treatments was expressed as per cent survival over the nontreated, nonfrozen control.

For regrowth, samples of 1 ml suspension in freezing solution (5% DMSO + 1 M sorbitol), containing about 0.3 g cells (fresh weight) were plated over pieces of sterilized filter paper (Whatman No. 1, 7 cm in diameter), which were placed over 20 ml B5 medium containing 1 mg/l 2,4-D, 1 g/l casein hydrolyzate, and 0.8% agar in a Petri dish. The regrowth technique was essentially the same as the double filter paper technique of Horsch and Jones (13). After 4 to 5 h, filter papers with cells were transferred to other Petri dishes containing the same medium. The Petri dishes were then sealed with Parafilm strips and kept inside a plastic bag to minimize water loss. They were incubated in a growth chamber at 26°C, and 16 h photoperiod. The weight of the wet filter paper discs (A) and the weight of filter papers plus cells (B) were measured during the growth period.

Alkaloid Extraction and Identification. The alkaloids were extracted and separated according to the procedure described earlier (4, 17). Alkaloid identification based on physical and spectral data have been described in a preceding publication (19).

RESULTS

The effect of DMSO alone and in combination with sugars and sugar alcohols on the survival of periwinkle cell line no. 200 at LN temperature is shown in Table I. In all experiments, 4-d-old cultures were precultured in culture medium containing 1 M sorbitol for 20 h prior to cryopreservation. With preculturing, the cell survival was marginal (<5%) after freezing and 1 h storage in LN. DMSO alone at concentration ranging from 5 to 15% showed very little cryoprotective effect. However, the combination of 5% DMSO with 0.5 or 1 M sugars or sugar alcohols dramatically improved survival of cells. For example, the combination of 5% DMSO and 1 M sorbitol enabled 61.6% of cells to survive the freezing and storage in LN. Among the sugars and sugar alcohols tested, the order of cryoprotection was sorbitol > mannitol, sucrose > glucose > trehalose. When these compounds were applied alone at concentrations of up to 1 M, cell survival rate was less than 10% (data not shown). Therefore, cryoprotectants used in the following experiments were 5% DMSO + 1 M sorbitol unless specified otherwise.

The optimal cooling rate appears to be 0.5°C/min for cell line no. 200 (Fig. 1). Cells cooled at 0.3°C/min exhibited an insignificantly lower viability than those cooled at 0.5°C/min. On the other hand, faster cooling rates greater than 1°C/min resulted in poor survival. At the optimal cooling rate of 0.5°C/min, the effect of the terminal freezing temperature prior to immersion in LN on cell survival is shown in Figure 2. A terminal freezing temperature of −35 to −40°C was optimum under experimental conditions given. Prefreezing of the specimens to a temperature above −35°C or below −40°C showed a decline in cell survival.

Table I. *Effects of DMSO, Sugars, and Sugar Alcohols on the Survival of Periwinkle Cells (Line No. 200) after Freezing and Storage at LN Temperature*

Cryoprotectant	Survival
	%
Control	7.2
5% DMSO	8.0
7.5% DMSO	8.0
10% DMSO	5.6
15% DMSO	5.6
5% DMSO + 0.5 M sucrose	36.0
5% DMSO + 0.5 M sorbitol	48.8
5% DMSO + 1.0 M sorbitol	61.6
5% DMSO + 0.5 M glucose	28.8
5% DMSO + 0.5 M trehalose	24.0
5% DMSO + 0.5 M mannitol	36.8
$LSD_{0.05}$	8.5

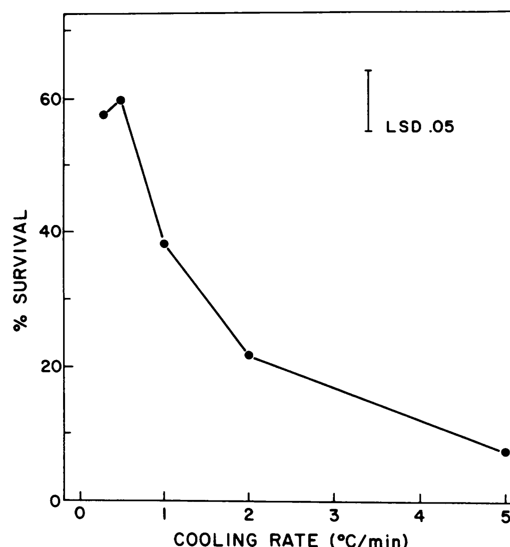

FIG. 1. Effect of cooling rate on the survival of periwinkle cell line no. 200 frozen and stored in LN for 1 h. Four-d-old cultures were precultured in culture medium containing 1 M sorbitol for 20 h, 1 h in 5% DMSO + 1 M sorbitol over ice, cooled at various cooling rates to −35°C and then immersed in LN.

