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IMPORTANCE OF SUCROSE FOR THE ACQUISITION OF TOLERANCE TO DESICCATION AND CRYOPRESERVATION OF OIL PALM SOMATIC EMBRYOS

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SUMMARY: In the present work, the importance of preculture with high sucrose concentration for the acquisition of resistance of oil palm somatic embryos to desiccation and cryopreservation was underlined. Survival of non-pregrown control embryos decreased for dehydration periods longer than 10 hrs. No survival was obtained after freezing in liquid nitrogen whatever the desiccation duration. With pregrown control embryos, 100% survival was noted whatever the dehydration period. Survival after freezing in liquid nitrogen was possible without dehydration (40%) but it was dramatically improved after 12 (70%) and 16 hrs of desiccation (80%). Thermal analysis revealed that crystallization still occurred in non-pregrown embryos after 16 hrs of dehydration. With pregrown embryos, a glass transition only was recorded after dehydration periods of 12 and 16 hrs which ensured the highest survival rates.

KEY WORDS: oil palm, somatic embryos, desiccation, cryopreservation, sucrose, thermal analysis.

INTRODUCTION

The original oil palm somatic embryo cryopreservation process was developed with a particular type of structures, i.e. clumps of shiny white embryos (1). It was improved recently by using standard embryos (2) and by adding to the 7-day pregrowth treatment on a medium containing 0.75 M sucrose a complementary partial dehydration before freezing. This dehydration was performed by placing the embryos either in the air current of the laminar flow or in an air-tight box containing silicagel (3).

It is well known that sucrose, like many other sugars, plays an important role in the mechanisms of resistance of plant tissues and organs to desiccation (4, 5, 6, 7). It is also one of the most frequently employed substances for cryopreservation of plant cells, tissues and organs (8).

Recently, cryopreservation procedures including a pregrowth combining pretreatment with high sugar concentration and partial dehydration have been developed, notably with coconut zygotic embryos (9), encapsulated carrot somatic embryos (10) and apices of various species, with (11, 12) or without (13) encapsulation in alginate beads.

In this paper, we investigated the effect of the pregrowth treatment with high sucrose concentration on the resistance of oil palm somatic embryos to desiccation and freezing.

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MATERIALS AND METHODS

Plant material

The clone of somatic embryos used in this study originated from an adult hybrid palm (Deli x La Mé).

Methods

Tissue culture

Embryos were produced according to the method of Pannetier *et al.* (14). They were subcultured monthly on a modified Murashige and Skoog medium (15) devoid of growth regulators and containing 0.1 M sucrose. Cultures were maintained at 27±1°C, with a photoperiod of 12 hrs light/12 hrs dark, with a light intensity of 40 µE. $m^{-2}.s^{-1}$.

Cryopreservation

Embryos were cryopreserved according to the method of Dumet *et al.* (3). Clumps of somatic embryos weighing 250 to 300 mg were dissected from standard cultures. They were pregrown for 7 days on a medium containing 0.75 M sucrose (5 clumps per 55 mm Petri dish containing 10 ml of medium). They were then dehydrated for 0 to 16 hours in air-tight boxes containing 40 g of silicagel. The embryos were then placed in 2 ml sterile cryotubes and immersed directly in liquid nitrogen where they were stored for 1 hour. They were thawed rapidly by plunging the cryotubes for 2 min in a 40°C water-bath. For recovery, the embryos were cultured for one week on a medium containing 0.3 M sucrose and 0.2 $mg.l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and then for 2 weeks on a medium containing 0.1 M sucrose and the same concentration of 2,4-D. They were then transferred onto standard medium devoid of growth regulators.

Water content of the embryos (expressed in g of H_2O/ g of dry weight) was measured on 10 clumps after each dehydration period. Controls consisted of embryos which were or not pregrown on high sucrose medium and were or not cryopreserved after the various dehydration periods. Recovery was measured 3 weeks after thawing. Clumps of embryos were considered surviving when proliferation recovery was observed. Ten clumps were used per treatment.

