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Short Communication

Trehalose as Cryoprotectant for the Freeze Preservation of Carrot and Tobacco Cells[1]

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ABSTRACT

Suspension cultures of carrot (*Daucus carota*, line C1), tobacco (*Nicotiana tabacum*, line TX1), and *Nicotiana plumbaginifolia* (line NP) were frozen under controlled conditions with trehalose as the sole cryoprotectant. Maximal post-thaw viability (71–74%), measured by phenosafranin dye exclusion, was obtained with the C1 cells following a 24-hour pretreatment with 5 or 10% trehalose and with 40% trehalose as the cryoprotectant during freezing. TX1 cells pretreated for 24 hours with 10% trehalose and cryoprotected with 40% trehalose during freezing showed 47% viability following thawing as determined by phenosafranin dye exclusion. The NP cells required a 3 to 6 day pretreatment with 10% trehalose and 40% trehalose as a cryoprotectant at the time of freezing for the recovery of viable cells. Growing cells were recovered when the C1 and NP cells treated as described were plated on agar-solidified medium following thawing.

The freeze preservation of plant cells is desired for efficient storage of germplasm sources. Successful recovery of viable tissues usually requires the use of a cryoprotectant like DMSO which itself can be very toxic. The use of naturally occurring compounds like proline (12) or trehalose which may not be toxic would be advantageous.

Freeze-damage of cells is generally attributed to freeze-induced cell dehydration which results in a higher solute concentration, reduced cell volume, and loss of structural water (2, 6, 11). Of these processes, structural water has been suggested to be of major importance for maintaining membrane integrity (6). Dehydration of a biological membrane results in irreversible loss of structural and functional integrity of the membrane (4). Chen *et al.* (2) concluded that cryoprotectants in general may have two roles. Cryoprotectants such as DMSO which penetrate the membrane and enter the cell may prevent the dehydration of the cells by maintaining structural water. Cryoprotectants used at a high osmotic concentration such as proline, sorbitol, and glycerol act by reducing the cellular water and also at the same time reduce both the extracellular and intracellular ice formation. The use of a mixture of cryoprotectants, therefore, elicits an additive response in terms of cryoprotection.

Anhydrobiotic organisms such as yeast, some other fungi, and nematodes can survive a high degree of desiccation. These organisms accumulate during dehydration high concentrations of trehalose, an α-α-linked glucose disaccharide, which is utilized during and after rehydration. In studies with artificial membranes, it appears that trehalose substitutes for water molecules in the membrane during dehydration and thus helps to maintain membrane integrity (5). Because of these observations, and because structural water is an important component of freeze-injury, we tried trehalose as a cryoprotectant.

MATERIALS AND METHODS

Carrot (*Daucus carota* L. var sativa, cv Danvers, root-derived, line C1) and tobacco (*Nicotiana tabacum* L. cv xanthi, pith-derived, line TX1) (10) were cultured in liquid Murashige and Skoog medium (8) containing 0.4 mg/L of 2,4-D as the only hormone on a 7-d subculture regime. The Erlenmeyer flasks with 50 ml of liquid medium were incubated on a reciprocating shaker at 80 rpm at 27 to 28°C. *Nicotiana plumbaginifolia* Viviani (line NP) was initiated from seedlings in 1982 and was maintained as above.

Trehalose (40% in culture medium) and DMSO (both purchased from Sigma Chemical Co.) were filter sterilized and added to the culture medium to final concentrations of 5 or 10%. Pretreatments began on day 4 for the C1 and TX1 cells and day 2 for NP cells after subculture.

The freezing and thawing protocol was as described earlier (7). Cells were collected from 10 ml medium by centrifugation at 100*g* for 10 min. The supernatant was removed and the cells were resuspended in 5 ml of the cryoprotectant solution. Two-ml aliquots of this suspension were then placed in Cryotubes (Neslab) which were placed in ice for 1 h prior to cooling at 1°C min^{-1} to −40°C. The tubes were then placed in liquid N_2 for 2 d before thawing in a 40°C water bath.