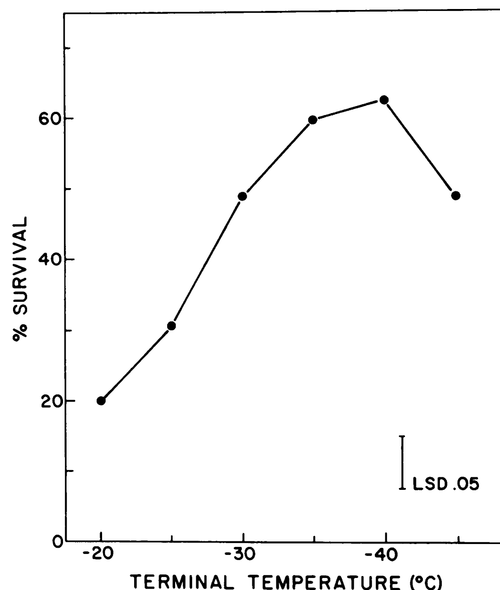

FIG. 2. Effect of terminal freezing temperature prior to immersion in LN on the survival of periwinkle cell line no. 200. Four-d-old cultures were precultured in culture medium containing 1 M sorbitol for 20 h, 1 h in 5% DMSO + 1 M sorbitol over ice, cooled 0.5°C/min to various temperatures from −20°C to −45°C and stored 1 h in LN.

From preliminary experiments, we found that preculture in nutrient media containing 0.5 to 1 M sorbitol for 17 h was critical for successful cryopreservation. In order to assess the optimal period of preculturing, cells of 4-d-old subcultures were transferred to nutrient media containing 1 M sorbitol for various periods of time from 0 to 48 h. As shown in Figure 3, the controls gradually lost their viability within the first 10 h of preculture and reached a level of about 80% viability. The survival of cells exposed to sorbitol, on the other hand, was markedly improved

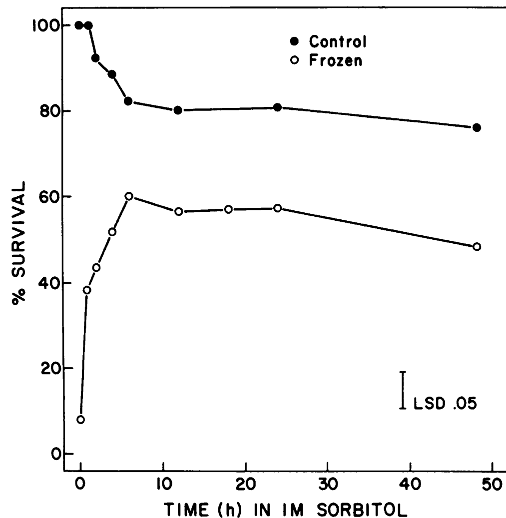

FIG. 3. Effect of the duration of preculturing in liquid media containing 1 M sorbitol on the viability of periwinkle cell line no. 200. After preculturing, the cell suspensions were incubated in liquid medium containing 5% DMSO + 1 M sorbitol in an ice bath for 1 h, cooled 0.5°C/min to −40°C and stored in LN for 1 h.

