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Effect of various sugars and polyols on the tolerance to desiccation and freezing of oil palm polyembryonic cultures

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Abstract

During a 7-day conditioning treatment of clumps of oil palm (*Elaeis guineensis* Jacq.) somatic embryos on a medium containing 0.75 M sucrose, the fructose and glucose concentrations remained constant, whereas a ten-fold and twenty-fold increase were noted for sucrose and starch concentrations, respectively. The only new sugar detected was arabinose which remained at a low concentration. After conditioning on media supplemented with various sugars and polyols at similar osmolarities, recovery of clumps of embryo growth was satisfactory except with ribose. After an additional desiccation period, survival was optimal with fructose, galactose, sucrose and glucose, intermediate with maltose and lower with other compounds. When embryos were cryopreserved without previous desiccation, survival was noted after conditioning treatment with sucrose only. In contrast, when freezing was performed after dehydration, survival could be obtained with several substances. It was optimal with sucrose, fructose, galactose and raffinose but was possible also with sorbitol and glucose. Intensity of recovery of proliferation was highest with embryos conditioned with sucrose.

Keywords: oil palm, clumps of somatic embryos, sugars, polyols, desiccation, cryopreservation.

Introduction

A new cryopreservation process developed recently for polyembryonic cultures of oil palm, *Elaeis guineensis* Jacq. (Dumet *et al.*, 1993a) was successfully applied to 39 different clones (Dumet *et al.*, 1993b). It comprised a conditioning treatment before freezing consisting of a 7-day culture of clumps of embryos on a medium with a high sucrose concentration followed by partial desiccation. During this work, the respective importance of a conditioning treatment with a high sucrose concentration and of desiccation became apparent (Dumet *et al.*, 1993c). Indeed, survival of non-conditioned clumps of embyros was very low after extended desiccation periods and no survival was obtained after freezing in liquid nitrogen whatever the desiccation period. On the contrary, embryos conditioned on a medium with a high sucrose concentration displayed 100% survival whatever the desiccation period. After cryopreservation, survival of conditioned embryos could be obtained after all dehydration periods but was optimal for the longest durations. Thermal analysis performed during freezing of embryos revealed that this increase in survival was correlated with the occurrence of a glass transition in embryos submitted to extended dehydration, whereas crystallization was noted after shorter durations (Dumet *et al.*, 1993c). Embryos not conditioned with sucrose displayed a crystallization peak under all experimental conditions.

Sugars are involved in the mechanisms of resistance of seeds to desiccation (Koster and Leopold, 1988; Blackman *et al.*, 1992). Their concentration increases dramatically during maturation of orthodox as well as of recalcitrant seed species (Farrant *et al.*, 1992). Sugars may act by substituting for water in stabilizing membranes in the dry state (Crowe and Crowe, 1986) and/or by inducing intracellular vitrification at ambient temperature, thus ensuring subcellular stability in the dry state (Williams and Leopold, 1989). Sucrose is generally the main soluble carbohydrate reserve found in dry seeds. It is generally associated with lesser amounts of oligosaccharides such as raffinose, stachyose or verbascose (Amuti and Pollard, 1977; Crowe and Crowe, 1986). Stachyose was found to be the predominant soluble sugar in seeds of the recalcitrant species *Avicennia marina* (Farrant *et al.*, 1992), and in axes of soybean seeds during induction of desiccation tolerance (Blackman *et al.*, 1992).

Sucrose is also the sugar most commonly employed in cryopreservation protocols. However, the use of other sugars (glucose, maltose) or polyols (mannitol,

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sorbitol) is also mentioned in the literature (Withers, 1992). A recent study performed with carrot somatic embryos showed that the specifity of sucrose as a cryoprotectant was low since a wide range of sugars and polyols allowed embryos to withstand freezing in liquid nitrogen (Tessereau, 1993). Sucrose is also commonly employed in maturation protocols for zygotic and somatic embryos (Xu *et al.*, 1990; Slawinska and Obendorf, 1991; Anandarajah and McKersie, 1992).

