Cryopreservation of oil-palm polyembryonic cultures

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Introduction

An oil-palm *in vitro* propagation process was set up by the ORSTOM/CIRAD research group in the early 1980s (Pannetier *et al.* 1981). In this process, the collections are maintained at the polyembryonic culture stage. To facilitate management of the *in vitro* collection so as to limit the risk of both somaclonal variation and contamination of cultures, a cryopreservation protocol has been developed.

The original cryopreservation method was set up for a particular type of embryo: shiny white, finger-shaped, 2–4 mm long, so-called "fusiform embryos". It was shown that these embryos presented high survival rates after cryopreservation when they were first pretreated for 1 week on a high-sucrose medium (0.75M) (Engelmann 1986). However, even if the appearance of fusiform embryos in the standard polyembryonic cultures were enhanced by subculturing them on a sucrose-enriched medium (0.3M), their production remained a limiting factor in the development of the cryopreservation process.

In this paper we describe the different steps of a desiccation-based cryopreservation protocol set up for standard polyembryonic cultures and we discuss its efficiency. High sucrose pretreatment plays a key role in the success of this protocol; sucrose improves both desiccation and cryopreservation tolerance of the polyembryonic cultures. When provided at high concentration it can either act *via* the osmotic pressure it develops on the tissue (non-specific action), or *via* its structural characteristics and potential metabolization (specific action). In a second part of this paper we investigate the mode of action of sucrose by replacing it with other saccharides and polyols. We then study the effect of a high sucrose treatment on the sugar contents of embryo clumps.

Materials and methods

Embryo clumps weighing 250–300 mg were dissected from standard polyembryonic cultures. They were then subcultured for 1 week on a 0.75M sucrose solid MS-based medium (Pannetier *et al.* 1981) before being desiccated for 16 h over silica gel (5 clumps per airtight box containing 40 g silica gel). Desiccated clumps were introduced in cryotubes which were plunged directly into liquid nitrogen. Thawing was performed quickly by plunging cryovials in a 40°C water-bath for 2 min. Embryo recovery was optimized by progressively decreasing sucrose concentration and by adding 0.2 mg/L of 2,4–

dichlorophenoxyacetic acid (2,4–D) in the recovery medium for 3 weeks.

The osmotic effect of sucrose was studied by comparing the tolerance of embryos to desiccation and cryopreservation after pretreatment on a medium containing sucrose and/or mannitol; mannitol was chosen because of its limited absorption by plant tissue (Cram 1984). Sucrose specificity was approached by replacing it with the following compounds: ribose, fructose, galactose, glucose, maltose, raffinose, mannitol and sorbitol. The osmotic pressure of a 0.75M sucrose medium measured with an osmometer is 1090 mOsm. Consequently, the concentrations of the compounds used in association or as a substitute for sucrose were such that the final osmotic pressure of different media was close to 1000 mOsm. Owing to its dilution limit, the maximal osmotic pressure tested with raffinose was 804 mOsm. For each pretreatment, survival after 16 h of desiccation and/or cryopreservation was recorded.

Sugar analysis was performed by ion chromatography HPLC/Dionex (BioLC unit, AG6 precolumn, ASG separation column) after extraction with 78% ethanol. Starch concentration was measured enzymatically using Boeringher detection test kits.

Application of the cryopreservation protocol

The cryopreservation protocol described above has been tested with oil-palm polyembryonic cultures of clones maintained *in vitro* either in France (ORSTOM/CIRAD) or in Côte d'Ivoire (IDEFOR). Depending on the test the average age of the polyembryonic culture, i.e. time since their last subculture, varied from 4 to 12 weeks.

Very high survival rates, ranging from 50 to 100% (Table 1), were obtained in the first test performed with 4-week-old cultures. The clones used in this case were those initially chosen to determine the optimal conditions of the cryopreservation process. An average of 27 and 31% survival was obtained in test nos. 2 and 4 performed with 4 and 5-week-old cultures, respectively. The 12-week-old cultures of test no. 3 showed lower tolerance to cryopreservation; indeed, their survival rate varied from as low as 2% up to 25% with an average value of only 11%.

Polyembryonic cultures of all the 39 clones tested withstood freezing in liquid nitrogen. However, their survival rate was greatly dependent on the age of the culture that embryos were excised from. Embryo clumps are highly heterogeneous as regards their histological structure. They consist of meristem areas organized in adventitious embryos at different developmental stages. In order to keep their embryogenic proliferation property, i.e. high density of meristematic areas, it is necessary to subculture them regularly every 4 weeks. In the case of oil-palm somatic embryos, it seems that the more meristematic the clump is, the higher its tolerance to desiccation and cryopreservation. Consequently, it is necessary to maintain or reactivate the proliferation potential of the polyembryonic cultures prior to freezing.

Table 1. Survival rate after cryopreservation of different polyembryonic cultures of the ORSTOM/CIRAD (France) and IDEFOR (Côte d'Ivoire) clone collections

Test no.	No. of clones tested	Culture age (weeks)	Location of the test	Survival rate (%)		
				Average	Lowest	Highest
1	3	4	France	73	50	100
2	7	4	France	27	13	53
3	19	12	France	11	2	25
4	10	5	Côte d'Ivoire	31	10	54

Osmotic effect and specificity of sucrose in desiccation and/or cryopreservation tolerance

Only 20% of the non-pretreated polyembryonic cultures (control) withstood the 16-h desiccation period (Table 2). When the pretreatment medium contained a high concentration of either sucrose or various monosaccharides such as fructose, galactose and glucose, desiccation tolerance increased considerably; indeed after such treatment 77–100% of the embryos withstood the 16-h desiccation period. Lower tolerance was obtained in the presence of the other compounds tested, ranging from 56% (maltose) to 18% (lactose). In the case of mannitol, which was used as a "strict" osmoticum, 40% survival was recorded after desiccation. This value dropped down to 0% when mannitol was combined with sucrose. The presence of ribose in the culture medium was lethal for the polyembryonic cultures.