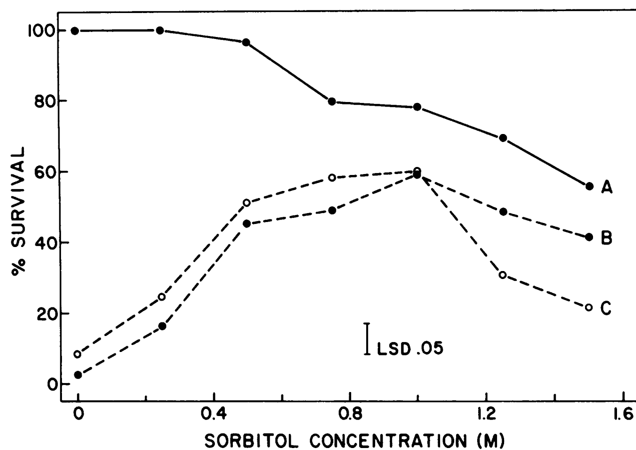

FIG. 4. Effect of sorbitol concentration for preculturing on the survival of periwinkle cell line no. 200 frozen and stored in liquid nitrogen for 1 h. A, Control; B, frozen in 5% DMSO + 1 M sorbitol; C, frozen in 5% DMSO + various sorbitol concentrations (5% DMSO plus the same concentration used for preculturing).

by such a preculturing treatment. One h preculture in 1 M sorbitol improved the rate of cell survival from 8% to about 40%, and the rate of survival reached a peak of 60% within 6 h. Extension of the incubation period from 6 to 20 h gave no increase in per cent survival. The 20-h preculturing period was adopted for routine cryopreservation for reasons of practical convenience.

Preculture in culture medium containing sorbitol concentrations higher than 1 M for 20 h, resulted in reduced viability even without freezing (Fig. 4). The viability was about 80%, 70%, and 60% for the cells precultured in media enriched with 1.0. 1.25, and 1.5 M sorbitol, respectively (line A). Line B shows the cells precultured in various sorbitol concentration from 0 to 1.5 M for 20 h and frozen in 5% DMSO + 1 M sorbitol. Under these conditions, a sorbitol concentration of 1 M was found to be optimal. Concentrations higher than 1.25 M or lower than 0.6 M were less effective. Line C is similar to line B except that after preculturing, cells were frozen in the same sorbitol concentration as those used for preculturing (0–1.5 M) with 5% DMSO. Again, preculturing in 1 M sorbitol for 20 h and frozen in 1 M sorbitol + 5% DMSO appeared to be optimum.

Four-d-old cultures were precultured in medium containing 1 M sorbitol for 20 h, and then chilled in an ice bath. DMSO solution (30% v/v in culture medium) was gradually added to the cell suspension to the final concentration of 0 to 10% and kept in an ice bath for 1 h. As shown in Figure 5, DMSO concentrations higher than 5% were found to be toxic (control). DMSO concentrations of 5% caused little effect on the viability of precultured cells and also retained maximum viability of cryopreserved cells.

Various compounds added to the preculture medium as osmotic agents for their effectiveness on cryopreservation were also tested (Table II). All the concentrations of tested compounds were adjusted to 1 Osmolar. $CaCl_2$ and KCl were found to be toxic to the cells (control) and less effective in improving the cell survival during cryopreservation. Sorbitol and glucose were less toxic than trehalose or sucrose. In terms of per cent cell survival after cryopreservation, the order of effectiveness were sorbitol > glucose > trehalose > sucrose.

Figure 6 shows the effect of age of subcultures on the cell survival of periwinkle cell line nos. 200, 615, and 91601 after cryopreservation. For cell line no. 200, a peak of cell survival was observed on the 4th d of transfer. For cell line no. 615, the peak was on the 3rd d. For cell line no. 91601, the per cent cell survival was almost the same from the 2nd to 5th d of subculture.

By gathering the information obtained from all experiments described above, the best conditions for successful cryopreser-

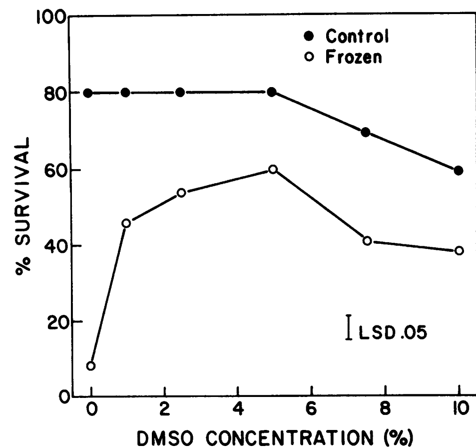

FIG. 5. Effect of DMSO concentration on the viability of periwinkle cell line no. 200. Control: 20 h precultured in 1 M sorbitol and 1 h in 1 M sorbitol plus various concentrations of DMSO. Frozen: same as control but cooled 0.5°C/min to −40°C and 1 h in LN.

