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Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) somatic embryos involving a desiccation step

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Summary: The standard cryopreservation process previously developed for oil palm clones using shiny white, finger-like somatic embryos could be applied in some cases to standard cultures. Its efficiency was markedly improved by completing the 7-day pregrowth period on 0.75 M sucrose by an additional dehydration period carried out either by placing the embryos in the air current of the laminar flow cabinet or in an air tight box containing silica gel. This improved process was successfully applied to 7 different clones. It will facilitate the routine use of cryopreservation for oil palm cultures.

Key words: oil palm, *Elaeis guineensis* Jacq., cryopreservation, sucrose, desiccation, somatic embryos.

Introduction

The cryopreservation process set up by our research team for oil palm cultures is now experimented on a large scale in Indonesia, Malaysia, Ivory Coast and France (Engelmann 1991a). It utilizes a particular type of material, consisting of shiny white, finger-shaped somatic embryos (Engelmann *et al.* 1985). However, the frequency of appearance of such embryos in standard cultures is very low and varies greatly between clones. Their production can be increased after a 2-month culture on a medium enriched with 0.3M sucrose (Engelmann and Dereuddre 1988) but it still remains hazardous and variable, depending on the clone considered and the physiological state of the material (Engelmann, 1991a). These problems considerably slow down the development of cryopreservation for this crop.

The progress made during the past two years in the area of cryopreservation of plant tissues and organs allowed to stress the fact that pregrowth is often the key step of a cryopreservation process (Engelmann 1991b). The pregrowth conditions must notably allow the decrease of as much as possible of the water level of the tissues, in order to avoid the detrimental formation of intracellular ice crystals in the tissues. However, it must be sufficiently tempered to avoid irreversible alterations of the regrowth capacities of the plant material caused by an excessive dehydration. In the case of embryos, pregrowth conditions generally differ for zygotic and somatic embryos (Engelmann 1992): somatic embryos are usually cultured on a high sugar medium (Engelmann *et al.* 1985), whereas zygotic embryos are only dehydrated in the air current of a laminar flow cabinet, without any cryoprotective treatment (Grout *et al.* 1983; Normah *et al.* 1986; Chin *et al.* 1988; Abdelnour-Esquivel *et al.* 1992). This technique has been applied to somatic embryos in the case of *Cucumis melo* only (Shiminoshi *et al.* 1991). A combination of both treatments (high sugar and dehydration) proved successful in the case of coconut embryos (Assy-Bah and Engelmann 1992).

With axillary buds of *Asparagus*, desiccation before cryopreservation was performed using silica gel (Uragami *et al.* 1990).

In this study, we tried firstly to apply the original cryopreservation process (Engelmann *et al.* 1985) to standard embryos. Secondly, we observed the effect of a modification of the pretreatment conditions on the recovery of cryopreserved standard embryos. In this aim, after their culture on a medium enriched with sucrose, the embryos were dehydrated either under the air current of a laminar flow cabinet, or using silica gel, before their immersion in liquid nitrogen.

Materials and Methods

Plant material

The clones (A to O) of somatic embryos used in this study were obtained from 15 adult hybrid palms from different genetic origins (14 Deli x La Mé, one Deli x Yangambi).

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Methods

- Tissue culture

The 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 12/24 hours, under a light intensity of 40µmol $m^{-2}s^{-1}$.

- Cryopreservation

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 (Fig. 1) (5 clumps per 55 mm Petri dish containing 10 ml of medium).

They were then either immersed directly in liquid nitrogen (standard process) or submitted to additional dehydration treatments before freezing:

- dehydration for 4 to 10 hours in the air current of a laminar flow cabinet.
- dehydration for 7 to 18 hours in air-tight boxes containing 40 g of silicagel. In that case, the boxes were placed in the dark at 27°C.