Thermal analysis

Thermal analysis was performed using a DSC apparatus (Mettler DSC 30) on fragments of clumps or whole clumps after each dehydration period for pregrown embryos, and after 0, 6 and 16 hrs of dehydration for non-pregrown embryos. Cooling and rewarming rates were $10K.min^{-1}$. Three to 6 samples per treatment were analysed.

RESULTS

The initial water content of the clumps of embryos was different between pregrown (4.0 g H_2O/g dw) and non-pregrown (14.6 g H_2O/g dw) (Table 1). Both categories of embryos reached a similar water level after 16 hrs of dehydration. Survival of pregrown and desiccated control embryos was 100 % whatever the dehydration duration. On the contrary, non-pregrown embryos showed a sharp decrease in survival rate after 12 and 16 hrs of dehydration (4/10 and 2/10, i.e. 40 and 20% respectively) and in proliferation intensity (Fig. 1). After freezing in liquid nitrogen, no survival was observed for non-pregrown embryos whatever the desiccation duration. In the case of pregrown embryos, survival after freezing in liquid nitrogen was possible without dehydration (4/10, i.e. 40% survival). A drastic drop in recovery rate was observed for 4 and 6 hrs of desiccation. Survival increased progressively afterwards up to 80 % (8/10 embryos) for 16 hrs of desiccation. Intensity of recovery followed a similar pattern (Fig. 2): it was very low for 0, 2 and 4 hrs of dehydration and progressively increased up to 16 hrs of dehydration.

Table 1: Effect of pregrowth and dehydration duration on water content and survival of control (-LN) and cryopreserved (+LN) embryos. (-: not measured).

	Without pregrowth			With pregrowth		
Dehydration duration (hrs)	Water content (g H2O/g dw)	Survival (-LN)	Survival (+LN)	Water content (g H2O/g dw)	Survival (-LN)	Survival (+LN)
0	14.6±1.7	10/10	0	4.0±1.0	10/10	4/10
4	7.8±0.9	10/10	0	2.1±0.2	10/10	1/10
6	5.3±1.2	10/10	0	1.6±0.3	10/10	1/10
8	5.0±0.8	10/10	0	1.3±0.4	10/10	4/10
10	2.9±1.1	10/10	0	0.8±0.3	10/10	4/10
12	-	4/10	0	0.7±0.1	10/10	7/10
16	0.7±0.4	2/10	0	0.5±0.4	10/10	8/10

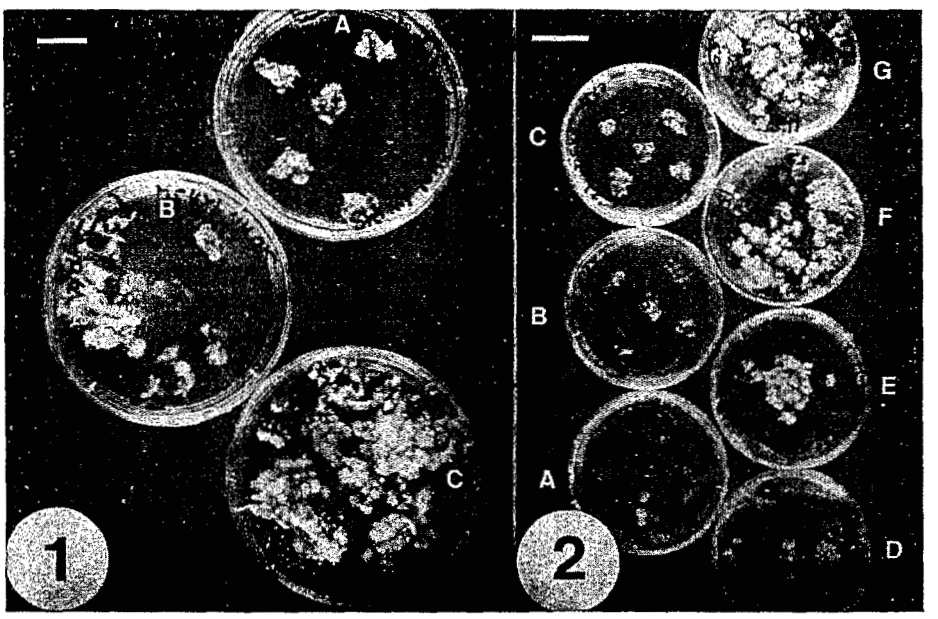


Figure 1: Effect of dehydration duration on the intensity of proliferation of control non-pregrown embryos. (A: 16 hrs; B: 12 hrs; C: 10 hrs). (Scale bar: 1 cm).