Table II. *Effects of Various Osmotic Agents for Preculturing on the Survival of No. 200 Periwinkle Cell Culture at LN Temperature*

The cells of 4-d-old culture were precultured in the culture medium containing 1 osmolar of various osmotica for 20 h prior to DMSO treatment and frozen, and stored in LN for 1 h.

Cryoprotectant	Survival	
	Control	Frozen
	%	
$CaCL_2$	18.1	6.5
KCl	43.7	25.0
Sorbitol	76.3	61.0
Trehalose	60.0	41.5
Glucose	76.4	54.3
Sucrose	51.0	41.1
$LSD_{0.05}$	6.5	5.2

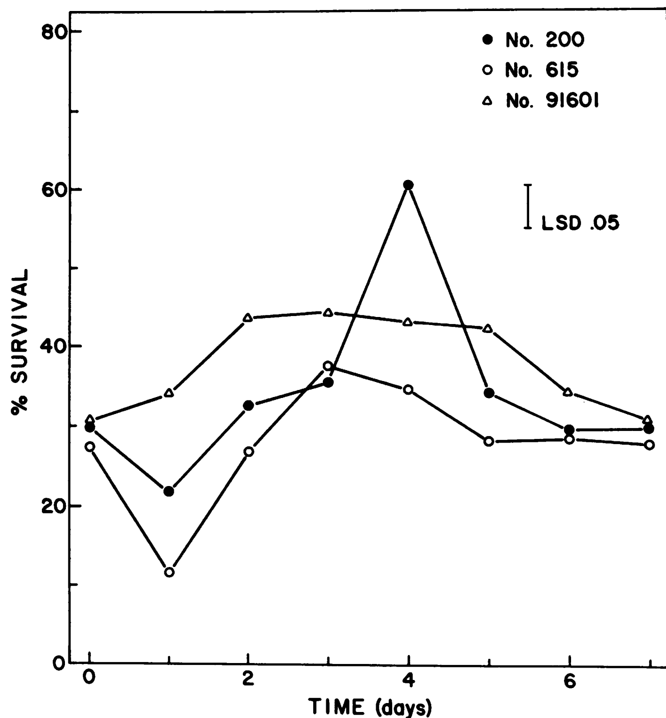

FIG. 6. Effect of the age of culture on the survival of cell line nos. 200, 615, and 91601 after freezing and storage in LN for 1 h.

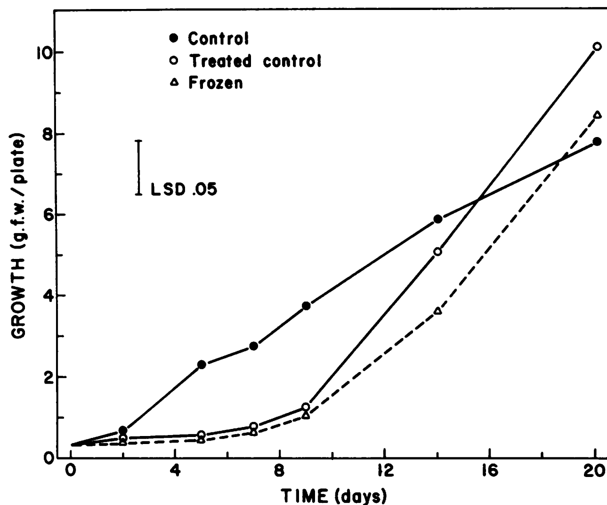

FIG. 7. Recovery growth of frozen and thawed cells of periwinkle cell line no. 200. Control: cultured without any treatment. Treated control: precultured in 1 M sorbitol 20 h, and in 5% DMSO + 1 M sorbitol 1 h. Frozen: same as treated control, but cooled 5°C/min to −40°C and stored 1 h in LN.

vation of these alkaloid producing periwinkle cell lines are: (a) 4-d-old cultures precultured in nutrient media containing 1 M sorbitol for 20 h, chilled over an ice bath in 1 M sorbitol + 5% DMSO for 1 h. (b) One ml of this cell suspension in each glass ampoule cooled at 0.5°C/min to −40°C followed by immersion in LN. (c) The ampoules rapidly thawed at 40°C and viability assayed immeditely after thawing but without washing away the cryoprotectants.