In the present work, we followed changes in the content of soluble sugars and starch in clumps of oil palm somatic embyros during conditioning on a medium with high sucrose content. We investigated the specifity of sucrose for the acquisition of tolerance to desiccation and freezing in oil palm somatic embryos by comparing its efficiency with that of various other sugars and polyols employed during conditioning.

Materials and Methods

Plant material

The clone of somatic embryos used in this study was obtained from an adult hybrid (Deli × La Mé) oil palm (*Elaeis guineensis* Jacq.).

Tissue culture

Somatic embryos were produced according to the method of Pannetier *et al.* (1981). They were subcultured monthly on a modified Murashige and Skoog medium (1962), devoid of growth regulators and containing 0.1 M sucrose. Cultures were maintained at 27 ± 1°C, with a photoperiod of 12 h light/12 h dark, under a flux density of 49 μmol m^{-2} s^{-1}.

Cryopreservation

Cryopreservation was carried out according to the method of Dumet *et al.* (1993a). Clumps of somatic embryos, consisting of embryos at various developmental stages, weighing 250–300 mg were dissected from standard cultures. They were conditioned for 7 d on media supplemented with various sugars or polyols (ribose, fructose, galactose, glucose, maltose, raffinose, sucrose, mannitol, sorbitol) or with a mixture of sucrose and mannitol. The osmolarity of the media was adjusted to the value of that of the standard conditioning medium (1090 mOsm) containing 0.75 M sucrose, using an automatic osmometer (Roebling, Germany).

Before cryopreservation, clumps were, or not, submitted to a desiccation period. Desiccation was performed by placing 5 clumps in an air-tight box containing 40 g of silica gel for 16 h. Embryos were then placed in 2-ml sterile polypropylene cryovials and immersed directly in liquid nitrogen. After a minimum of one hour in liquid nitrogen, they were thawed by plunging the cryotubes for 2 min in a 40°C water-bath. For growth recovery, embryos were cultured for one week on a medium containing 0.3 M sucrose and 0.2 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/litre and then for 2 weeks on a medium containing 0.1 M sucrose and the same concentration of 2,4-D (Englemann *et al.*, 1985). They were then transferred to the standard medium devoid of growth regulators.

For each conditioning condition, the following treatments were made: non-frozen embryos, cryopreserved embryos, desiccated non-frozen embryos, desiccated and cryopreserved embryos. The water content of the embryos (expressed in g H_2O/g dry weight) was measured in each conditioning condition, after conditioning and after desiccation, using 5 clumps of embryos. Survival was assessed 3 weeks after thawing. Clumps of embryos were considered as surviving when proliferation recovery, i.e. the appearance of neoformed adventitious embryos, was observed. Five to 49 clumps of embryos per condition were used.

Measurement of sugar concentration

For sugar analysis, clumps of embryos (250 mg fresh weight) were briefly rinsed in alcohol and then ground in 60 ml of 78% ethanol. The solution was filtered and 25 μl was injected in an ion chromatography HPLC/Dionex system (BioLC unit, AG6 precolumn, ASG separation column). The eluent solution was sodium hydroxide (140 mM) and the flow rate 1 ml/min. The following sugars were identified and quantified in comparison with standards injected at known concentrations: arabinose, fructose, glucose, raffinose, rhamnose, sucrose and trehalose.

Sugar analyses were performed on three clumps of embryos after various durations on conditioning medium (0, 1, 2, 3, 4, 5 and 7 d).

Measurement of starch concentration

Starch concentration was measured enzymatically using Boeringher detection test kits. Analyses were performed on two clumps of embryos after various culture periods on conditioning medium (0, 1, 3, 4, 5, 7 d).