After the various pretreatments, the embryos were treated according to the original cryopreservation process (Engelmann *et al.* 1985). They were placed in 2 ml sterile cryotubes and immersed directly in liquid nitrogen. The embryos were stored at -196°C for a minimum of 1 hour. They were then thawed rapidly by plunging the cryotubes for 2 min in a 40°C water-bath. For growth 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 to the standard medium, devoid of growth regulators.

The water content of the embryos (expressed in % of their fresh weight) was measured after each dehydration period. The controls corresponded to clumps of embryos which were pregrown on the medium with a high sucrose level, dehydrated for various durations but not cryopreserved. Survival was measured 3 weeks after thawing. Clumps of embryos were considered surviving when proliferation recovery was observed. The first experiment (use of the standard cryopreservation process) was performed with 9 clones (A to I), the second one (desiccation under the laminar flow) with 4 clones (A, C, E and H), the third one (desiccation with silica gel) with three clones (C, E, H). In the fourth experiment, the modified technique was tested with 6 additional clones (J to O) and clone D. Twenty to thirty clumps per condition were used in the first and fourth experiments, nine to ten in the second and third.

Table 1: Water content after one week pregrowth on a medium containing 0.75 M sucrose and survival after cryopreservation of oil palm somatic embryos of 9 different clones (standard process).

Clone	Water content (%)	Survival
A	77±2	0/30 (0%)
B	78±3	7/30 (23%)
C	79±1	0/30 (0%)
D	75±2	0/30 (0%)
E	76±2	6/30 (20%)
F	73±3	0/35 (0%)
G	76±1	5/25 (20%)
H	77±1	0/20 (0%)
I	73±1	3/29 (10%)

Results

The water content of the embryos varied between 73 and 79% after the pregrowth treatment on 0.75 M sucrose (Table 1). Only 4 out of the 9 clones tested survived after freezing in liquid nitrogen, with relatively low survival rates ranging between 10 and 23%. No direct correlation could be made between the water content of the embryos and their resistance to cryopreservation.

After 10 hours of dehydration under the laminar flow, the water content of the embryos decreased to 37-44% (Table 2). Survival of unfrozen controls was affected for dehydration periods longer than 4 or 6 hours for clones A, H and C, E respectively. When the water content was lower than 50%, irreversible damage caused by dehydration led to death of some clumps. However, this dehydration was indispensable in most cases to achieve survival after cryopreservation. A 6-hour dehydration seemed optimal, since it allowed on one hand to obtain survival of clones A and H (recalcitrant until now to cryopreservation) and on the other hand to greatly improve the survival of clone E. However, no survival was noted with clone C.

When dehydration was performed with silica gel, the water content of the embryos was around 30% after 18 hours of dehydration (Table 3). For the longest desiccation periods (16 and 18 hr), survival of cryopreserved clumps was sometimes higher than that of controls but no conclusion could be drawn from this result due to the low number of clumps used in this experiment. Survival of unfrozen controls was decreased when water level was around 30-34%. The dehydration treatment increased the resistance of the embryos to freezing in liquid nitrogen. Survival of clone C, which was previously 0% (Tables 1 and 2) was maximal (10 clumps/10) after a 16-hour dehydration. Survival of clone E was improved since 9 clumps out of 10 recovered after 18 hours of dehydration. However, survival of clone H was not improved but remained high (7 clumps/10). It is interesting to note that the behaviour of this clone varied with time. Indeed, during the first two experiments, no survival could be obtained after pregrowth with sucrose without desiccation (Tables 1 and 2), whereas 6 clumps out of 10 survived during this third trial which comprised a desiccation treatment. This may be due to the fact that at the time of the third experiment, this clone had produced many finger-shaped embryos.

These new pretreatment conditions (culture on 0.75M sucrose followed by 16 hours of dehydration with silica gel) were applied to clone D and 6 additional clones (Table 4). The survival rates of the clumps after freezing in liquid nitrogen varied between 13 and 53%, depending on the clone.