Cells of line no. 200 have been frozen following this procedure and grown on filter paper discs over agar medium. Figure 7 shows the regrowth of the control, treated control (see legend) and frozen cells of line no. 200. The control cells grew immediately after plating, whereas the treated control cells started the growth after a lag period of 5 d, and rapid growth was observed after 7 d of plating. The regrowth of the LN-stored cells followed a similar pattern as the treated control, but at a slightly lower rate. The average growth as expressed by fresh weight (g) per plate, 20 d after plating, was greater for treated control or frozen samples than for the control.

Figure 8 shows the examples of regrowth of cell line nos. 200, 615, and 91601 cells on filter paper after 20 d of plating. The frozen (LN) and thawed samples of all three cell lines did grow to the extent of control (no. 200) or lesser than control (nos. 615 and 91601). Cells grown on filter paper were transferred to liquid media to re-establish the suspension cultures. Alkaloid analysis (Table III) revealed that the total alkaloid production of cells recovered after storage is essentially the same as that of the treated control; however, slight qualitative shifts in the alkaloids produced from the cryopreserved cells have been observed.

DISCUSSION

Studies of plant cell suspensions indicate the importance of the phase in the growth cycle for successful cryopreservation (14, 27, 29, 30). For example, Withers and Street (30) reported that with carrot suspension culture cells in late lag phase or exponential growth were most likely to recover rapidly after freeze preservation. In suspension cultures of *Acer pseudoplatanus*, cells in G_1 have an increased capacity to survive freezing and thawing (29). With periwinkle suspension cultures, 3- to 4-d-old cultures were most suitable for cryogenic manipulation; this observation was found with both the nonproducing cell line no. 916 (14) as well as with the alkaloid-producing lines used in this study. This is probably because 3 to 4 d after subculture, cells had undergone active division and appeared less vacuolated and displayed dense cytoplasm (14).

Supplementation of culture medium prior to freezing the cells with such additives as mannitol, sugar or proline was found to be an effective method for improving the recovery of LN-stored cells (26, 27, 30). Kartha *et al.* (14) reported that preculturing periwinkle cells of line no. 916 in culture medium containing 5% DMSO for 24 h dramatically improved their survival in cryogenic storage. Weber *et al.* (26) reported that preculture in medium containing 1 M sorbitol for 16 h and frozen in the same solution permitted sorbitol to replace DMSO as cryoprotectant. All previous protocols have been tried with alkaloid-producing PRL cell lines without success. Preculture of alkaloid producing cell line no. 200 in 5% DMSO for 24 h resulted in about 90% loss of viability. Precultured in 1 M sorbitol and frozen in the same solution, the cells did not survive better than without such preculture. However, when preculture was followed by freezing in 5% DMSO + 1 M sorbitol, cells did survive freezing and LN storage.

If such a pretreatment were purely due to the dehydration effect as proposed (26), one would expect that various agents of equal osmolarity should give similar results. Withers (27) stated that "although cell size reduction may result from the medium supplementation, this is unlikely to explain totally the improvement in survival potential." She rather speculated that the effect of supplementation of osmotically active compounds in the growing medium may in fact trigger some cellular changes which mimic the natural cold hardening process (27). Our results indicated that different agents of the same osmolarity show both different preculturing and different cryoprotective effects during freezing. We also speculate that those osmotic agents may not act simply through dehydration.