Statistical analysis of the results

In Figure 1, each point corresponds to the average value of three replicates for sucrose (Fig. 1A) and arabinose (Fig. 1B), and two replicates for starch (Fig. 1C). A one-way analysis of variance was applied to test the effect of conditioning duration on glucose, fructose, arabinose, sucrose and starch concentrations in clumps of embryos (Table 1). Conditioning

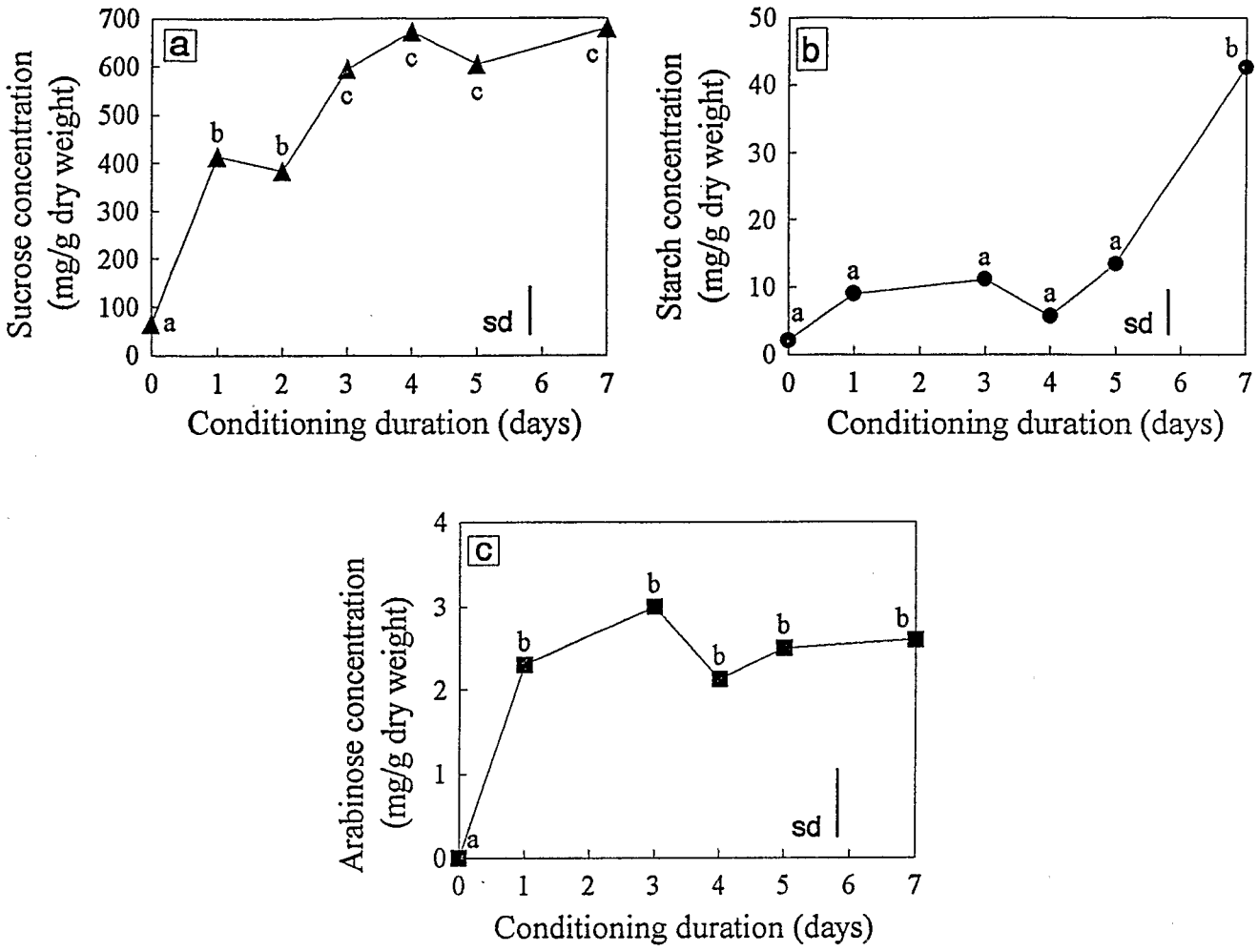

Figure 1. Sucrose (A), starch (B) and arabinose (C) concentrations in clumps of oil palm somatic embryos during conditioning on medium containing 0.75 M sucrose. Points followed by the same letter are not significantly different at the 0.05 probability level. (sd: estimated standard deviation).