Cell viability was measured by counting cells which had excluded phenosafranin (9) (about 150 cells were observed for each treatment). The remaining cells were plated on 20 ml of the culture medium solidified with 0.8% Bacto agar in 10-cm plastic Petri dishes which were then incubated at 27 to 28°C.

RESULTS AND DISCUSSION

Initial experiments using trehalose as the sole cryoprotectant during the freezing period without pretreatment did not allow the recovery of any viable cells following thawing with lines C1, TX1, and NP. However, the pretreatment of C1 cells with 5 or 10% trehalose for 24 h prior to freezing in 20 or 40% trehalose gave more than 50% viability (Table I; Fig. 1A). The cells were capable of continued growth when plated on agar-solidified medium (Fig. 1A). The use of 5% DMSO plus 10 or 20% trehalose did not increase the post-thaw viability and did prevent further cell growth in the case of 5% DMSO plus 20% trehalose (Table I).

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Table I. *Effect of Trehalose Pretreatment (1 Day) on Post-Thawing Viability (%) and Further Growth of Daucus carota Line C1 Cells with Trehalose or Trehalose and DMSO as Cryoprotectants*

Cell viability was determined by phenosafranin dye exclusion. Data are from one of two experiments which gave similar results. Cells were pretreated for 24 h with 5 or 10% trehalose added to the culture medium. The cell viabilities following the pretreatments were 93 and 81%, respectively. The cells were centrifuged, the medium poured off, and the cells were resuspended in 10, 20, or 40% trehalose or trehalose plus DMSO. Two-ml aliquots were placed in cryotubes on ice for 1 h prior to cooling at 1°C min^{-1} to −40°C when they were placed in liquid N_2. After 2 d, the cells were thawed in a 40°C water bath.

Pretreatment	Post-Thaw Viability					
	Cryoprotectant added					
	0	10% T[a]	20% T	40% T	10% T + 5% DMSO	20% T + 5% DMSO
				%		
0	0	0	0	0	5	7
5% T	5	11	53[b]	71[b]	51[b]	66
10% T	11	16	66[b]	74[b]	66[b]	71

[a] Trehalose. [b] Cell growth was observed within 15 d on plates.

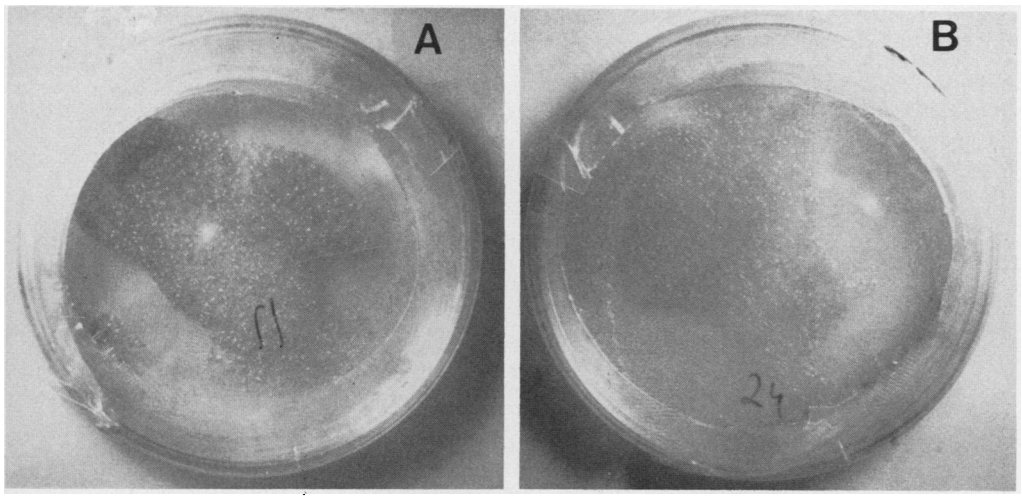

FIG. 1. Growing C1 (A) and NP (B) cells following thawing of cells stored in liquid N_2 for 2 d and plating on agar-solidified Murashige and Skoog culture medium. Photograph taken 15 and 20 d following plating, respectively. The C1 cells were pretreated for 1 d with 5% trehalose and were frozen in 40% trehalose while the NP cells were pretreated for 4 d with 10% trehalose and were frozen in 40% trehalose.