No direct relationship was found between the water content of the embryos and their survival after cryopreservation. Even though the clumps of embryos seemed to be severely damaged by the pregrowth and

Table 2: Water content of oil palm standard somatic embryos and survival of unfrozen control (-LN) and cryopreserved (+LN) embryos of 4 clones, as a function of the desiccation duration (DD) performed under a laminar flow cabinet. (10 clumps/condition).

	CLONE A			CLONE C			CLONE E			CLONE H		
DD (hrs)	Water Content (%)	Survival		Water Content (%)	Survival		Water Content (%)	Survival		Water Content (%)	Survival	
		- LN	+ LN		- LN	+ LN		- LN	+ LN		- LN	+ LN
0	77±2	10/10	0/10	76±5	10/10	0/10	78±9	10/10	2/10	77±1	10/10	0/10
4	56±4	10/10	0/10	62±5	10/10	0/10	58±5	10/10	4/10	58±7	10/10	0/10
6	50±5	7/10	2/10	50±9	10/10	0/10	54±1	10/10	7/9	47±8	8/10	7/10
8	40±7	5/10	2/10	50±5	8/10	0/10	49±4	9/10	4/9	43±9	9/10	3/10
10	39±7	6/10	0/10	43±3	4/10	0/10	37±8	7/10	5/9	44±6	7/10	1/9

Table 3: Water content of oil palm standard somatic embryos and survival of unfrozen control (-LN) and cryopreserved (+LN) embryos of 4 clones, as a function of desiccation duration (DD) using silica gel. -: not measured.

	CLONE C			CLONE E			CLONE H		
DD (hrs)	Water content (%)	Survival		Water content (%)	Survival		Water content (%)	Survival	
		- LN	+ LN		- LN	+ LN		- LN	+ LN
0	76±5	10/10	1/7	78±2	10/10	6/10	74±2	10/10	6/10
7	62±4	10/10	0/10	60±3	10/10	7/10	49±7	10/10	5/10
16	30±5	6/10	10/10	-	-	7/10	34±6	8/10	5/10
18	29±2	5/10	8/10	30±6	4/10	9/10	30±6	4/10	7/10

Table 4: Water content of oil palm standard somatic embryos and survival of unfrozen control (-LN) and cryopreserved (+LN) embryos of 7 clones. Pregrowth consisted of a 7 day culture on a medium containing 0.75M sucrose followed by 16 hours of dehydration with silica gel.

	CLONE						
	D	K	J	L	M	N	O
Water content (%) after pregrowth	75±2	78±1	78±2	73±1	81±2	75± 2	75± 2
Water content (%) after pregrowth + dehydration	23±1	32±3	36±4	19±2	35±2	20±5	28±1
Survival (-LN)	15/30(50%)	20/30(66%)	4/30(13%)	21/30(70%)	15/30(50%)	6/30(20%)	12/30(40%)
Survival (+LN)	5/30(16%)	4/30(13%)	5/30(16%)	16/30(53%)	13/30(46%)	11/30(37%)	4/30 (13%)

desiccation treatments (Fig. 2), proliferation recovery of the clumps surviving after cryopreservation was very rapid and intense (Fig. 3).

The best survival rates were obtained with clones L, M and N (37 to 53%). Poorer results (13 to 16% survival after freezing in liquid nitrogen) were obtained with the other clones. The unfrozen controls of these latter clones withstood differently the pregrowth and desiccation treatments: survival rate was only 13% with clone J, whereas it was comprised between 40 and 66% for clones D, K and O. Therefore, the dehydration duration chosen (16 hours) was not optimal for all clones.