Figure 2: Effect of dehydration duration and cryopreservation on the intensity of proliferation of pregrown embryos. A: 0 hrs; B: 4 hrs; C: 6 hrs; D: 8 hrs; E: 10 hrs; F: 12 hrs; G: 16 hrs). (Scale bar: 3 cm).

Pregrown embryos displayed a lower enthalpy variation during freezing and rewarming (Table 2) than non-pregrown ones (Table 3) without desiccation, thus reflecting their different water level. The value of enthalpy variation during crystallization or melting decreased in line with the desiccation duration and no enthalpy variation could be detected for desiccation durations longer than 8 hrs in the case of pregrown embryos (Table 2). With both categories of embryos, the temperature of crystallization decreased progressively in line with the desiccation duration. In the case of pregrown embryos, the pattern of thermograms varied with the desiccation duration: a peak of crystallization or melting only (Fig. 3A and B) was observed in all samples without dehydration. After 8 hrs of dehydration, crystallization and melting peaks of lower intensity were noted in two samples and a glass transition in the third one (Fig. 3C

and D). Clumps of embryos desiccated for 10 hrs displayed a glass transition or a drastically reduced peak of crystallization (Fig. 3E and F). After 16 hrs of dehydration, only glass transitions were noted upon cooling and rewarming (Fig. 3G and H). In the case of non-pregrown embryos, only crystallization and melting peaks were noted without dehydration (Fig. 3I and J) and after 16 hrs of dehydration (Fig. 3K and L).

Table 2: Onset temperature, enthalpy variation and type of event (C: crystallization, GT: glass transition, M: melting) observed during thermal analysis of embryos pregrown with sucrose, after various dehydration durations.

	Cooling			Rewarming		
dehydration duration (hrs)	Onset temp. (°C)	Enthalpy variation ($J.g^{-1}$ fw)	Type of thermal event	Onset temp. (°C)	Enthalpy variation ($J.g^{-1}$ fw)	Type of thermal event
0	-12.7	166.4	C	-16.4	195.0	M
	-14.4	164.2	C	-18.3	170.6	M
	-13.2	139.7	C	-19.9	153.3	M
4	-29.7	86.4	C	-25.8	108.8	M
	/	54.0	C	-30.0	63.0	M
	-21.6	111.3	C	-22.7	120.7	M
6	-30.2	38.4	C	-32.4	57.4	M
	-26.9	16.6	C	-31.5	39.8	M
	-19.0	15.8	C	-32.6	27.8	M
8	-45.9	/	GT	-58.2	/	GT
	-35.7	26.6	C	-30.3	42.5	M
	-51.0	/	GT	-59.8	/	GT
10	-36.8	/	GT	-53.4	/	GT
	-41.3	/	GT	-44.3	/	GT
	-48.8	/	GT	-34.1	/	GT
16	-11.5	/	GT	-30.6	/	GT
	-19.6	/	GT	-51.8	/	GT
	-15.3	/	GT	-32.7	/	GT
	-21.4	/	GT	-43.7	/	GT

Table 3: Onset temperature, enthalpy variation and type of event (C: crystallization, GT: glass transition, M: melting) observed during thermal analysis of non-pregrown embryos, after various dehydration durations.