duration was a fixed-effect factor. In cases where the test for the conditioning duration effect was significant, Newman and Keul's test was used for multiple comparison of categorical means (Newman, 1939; Keuls, 1952). On the graphs, points followed by the same letter are not significantly different at the 0.05 probability level, as determined by the Newman and Keul's test.

A two-way analysis of variance was applied to test the effects of conditioning duration, medium composition and of interaction between conditioning duration and medium composition on the growth of

Table 1. Effect of duration of conditioning treatment on the arabinose, fructose, glucose, starch and sucrose concentrations in clumps of oil palm somatic embryos

		Arabinose concentration	Fructose concentration	Glucose concentration	Starch concentration	Sucrose concentration
Conditioning	F	4.79	1.41	1.25	12.91	16.66
Duration effect	*P*	0.0074	0.2789	0.3413	0.0037	0.0000
		**	NS	NS	**	***

Results of one-way analysis of variance. F (observed value of F-test) and *P* (observed probability) values are given for each studied trait. NS: non significant; **: highly significant; ***: very highly significant. Cryopreservation was in liquid nitrogen.

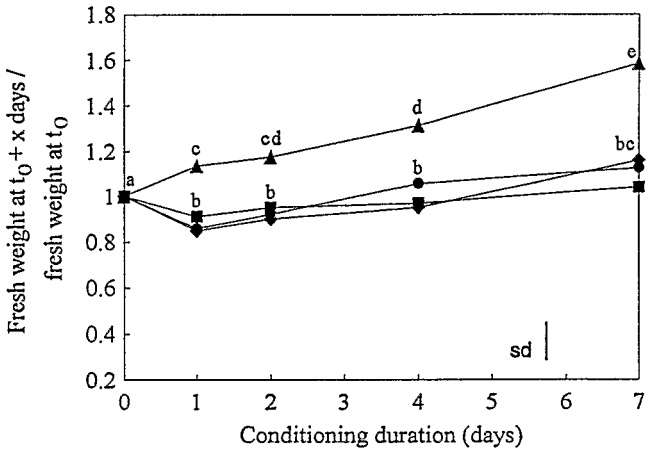

Figure 2. Fresh weight (in % of initial fresh weight) of clumps of oil palm somatic embryos during conditioning on media with various concentrations of sucrose and mannitol: 0.1 M sucrose (▲), 0.75 M sucrose (●), 0.1 M sucrose + 0.75 M mannitol (◆), 0.9 M mannitol (■). Initial fresh weight (at t_0) was 250 mg. Points followed by the same letter are not significantly different at the 0.05 probability level. (sd: estimated standard deviation).

embryos (Table 3). A t test was applied for comparing the average value at day 1 to the constant value at day 0 (0.05 probability level). In Figures 2 and 3, each point corresponds to the average value of ten replicates. Conditioning duration and medium composition were fixed-effect factors. Newman and Keul's test was used for multiple comparison of categorical means.

On each graph, a bar indicates the standard deviation (sd) estimated by the square-root of the residual mean square of the ANOVA.

In Tables 2 and 4, χ^2 test was used to test the effect of the composition of the conditioning medium on survival of embryos. When the expected frequency of a treatment was lower than 3, the χ^2 test could not be used. Therefore, the Fisher's exact test was applied. In cases where the effect of the composition of the conditioning medium was significant, Ryan's test (1960) was used for multiple comparison of frequencies. In Tables 2 and 4, frequencies followed by the same letter are not significantly different at the 0.05 probability level, as determined by Ryan's test.

