Factors affecting the cryopreservation of coffee, coconut and oil palm embryos

F. Engelmann[1,2], D. Dumet[1], N. Chabrillange[1], A. Abdelnour-Esquivel[3], B. Assy-Bah[4], J. Dereuddre[5] and Y. Duval[1]

[1] ORSTOM, BP 5045, 34032 Montpellier Cédex 1, France
[2] IPGRI, Via delle Sette Chiese 142, 00145 Rome, Italy (present address)
[3] CATIE, Turrialba, Costa Rica
[4] IDEFOR-DPO, Station Principale de La Mé, 13 BP 969 Abidjan 13, Côte d'Ivoire
[5] Université Paris 6, Cryobiologie Végétale, 12 rue Cuvier, 75230 Paris Cédex 05, France

Summary

This paper describes the importance of various parameters which can significantly influence the cryopreservability of zygotic and somatic embryos of several recalcitrant and intermediate seed species (coffee, coconut, oil palm). Embryos should be used only when they are in an optimal physiological state as regards notably their maturity and metabolic status. Modifications of recovery conditions can greatly increase the survival rate of zygotic embryos. In the case of oil palm somatic embryos, pregrowth on medium with high sucrose concentration is necessary to induce tolerance to desiccation and cryopreservation.

Introduction

Seeds of a large number of tropical, subtropical and temperate species have been termed recalcitrant (Roberts 1973) since they are sensitive to desiccation and can thus be conserved for short periods only (weeks-months) even in the optimal moisture conditions. Careful adjustment of the storage environment (humidity, temperature) led to improvements in the conservation duration for several of these species such as oil palm and coffee (Ellis *et al.* 1990, 1991) which are now considered intermediate in their seed storage behaviour. Nevertheless, long-term storage of these seeds still remains impossible.

Large-scale multiplication processes by means of somatic embryogenesis in liquid medium are being set up for some species of commercial importance with recalcitrant or intermediate seed storage behaviour, such as oil palm and coffee (Vasil 1991). This leads to the development of an increasing number of strains, which creates laboratory management problems. Moreover, the risks of somaclonal variation increase in line with the duration of *in vitro* culture. Cryopreservation (liquid nitrogen, -196°C) currently represents the only long-term conservation option for zygotic and somatic embryos of these species.

Various cryopreservation protocols have been developed for zygotic embryos of a large number of species, among them many with recalcitrant seed storage behaviour (Table 1). This is not the case with somatic embryos, for which only a limited number of studies have been conducted until now. Research performed on cryopreservation of embryos presently concerns mostly methodological aspects and only a limited amount of work deals with the understanding of biological mechanisms in relation to cryopreservation. Various methods are employed for freezing embryos (Engelmann 1992): classical protocols, comprising pregrowth with cryoprotectants and slow freezing, and encapsulation/dehydration (Dereuddre *et al.* 1991) are used for somatic embryos. Most zygotic embryos are frozen rapidly after partial desiccation. Finally, a new desiccation technique termed flash-drying has been developed with zygotic embryos of *Landolphia kirkii* (Berjak *et al.* 1990). Flash-drying was followed by freezing at an intermediate (Vertucci *et al.* 1991) or ultra-rapid rate (Wesley-Smith *et al.* 1992).

In this article, we present results concerning the importance of various parameters on the cryopreservation of zygotic and somatic embryos of several species with recalcitrant or intermediate seeds and discuss their practical implications. This paper includes results already obtained by the ORSTOM research team either in Montpellier or in collaboration with other institutes.

Cryopreservation of zygotic embryos: coffee, coconut and oil palm

The experiments performed with coffee, coconut and oil palm zygotic embryos aimed at setting up cryopreservation processes for these materials. However, they also allowed the determination of the importance of various parameters such as the maturity and physiological stage of embryos before cryopreservation and the recovery medium.

Embryos of *C. arabica* were frozen at two different maturity stages, as determined by the colour of the fruit: immature embryos extracted from green fruits (i.e. 2

Table 1. Present application of cryopreservation for somatic and zygotic embryos of plant species