	Cooling			Rewarming		
dehydration duration (hrs)	Onset temp. (°C)	Enthalpy variation ($J.g^{-1}$ fw)	Type of thermal event	Onset temp. (°C)	Enthalpy variation ($J.g^{-1}$ fw)	Type of thermal event
0	-15.4	254.2	C	-4.3	293.3	M
	-9.4	258.8	C	-4.5	288.5	M
	-12.4	246.1	C	-6.1	283.9	M
16	-29.7	23.5	C	-29.1	35.4	M
	-27.7	24.1	C	-27.6	32.3	M
	-22.5	80.3	C	-26.0	104.6	M
	-18.4	137.9	C	-18.6	161.3	M

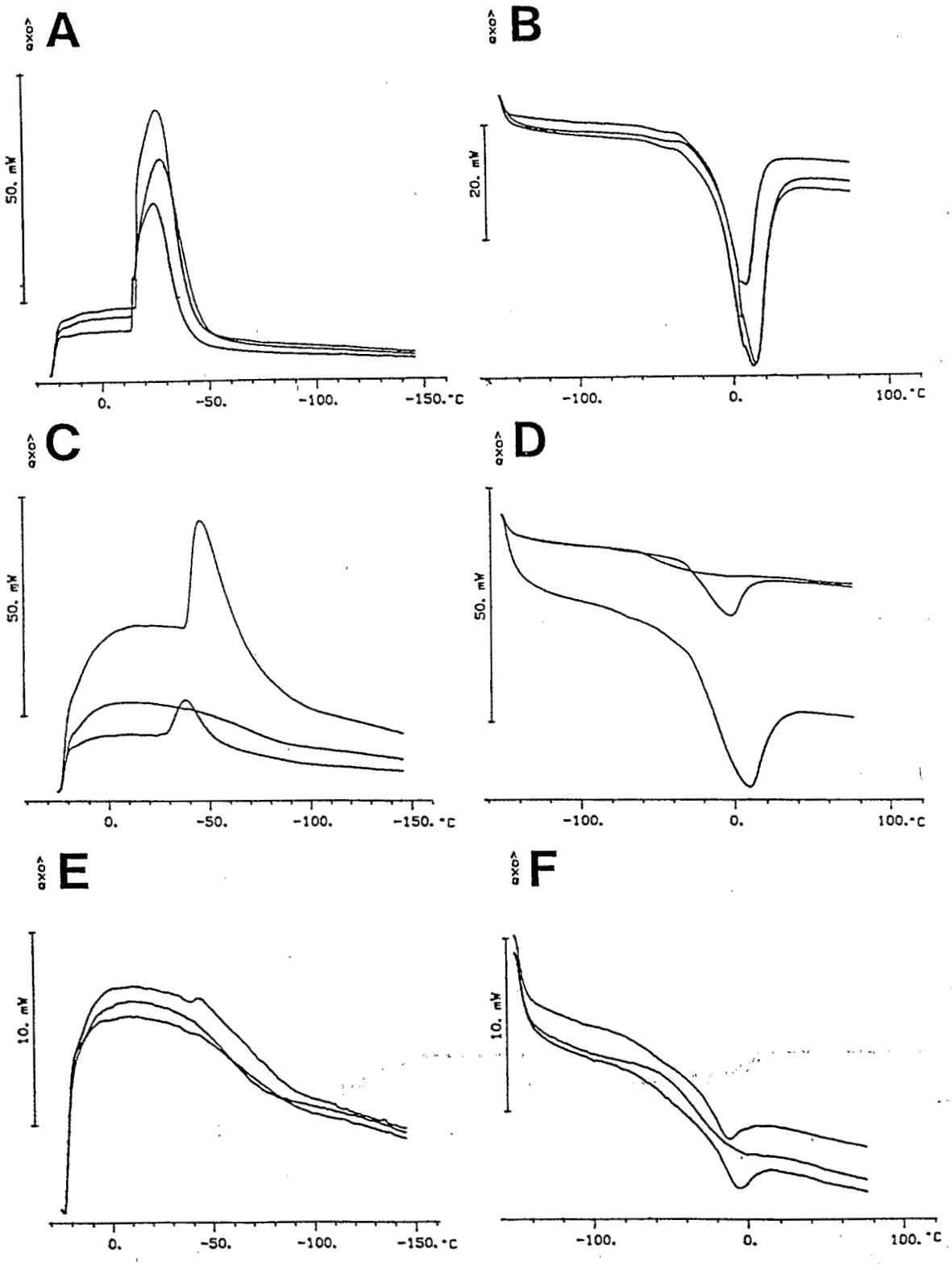
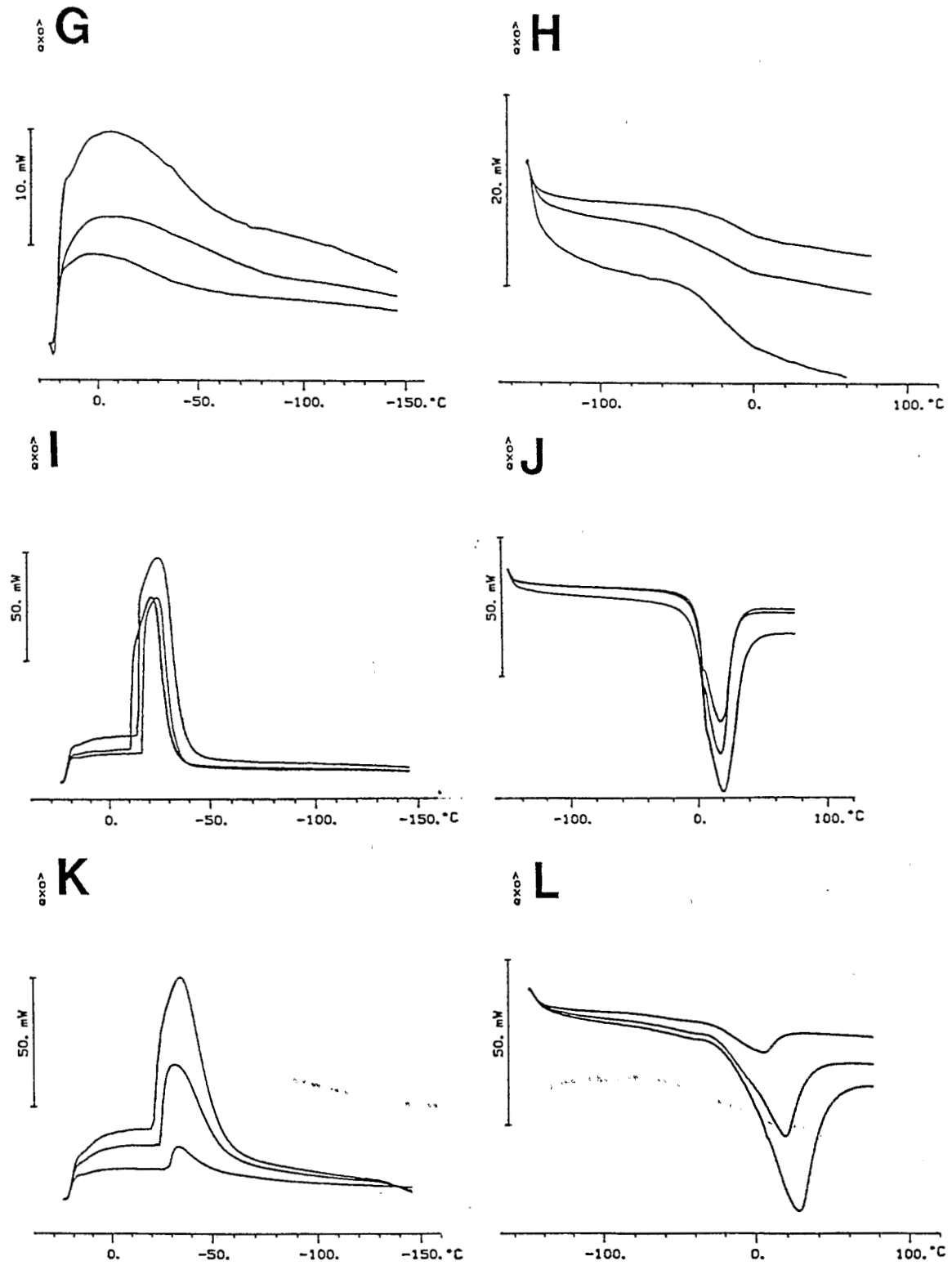