Results

No significant modification in glucose and fructose concentration was noted during conditioning of embryos, whereas there was a significant effect of the conditioning duration on sucrose, arabinose and starch concentration (Table 1). Sucrose concentration dramatically increased during the first day, reaching a maximum of 679 mg/g DW (ten-fold increase in comparison with its initial concentration) after 7 d (Fig. 1A). Starch was present in embryos at a low concentration before conditioning (2.12 mg/g DW) (Fig. 1B). Its concentration increased significantly after 5 d of conditioning, reaching 45.58 mg/g DW, i.e. a twenty-fold increase. Arabinose, which was not detected initially in embryos, was observed at low concentration within one day of conditioning which remained stable afterwards (Fig. 1C).

Conditioning of embryos on media supplemented with high concentrations of sucrose, mannitol or a mixture of sucrose (0.1 M) and mannitol (0.75 M) in comparison with conditioning on standard medium (0.1 M sucrose) displayed no toxicity since survival of conditioned controls was 100% in all cases (Table 2). Culture on media with high osmotic potential reduced the water content to 3–4 g H_2O/g DW, in comparison with 14.6 g H_2O/g DW on standard medium. After

Table 2. Effect of the composition of conditioning medium on water content and survival (%) of clumps of oil palm somatic embryos after various treatments

Medium	Water content (g/g DW)		Survival (%)			
	Conditioning	Conditioning +Desiccation	Conditioning	Conditioning +Desiccation	Conditioning +LN	Conditioning +Desiccation +LN
Sucrose 0.1 M	14.60	0.70	100	20 a	0 a	0 a
Sucrose 0.75 M	4.00	0.50	100	100 b	40 b	93 b
Mannitol 0.9 M	4.80	0.56	100	42 a	0 a	0 a
Mannitol 0.75 M + sucrose 0.1 M	3.50	0.24	100	0 c	33 b	0 a
χ^2	—	—	—	27.212		64.167
P	—	—	—	0.0000 ***	(Fisher's exact test)	0.0000 ***

Duration of conditioning treatment was 7 days. Percentages followed by the same letter are not significantly different at the 0.05 probability level. ***: very highly significant; χ^2: observed value of chi-squared test; *P*: observed probability. (LN: Cryopreservation in liquid nitrogen).

Table 3. Effect of medium composition, duration of conditioning treatment and interaction between both effects on the growth of clumps of oil palm somatic embryos

		Growth Fresh Weight	Growth Dry Weight
Medium composition effect	F	31.45	63.85
	P	0.0000 ***	0.0000 ***
Conditioning duration effect	F	11.83	11.83
	P	0.0000 ***	0.0000 ***
Medium × conditioning duration interaction	F	1.81	3.12
	P	0.0719 *	0.0018 NS

Medium compositions: sucrose 0.1 M, 0.75 M, mannitol 0.9 M, mannitol 0.75 M + sucrose 0.1 M. Duration of treatment: 0 to 7 days. Results of two-way analysis of variance. F (observed value of F-test) and *P* (observed probability) values are given for each effect (rows) and trait (columns) studied. NS: non significant; *: significant; ***: very highly significant.