Table 2. Survival rate (%) after 16-h desiccation and/or cryopreservation of polyembryonic cultures pretreated for 7 days in presence of various sugars and polyols at different concentrations

Sugars/polyols concentration during pretreatment	Survival rate (%) after		
	Pretreatment + Desiccation	Pretreatment + Cryopreservation	Pretreatment + Desiccation + Cryopreservation
Sucrose 0.10M	20	0	0
Sucrose 0.75M	100	40	93
Mannitol 0.90	42	0	0
Sucrose 0.10M + Mannitol 0.75M	0	33	0
Sucrose 0.75M	77	20	57
Ribose 0.93M	0	0	0
Fructose 0.90M	100	0	53
Galactose 0.90M	100	0	47
Glucose 0.90M	92	0	12
Lactose 0.70M	18	0	0
Maltose 0.80M	56	0	0
Raffinose 0.24M	38	0	21
Sorbitol 0.90M	32	0	12

Survival after cryopreservation of the non-desiccated embryo clumps was recorded only when the pretreatment medium contained sucrose, associated or not with mannitol, with 20–40% and 33% survival, respectively. By contrast, when embryo clumps were frozen after desiccation, various compounds such as fructose, galactose and to a lesser extent, glucose, raffinose and sorbitol, allowed clumps to survive after cryopreservation, with survival rates ranging from 12 to 53%.

High sucrose pretreatment improved desiccation tolerance of the polyembryonic cultures and allowed them to survive after cryopreservation. The high osmotic pressure developed by such a medium is likely partly responsible for the improvement of their desiccation tolerance. Indeed, cultures maintained on a standard medium withstand less dehydration than those maintained in the presence of a high osmoticum concentration such as mannitol. Sucrose specificity was very low as regards desiccation tolerance as well as cryopreservation tolerance at low water content, as several compounds could mimic its effect. However, sucrose acted in a very specific way when embryo clumps were not desiccated prior to freezing. In that case, only a high osmotic pressure medium containing sucrose allowed embryonic tissues to withstand cryopreservation.

Changes in sugar profiles induced by high sucrose pretreatment

Four different sugars – sucrose, glucose, fructose and starch – were detected initially in the polyembryonic clumps (Table 3). A 7-day high sucrose pretreatment resulted in a drastic increase in sucrose and starch concentrations which were multiplied by 10- and 20-fold respectively. No significant modifications were noticed in glucose and fructose concentrations (data not shown). The only qualitative change recorded was the appearance of arabinose, whose concentration remained very low (around 2 mg/g dry weight [DW]) throughout the pretreatment. The acquisition of high tolerance to desiccation and cryopreservation of the polyembryonic clumps was associated with an increase in their sucrose and starch concentrations.

Table 3. Sugar profile of polyembryonic cultures before and after high sucrose pretreatment

	Sugar concentration (mg/g DW)	
Sugars	**Before**	**After**
Sucrose	64	679
Glucose	54	30
Fructose	43	35
Arabinose	0	2
Starch	2	45

Discussion

The cryopreservation protocol described in this paper is now used for oil-palm germplasm banking. In order to obtain satisfactory survival rates after cryopreservation it is essential to freeze only regularly subcultured polyembryonic cultures.

High sucrose pretreatment played an important role in the success of this protocol. One of its consequences was a drastic increase in the saccharose concentration of the polyembryonic cultures. Whether this uptake is apoplastic or symplastic remains unclear; however, starch accumulation at the end of the pretreatment reflects that, probably, a large quantity of sucrose was absorbed and metabolized. According to Sagishima *et al.* (1989), sucrose is first hydrolyzed into glucose and fructose before being incorporated into the cell *via* invertase activity. However, for Stranzel *et al.* (1988), sucrose incorporation depends on its concentration in the medium; invertase activity would be necessary at low concentration while sugar absorption would be passive through hydrophilic domains at high concentration. Sucrose is well known for its implication in desiccation tolerance of plant tissues (Koster and Leopold 1988; Leopold 1990). Sucrose could act either by replacing the water molecules involved in the strutural maintenance of macromolecules, or by inducing vitrification of the intracellular medium (Crowe *et al.* 1988; Williams and Leopold 1989). In the case of oil-palm somatic embryos, other sugars such as fructose, galactose and glucose were able to mimic a sucrose effect as regards desiccation tolerance. Their efficiency could depend on their ability to be both absorbed into the symplastic compartment and metabolized into sucrose. Other studies have shown that as the crystallisable water is removed from the high sucrose pretreated polyembryonic culture their survival after cryopreservation increased (Dumet *et al.* 1993). It is likely that a similar effect occurred with sugars other than sucrose, allowing desiccated embryo clumps to withstand cryopreservation. By contrast, only sucrose provided cryoprotection when polyembryonic culture water content was relatively high. Our hypothesis is that the presence of sucrose in the apoplast compartment prevents lethal extra-cellular ice formation which may occur in relatively highly hydrated embryo clumps.

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