Figure 3: Thermograms recorded during cooling (A, C, E, G, I, K) and rewarming (B, D, F, H, J, L) of 3 clumps of oil palm somatic embryos, with or without pregrowth treatment, after various desiccation durations. A, B: 7-day pregrowth treatment, no desiccation; C, D: 7-day



pregrowth treatment, 8 hrs of desiccation; E, F: 7-day pregrowth treatment, 10 hrs of desiccation; G, H: 7-day pregrowth treatment, 16 hrs of desiccation; I, J: no pregrowth treatment, no desiccation; K, L: no pregrowth treatment, 16 hrs of desiccation.

DISCUSSION

In this paper, the role of sucrose in the resistance of oil palm somatic embryos to dehydration and freezing in liquid nitrogen was underlined. The microcalorimetric studies showed that resistance to freezing of embryos pregrown with sucrose increased when they were drastically dehydrated, i.e. when all freezable water had been extracted and when a glass transition only was observed.

The critical water level for non-pregrown embryos was 2.9 g H2O/g dw since a large number of embryos were killed below this water content. Moreover, no survival was obtained after freezing in liquid nitrogen, whatever the water level of clumps. On the contrary, in the case of embryos pregrown with sucrose, survival of controls was 100% whatever the water level and recovery after freezing in liquid nitrogen was optimal for a water content of 0.7 g H_2O/g dw. It is interesting to note that a similar water content was also optimal for the cryopreservation of oil palm zygotic embryos (16). This improvement in resistance to desiccation and freezing in liquid nitrogen was due to the presence of sucrose during pregrowth. Contrarily to previous hypotheses on the mode of action of sucrose which mentioned an osmotic effect only (8), this compound enters cells in large quantities. This was shown indirectly by observation of intracellular accumulation of starch during pregrowth with date palm apices (17) and oil palm somatic embryos (18) and directly by measuring the sucrose concentration in oil palm somatic embryos (19) and *Asparagus* apices (13). Sucrose acts by stabilizing membranes and proteins during desiccation (20). Experiments are under way to determine its specificity by comparing its effect with that of other compounds during pregrowth of oil palm somatic embryos. Indeed, various authors (21, 6) showed that many excellent cryoprotectants such as glycerol, proline or glucose had no stabilizing effect during extreme dehydration. The only solutes that did have such properties were disaccharides (trehalose, sucrose, maltose). Moreover, Tessereau (22) noted that survival of carrot somatic embryos varied according to the type of cryoprotective substance employed, a triholoside (raffinose) and diholosides (sucrose, maltose, trehalose, lactose) giving better results than monosides, polyols, dimethylsulfoxide, polyethyleneglycol or proline.

Microcalorimetric studies showed the necessity of removing the crystallizable water until a glass transition only was recorded during cooling and rewarming, in order to increase survival and growth recovery of oil palm clumps after freezing in liquid nitrogen. This was also observed notably with carrot somatic embryos (23) and with various plant materials cryopreserved using the technique called vitrification (see 11, for a review).

With the clone used in this study, it was possible to obtain low survival and growth recovery after pregrowth without desiccation, which indicated that treatment with high sucrose partially protected the cells against freezing in liquid nitrogen. A drastic drop in survival after cryopreservation was observed afterwards for dehydration durations corresponding to a cristallization peak of low intensity or a crystallization and a glass transition. During this period, cells may be in an unstable state as regards their water status, freezing leading therefore to very severe cellular damages. In previous studies performed with several oil palm clones (2, 3), it was shown that dehydration induced a similar water level in all clones. However, optimal survival rates after freezing in liquid nitrogen were obtained for different dehydration durations. This may be due to physiological differences among clones: they may absorb sucrose at different rates during pregrowth or up to different levels and may have different quantities of crystallizable water. Additional experiments including measurement of sucrose uptake during pregrowth and thermal analysis after various desiccation durations may contribute to explain these differences.

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