One of the critical factors in cryopreservation is the cryoprotectant and its concentration. The use of a mixture of cryoprotectants in general is better than using a single cryoprotectant (9, 10, 15, 25, 27). With periwinkle cells, both DMSO and sorbitol broadened the range of temperature from initiation to comple-

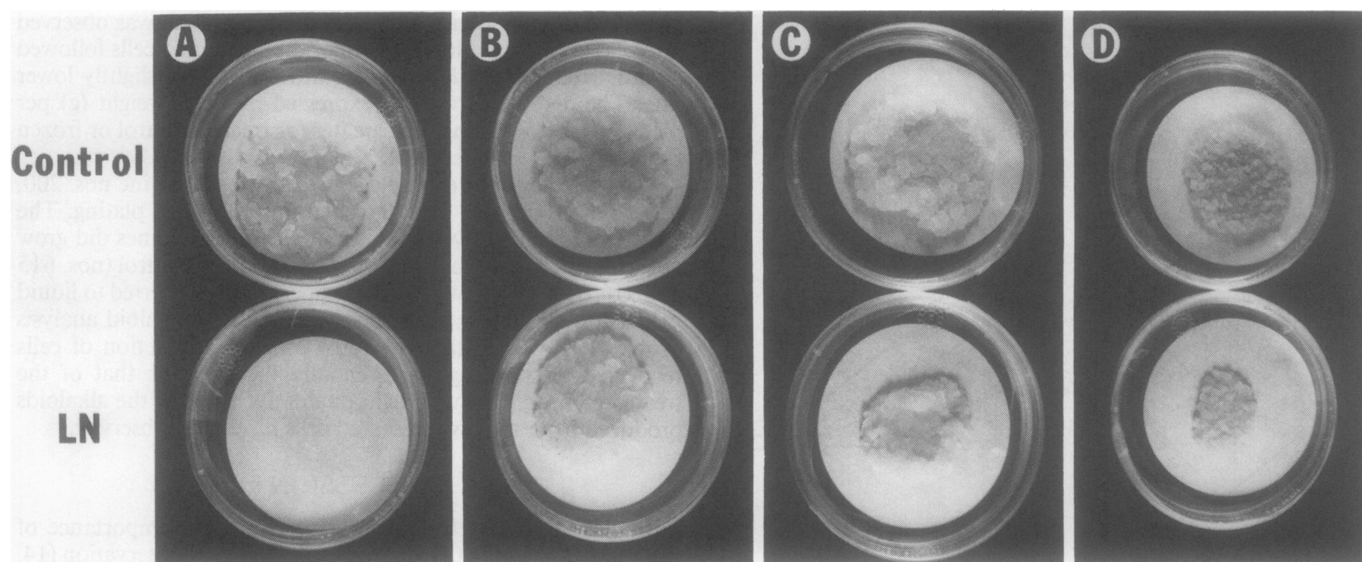

FIG. 8. Regrowth of periwinkle cell line nos. 200, 91601, and 615, frozen and stored in LN for 1 h. A, no. 200 without preculture; B, no. 200 precultured in 1 M sorbitol for 24 h; C, no. 91601 precultured in 1 M sorbitol for 24 h; D, no. 615 precultured in 1 M sorbitol for 24 h. After precultured for 20 h in 1 M sorbitol (except A), cells were incubated 1 h in 5% DMSO + 1 M sorbitol, cooled 0.5°C/min to −40°C, and stored 1 h in LN. The picture was taken 20 d after plating onto the filter paper over agar medium.

Table III. *Alkaloid Production from Suspension Cultures of Three Periwinkle Cell Lines before and after LN Storage*

Cell Line No.	Treatment			Alkaloids				Total Alkaloid
	1 M sorbitol	5% DMSO + 1 M sorbitol	LN[a]	Strictosidine[b]	Ajmalicine	Hörhammericine	Hörhammerinine	
		h						*mg/ml culture*
200	0	0	0	+	+	+	+	0.193[c]
200	20	1	0	+	+	+	−	0.201
200	20	1	1	+	+	+	−	0.186
91601	0	0	0	++	++	++	+	0.242
91601	20	1	0	++	++	++	+	0.273[c]
91601	20	1	1	++	++	++	+	0.289
615	0	0	0	+	+	+	+	0.209[c]
615	20	1	0	+	+	++	+	0.197
615	20	1	1	+	+	++	−	0.185

[a] Cooled at 0.5°C/min. to −40°C prior to immersion in LN.
[b] Identified as Strictosidine lactam.
[c] Not significantly different within each cell line by *t* test.

tion of ice crystallization. As shown previously, DMSO treatment has an additional effect in reducing the amount of ice crystallized at −30°C to −40°C (3), and the combination of DMSO and sorbitol affects the rate of ice crystallization and the amount of water frozen at sub-zero temperature. This observation may explain why DMSO and sorbitol in combination was more effective as a cryoprotectant than when used alone.