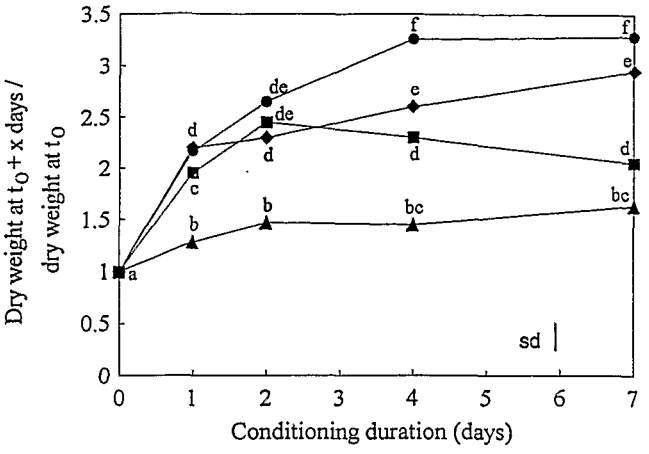

Figure 3. Dry weight (in % of initial dry weight) of clumps of oil palm somatic embryos during conditioning on media with various concentrations of sucrose and mannitol: 0.1 M sucrose (▲), 0.75 M sucrose (●), 0.1 M sucrose + 0.75 M mannitol (◆), 0.9 M mannitol (■). Initial dry weight (at t_0) was 17.5 mg. Points followed by the same letter are not significantly different at the 0.05 probability level. (sd: estimated standard deviation).

the desiccation treatment, the water content of embryos decreased drastically, reaching a minimal value of 0.24 g H_2O/g DW after conditioning on sucrose + mannitol. This condition was ineffective since no survival was noted after dehydration. In the other conditions, water content varied between 0.5 (high sucrose) and 0.7 g H_2O/gDW (standard medium). Survival after desiccation ranged from 0% for embryos conditioned on standard medium to 100% after conditioning on high sucrose medium. Only embryos conditioned on media containing high sucrose concentration or low sucrose associated with mannitol withstood cryopreservation without previous dehydration, with survival rates of 33 and 40%, respectively. When conditioning was followed by desiccation, only embryos cultivated on high sucrose medium withstood cryopreservation, with a survival rate of 93%.

The fresh and dry weight of clumps of embryos placed on these different media was followed during conditioning. Medium composition and conditioning duration had a significant effect on the evolution of fresh and dry weight (Table 3). The fresh weight of embryos placed on standard medium (0.1 M sucrose) increased significantly during the conditioning treatment (Fig. 2). The fresh weight of clumps of embryos placed on media with high osmotic potential, which was comparable among the three conditions tested, had a different pattern. It decreased after the first 24 h and then increased slowly, reaching values only slightly above that measured at the beginning of conditioning. On the other hand, the dry weight increase of embryos placed on standard medium was slight but progressive throughout the whole conditioning (Fig. 3). On the contrary, embryos placed on media with high osmotic potential displayed a dramatic increase in dry weight within 48 h of conditioning. An increase in dry weight was noted up to the fourth day with embryos cultivated on high sucrose and sucrose + mannitol, whereas that of embryos placed on medium with mannitol only reached a maximum after 2 d of conditioning.

After conditioning on media supplemented with various sugars or polyols at similar osmolarities, the water content of embryos ranged between 3.54 (lactose, maltose) and 6.69 g/gDW (ribose) (Table 4). Growth recovery of conditioned embryos was noted with all substances except ribose, which proved to be highly toxic (0% survival). After desiccation, survival was noted in all conditions, but it varied depending on the substances used during conditioning. Survival was optimal with fructose, sucrose, galactose and glucose (77–100%), intermediary with maltose (56%) and lower with the other compounds, ranging between 18 (lactose) and 38% (raffinose). When embryos were cryopreserved without previous desiccation, survival was noted after conditioning with sucrose only. When freezing was performed after dehydration, survival was optimal with sucrose, fructose, galactose and raffinose but it was also possible to achieve it with other compounds such as sorbitol and glucose (12%). It was observed that conditioning conditions greatly influenced the intensity of proliferation recovery after cryopreservation, which was highest for embryos conditioned with sucrose.

Table 4. Effect of sugars and polyol employed during conditioning of clumps of oil palm somatic embryos on their water content and on their survival rate after various treatments