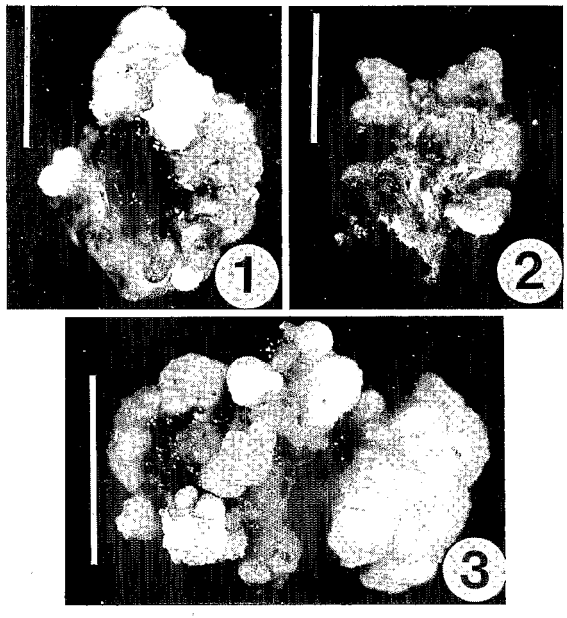

Fig. 1: Clumps of somatic embryos after one week pregrowth on a medium containing 0.75 M sucrose. (scale bar: 5 mm).

Fig. 2: Clumps of somatic embryos after one week pregrowth and 16 hours of dehydration with silica gel. (scale bar: 5 mm).

Fig. 3: Proliferation recovery of clumps 3 weeks after cryopreservation. (scale bar: 1 cm).

Discussion

The pretreatment conditions ensuring survival after cryopreservation differed among clones. Indeed, for some of them, pregrowth with sucrose only was sufficient, whereas, with other clones, it was necessary to complete this treatment with dehydration using a laminar flow or silica gel. In all these experiments, no relationship could be established between the growth behaviour of clones and their ability to survive.

Partial desiccation, which is usually necessary to achieve the maturation of single somatic embryos (Redenbaugh *et al.* 1991), was employed in this study in order to increase the resistance to cryopreservation of the embryo clumps. The tolerance of the embryos to desiccation seemed higher when it was performed with silica gel in comparison with the laminar flow cabinet. Indeed, regrowth could be obtained with embryos with a lower water content. Dehydration with silica gel may be slower than that achieved under the laminar flow and thus allowed the tissues to adapt to the water stress. However, this is in contradiction with the observations of Vertucci *et al.* (1991) who showed that, with zygotic embryos of various species, rapid dehydration induced extended freezing tolerance. It would be interesting to observe if addition of abscisic acid to the pregrowth medium would increase the resistance of the embryos to desiccation, as noted for other systems (Gray 1987;Kim and Janick 1989).

The clumps of embryos used in this study are highly heterogenous as regards their size and histological structure. They consist of adventitious embryos at various developmental stages. The most immature ones are predominantly composed of highly meristematic cells, with a high nucleocytoplasmic ratio. More mature ones are more differentiated, with a haustorium composed of parenchymatous cells and vascular bundles. The differential reaction between clones to cryopreservation could not be explained by a different hydration level of the tissues after the sucrose treatment since, whatever the clone, their water level was similar. The nature of the tissue is certainly of paramount importance for the acquisition of resistance to dehydration. Our hypothesis is that the more meristematic the tissue, the more it can be dehydrated without major alterations and the better is its resistance to low temperatures. The closeness of meristematic zones may also be a factor in resistance to freezing in liquid nitrogen. Indeed, we observed frequently that after cryopreservation, some clumps which had shown a limited number of surviving areas did not develop further or gave rise to shoots only. It seems, thus, that in the absence of a sufficient number of other living meristematic zones, a single meristematic area is not able to restart adventitious embryogenesis and will produce one shoot only or die. This hypothesis could be verified by a histological study of the dehydrated and cryopreserved clumps.

From an applied point of view, the improvements made to the original cryopreservation technique will facilitate its routine application. Indeed, due to the possibility of using standard somatic embryos, we are no longer limited by the production of finger-like embryos. Moreover, desiccation with silica gel will ensure more reproducible dehydration conditions than those obtained with a laminar flow cabinet which can vary, depending notably on the air humidity. However, it will be necessary for each clone to determine its optimal dehydration period before its cryopreservation and its long-term conservation. This improved technique will now be experimented on a large scale in the different industrial units producing clonal oil palms.

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