In most cases of cryopreservation of plant cell cultures, slow freezing to an appropriate freezing temperature prior to transferring into LN is recommended. It is generally believed that during relatively slow freezing, the extracellular medium freezes at a higher temperature, whereas the freezing of intracellular water will initiate at lower temperatures. Therefore, a continuous flow of water occurs out of the supercooled cytoplasm to the surrounding ice due to a vapor pressure deficit. Such partially dehydrated cells would then be able to survive the LN temperature. Too rapid freezing will prevent the dehydration resulting in intracellular freezing and consequently cell death. There is no report of successful cryopreservation of cells from suspension cultures by freezing at a cooling rate greater than 10°C/min (27), which is probably due to the extremely high water content of cells grown in liquid media. For cryopreservation of cells from suspension cultures, the optimal cooling rate was found to be in the range of 0.5 to 2°C/min (15, 27). For the alkaloid-producing periwinkle cell lines, the maximal cell survival was achieved by slowly freezing at 0.5°C/min to −40°C prior to immersion in LN (Figs. 1 and 2), similar to that reported for cell line no. 916 (14).

Post-thaw removal of the cryoprotectants has been prescribed in most protocols (10, 13, 14, 21, 22, 27, 30). However, such an operation may lead to deplasmolysis injury, the removal of lost solutes, and may reduce the proportion of viable cells (27). Withers and King (28) found that suspension-cultured cells of *Zea mays* protected with DMSO, glycerol, sucrose, or proline recovered growth only when layered over a semi-solid medium without washing. Washing at 0°C, the most frequently used condition, has been found to reduce the viability of cryopreserved

rice and sugarcane cells (10). Therefore, the washing step may in fact lead to unnecessary loss of viability of otherwise successfully cryopreserved cells. Washing step may be even more harmful when higher concentrations of osmotic agents are incorporated in the freezing solution. A two-step dilution beginning with using 0.6 M sorbitol and followed by using sorbitol-free medium was employed by Weber *et al.* (26). With alkaloid-producing periwinkle cell lines, about 60% of cells were viable immediately after thawing without washing. When washed according to the procedure for the nonalkaloid-producing cell line no. 916 (14), the viability was less than 10% and no regrowth was observed after plating on filter paper discs. By plating the thawed cells over agar medium without washing, some callus growth was observed, but the rate of regrowth was very slow with a lag period of 3 to 4 weeks. Plating the thawed cells onto filter paper over agar medium without washing for 4 to 5 h may have enabled the cryoprotectants to leak into agar medium slowly. This not only simplified the post-thaw handling, but also avoided unnecessary washing damage. The regrowth technique practiced here was thought to be considerably better than washing and growing the thawed cells in liquid medium (21, 22), step wise lowering the concentration of the osmoticum (26), or plating the cells on semi-solid agar medium (27, 28).

The regrowth of precultured or LN-stored cells was better than controls during a 20-d regrowth period. There is no explanation for this observation, except that the cryopreservation may eliminate certain subpopulations of cells in the culture. Cryopreservation may have effected selection of a subpopulation of cells at particular stages of the cell cycle and levels of ploidy (29). For example, preculturing caused 20% loss of viability. Such loss may have represented cells more susceptible to osmotic stress. Similarly, during freezing and LN storage, another 20% viability loss may be due to elimination of another subpopulation of the cells. This may have resulted in the accumulation of cells which survived the cryopreservation expressing more vigorous growth than the control. This may also explain the observed qualitative changes in alkaloid spectra produced by the recovered cell cultures as compared to the controls.

The cryopreserved cells retained their alkaloid-producing capability. Selected constituents of the alkaloid spectra of cell lines nos. 200, 615, and 91601, *i.e.* corynanthe—and aspidosperma-type alkaloids, were detected prior to and after cryopreservation, and the total alkaloid content of cultures remained basically unchanged. Furthermore, the capacity for catharanthine accumulation was redetected in cryopreserved cells of line no. 200 (data not shown in Table III). The protocol reported here appears suitable for the cryopreservation of selected lines of *C. roseus.*

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