Table II. *Effect of 10% Trehalose Pretreatment on Post-Thawing Viability and Further Growth of NP and TX1 Cells with Trehalose as Cryoprotectant*

Freezing protocol was the same as in Table I. Cell viability was determined by phenosafranin dye exclusion. Data is from one of two experiments which gave similar results. The values in parentheses are the cell viabilities following pretreatment.

Cell Line	Pretreatment Time	Post-Thaw Viability		
		None	20% Trehalose	40% Trehalose
	d		%	
NP	0 (84)	0	0	0
NP	3 (85)	0	0	31[a]
NP	4 (81)	0	0	37[a]
NP	5 (83)	0	0	45[a]
NP	6 (73)	0	0	35[a]
TX1	0 (92)	0	0	0
TX1	1 (91)	0	0	47[b]

[a] Cell growth was observed within 20 d on plates. [b] Regrowth was not reproducible and only a few colonies were usually observed.

Since a 1-d preincubation of NP cells with 5 or 10% trehalose did not produce viable cells following freezing and thawing, a longer preincubation period was used. The recovered cell viabilities ranged from 31 to 45% with pretreatment periods of 3 to 6 d with 10% trehalose and with 40% trehalose as the sole cryoprotectant (Table II). Cell regrowth could be observed within 20 d in all cases (Fig. 1B). No viable cells were recovered in these experiments when no cryoprotectant was used or when 20% trehalose was used as the cryoprotectant.

When TX1 cells were pretreated for 1 d with 10% trehalose and frozen with 40% trehalose, the post-thaw viability was 47% (Table II) but the recovery of growing cells upon plating on agar-solidified medium was sporadic. Viable cells could not be recovered when soybean suspension cultured cells were pretreated with trehalose and frozen with trehalose or trehalose and DMSO (data not shown).

No previous reports have shown that trehalose can be used as a sole cryoprotectant. Chen *et al.* (3) did use trehalose along with other sugars and sugar alcohols with DMSO as cryoprotectants when freezing sorbitol pretreated *Catharanthus roseus* cell cultures. In this species, cell viabilities of 24% were obtained with DMSO plus 0.5 M trehalose which was lower than the values obtained with some other sugars and sugar alcohols. Trehalose pretreatment of the *C. roseus* cells for 20 h prior to freezing with DMSO allowed the recovery of viable cells but the percentage was no better than that obtained with sorbitol, glucose, or sucrose.

Previous work in this laboratory (7) described the recovery of frozen C1 and TX1 cells using DMSO and glycerol as cryoprotectants. These cells would grow only if placed on feeder plates following thawing. Thus, the method described in this paper

where growth of C1 and NP cells can be obtained on agar-solidified medium is an advance in this technology. Bajaj (1) reported the recovery of growing tobacco cells after thawing when plated on solid medium after periods of 4 to 6 months. This long time period is inconvenient and indicates that extreme selection has occurred due to the low number of cells capable of further growth. In experiments with carrot cells, Withers and Street (13) also reported that only a small proportion of the frozen and thawed cells could regrow even though a large proportion of the cells were viable as determined by fluorecein diacetate staining.

The observation that trehalose is effective as a cryoprotectant only following pretreatment of cells with trehalose indicates that the cells need to be conditioned prior to freezing or that trehalose must also be taken up and be inside the plasmalemma to be effective. In addition, high external trehalose concentrations may be necessary as an osmoticum.

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