Medium	Water content (g/g DW)		Survival (%)			
	Conditioning	Conditioning + Desiccation	Conditioning	Conditioning + Desiccation	Conditioning + LN	Conditioning + Desiccation + LN
Sucrose	4.00	0.70	100 a	77 ab	20 a	57 a
Ribose	6.69	0.71	0 b	0 d	0 b	0 c
Fructose	4.00	0.34	100 ab	100 ab	0 b	53 ab
Galactose	4.80	0.30	100 a	100 ab	0 b	47 ab
Glucose	5.25	0.47	90 a	92 ab	0 b	12 b
Lactose	3.54	0.27	100 a	18 c	0 b	0 c
Maltose	3.54	0.52	100 a	56 bc	0 b	0 c
Raffinose	6.14	0.41	80 a	38 c	0 b	21 ab
Sorbitol	4.80	0.46	80 a	32 c	0 b	12 b
χ^2	—	—	—	109.18	—	75.89
P	—	—	(Fisher's exact test)	0.0000 ***	(Fisher's exact test)	0.0000 ***

Conditioning, 7 days; Desiccation, 16 hours. LN: cryopreservation in liquid nitrogen. Percentages followed by the same letter are not significantly different at the 0.05 probability level. ***: very highly significant; χ^2: observed value of chi-squared test; *P*: observed probability.

Discussion

In these experiments, sucrose appeared as the compound allowing the highest survival rates of clumps of oil palm somatic embryos after cryopreservation. Sucrose was employed both as osmotic agent and as carbon source for oil palm somatic embryos. It is important to know if the resistance of oil palm somatic embryos to desiccation and cryopreservation is linked with the osmotic effect of sucrose and/or its absorption by cells. Sucrose employed as a cryoprotectant was considered as having mainly an osmotic action (Finkle *et al.*, 1985). Various hypotheses have been proposed regarding the possible mechanisms of intracellular accumulation of sucrose. Sagishima *et al.* (1989) suggested that sucrose was not incorporated directly into cells but was first hydrolysed into glucose and fructose by an invertase. This enzyme could be either bound to the plasma membrane (Stranzel *et al.*, 1988a,b) or secreted into the cell wall (Sagishima *et al.*, 1989). Stranzel *et al.* (1988a,b) demonstrated that the process of sucrose accumulation depended on its concentration in the medium: at concentrations lower than 10^{-3} M, sucrose was hydrolysed before absorption, whereas at higher concentrations, sucrose uptake proceeded through hydrophilic membrane domains.

During conditioning on a medium with a high sucrose or mannitol concentration, the water content of embryos decreased and their dry weight increased drastically, reaching similar values in both conditions. Since mannitol is not absorbed or poorly absorbed by plant cells (Cram, 1984), it should be present in the apoplastic compartment only during preconditioning with this substance. When sucrose was the conditioning agent, it is likely that during the first 48 h of conditioning, the sugar was predominantly accumulated in the apoplast. It would then be progressively accumulated intracellularly, since absorption mechanisms for this compound exist (Stranzel *et al.*, 1988a,b; Sagishima *et al.*, 1989) and then stored partly in the form of starch which was mostly accumulated at the end of the conditioning period.

The specificity of sucrose was very low as regards tolerance to dehydration. In contrast, when embryos were frozen without previous desiccation, survival was observed only after conditioning on a medium containing sucrose. Therefore, sucrose appears to have a very specific action for the acquisition of tolerance to freezing in liquid nitrogen with tissues at intermediate water contents. Intracellular concentration of sucrose may be sufficient to protect cell structures, either by allowing vitrification of intracellular solutes at positive temperature (Williams and Leopold, 1989) or by stabilizing membranes and proteins (Crowe and Crowe, 1986). When embryos were dehydrated before freezing, survival could be obtained with several other substances. One hypothesis may be that these substances can be absorbed by cells during conditioning and then at least partially converted into sucrose. The total amount of sucrose at the end of the conditioning period may be sufficient to ensure survival of embryos after dehydration to a low water level. Differences in the resistance to freezing after conditioning with these various substances may depend on the rate of their absorption by cells and of their conversion into sucrose. In this way it is interesting to note that

fructose was as efficient as sucrose for ensuring resistance to cryopreservation of desiccation embryos. Indeed, this could be linked with the fact that this monosaccharide may be more readily used for sucrose synthesis, as observed by Sagishima *et al.* (1988) in the case of cells of *Catharanthus roseus*.

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