Species	Reference
Somatic embryos	
Brassica napus	Uragami *et al.* 1993
Citrus sinensis	Marin and Duran Vila 1988; Marin *et al.* 1993
Coffea arabica	Bertrand-Desbrunais *et al.* 1988
Coffea canephora	Bertrand-Desbrunais 1991; Hatanaka *et al.* 1994; Tessereau *et al.* 1994
Cucumis melo	Shimonishi *et al.* 1991
Daucus carota	Withers 1979; Tessereau *et al.* 1994
Elaeis guineensis	Engelmann *et al.* 1985; Dumet *et al.* 1993a
Juglans	de Boucaud *et al.* 1994
Manihot esculenta	Sudarmonowati and Henshaw 1990
Xanthosoma	Zandvoort 1987
Zygotic embryos	
Aesculus hypocastanea	Pence 1990
Arachis hypogaea	Runthala *et al.* 1993
Araucaria excelsa	Pritchard and Prendergast 1986
Artocarpus heterophyllus	Krishnapillay 1989
Brassica napus	Withers 1982
Carva	Pence 1990
Camellia sinensis	Chaudhury *et al.* 1991
Castanea	Pence 1990
Citrus sinensis	Radhamani and Chandel 1992
Cocos nucifera	Chin *et al.* 1989; Assy-Bah and Engelmann 1992b
Coffea	Normah and Vengadasalam 1992; Abdelnour *et al.* 1992
Corylus avellana	Gonzales-Benito and Perez 1994; Normah *et al.* 1986; Reed *et al.* 1994
Elaeis guineensis	Grout *et al.* 1983
Fagus	Pence 1990
Hevea brasiliensis	Normah *et al.* 1986
Hordeum vulgare	Withers 1982
Howea fosteriana	Chin *et al.* 1988
Juglans	Pence 1990
Landolphia kirkii	Vertucci *et al.* 1991
Manihot esculenta	Marin *et al.* 1990
Musa	Abdelnour *et al.* 1992
Olea europaea	Gonzales-Rio *et al.* 1994
Phaseolus vulgaris	Zavala and Sussex 1986
Pisum	Mycock *et al.* 1989
Poncirus trifoliata	Radhamani and Chandel 1992
Prunus amygdalus	Chaudhury and Chandel 1994
Prunus persica	de Boucaud and Brison 1991
Quercus	Pence 1990
Triticum	Bajaj 1983
Triticale	Bajaj 1983
Theobroma cacao	Pence 1991
Veitchia merrillii	Chin *et al.* 1988
Zea mays	Delvallée *et al.* 1989

months before harvest) and mature embryos (i.e. 1 week before harvest). Even though the desiccation period ensuring the highest survival rates was similar for both categories of embryos (Table 2), 96% of mature embryos withstood cryopreservation but 50% only of immature ones (Abdelnour *et al.* 1992). However, the lower survival of immature embryos could be almost totally overcome by placing them for recovery on a modified medium supplemented with 100 mg/l gibberellic acid (GA_3). In-

Table 2. Effect of maturity stage and desiccation period on the survival of control (-LN) and cryopreserved (+LN) zygotic embryos of *Coffea arabica* (from Abdelnour *et al.* 1992)

Desiccation (hours)	Survival (%)			
	Immature embryos		Mature embryos	
	-LN	+LN	-LN	+LN
0.0	100	0	100	0
0.5	80	50	100	96
1.0	53	34	56	42
1.5	25	14	28	19
2.0	6	0	8	0

deed, in these conditions, survival of cryopreserved immature embryos increased up to 83%. This result demonstrates that the difference in survival noted between the two categories of embryos was not due to a greater sensitivity to desiccation and freezing of immature embryos but to the fact that they were placed for recovery in non-optimal conditions.

Experiments performed with coconut embryos confirmed these observations. High survival rates could be obtained after freezing immature embryos (7-8 months after pollination) but only a limited number of them could develop into plantlets (Assy-Bah and Engelmann 1992a). On the other hand, most mature embryos (11-12 months after pollination) withstood cryopreservation and gave rise to whole plants after freezing (Assy-Bah and Engelmann 1992b). This was due to inadequate recovery conditions for immature embryos (Assy-Bah 1992).

Partial desiccation of zygotic embryos is necessary to obtain survival after cryopreservation. This treatment generally induces a drop in survival in comparison with untreated control embryos. In some cases, modifications in the regrowth pattern of cryopreserved embryos are also observed, such as the non-development of the haustorium of *Veitchia* and *Howea* (Chin *et al.* 1988) and coconut embryos (Assy-Bah and Engelmann 1992b). Experiments performed with oil palm zygotic embryos extracted from hydrated seeds showed that damage could be more severe than those observed with *Veitchia*, *Howea* or coconut embryos (Engelmann *et al.* 1995). Embryos desiccated down to 0.3 g H_2O/g dry weight (DW) and frozen in liquid nitrogen showed high survival rates (90%). However, desiccation induced irreversible damages to some embryos since only 60% of them could develop into plantlets, the others displaying abnormal development (callusing, development of root pole or haustorium only). Another experiment performed with oil palm zygotic embryos showed the importance of their physiological state before cryopreservation. Embryos extracted from rehydrated seeds were desiccated down to the water level of embryos in dry seeds (0.12 g/g DW). In parallel, dry seeds were frozen directly or after partial rehydration

until the water content of embryos reached 0.3 g/g DW, which ensured the highest survival rate with embryos extracted from rehydrated seeds and desiccated before cryopreservation. Embryos were considered surviving when they showed any sign of regrowth, whereas only embryos which developed into a whole plantlet were considered recovered. Survival of embryos was high in all conditions (70-96%). However, recovery of embryos frozen with a water content of 0.12 g/g DW, either extracted from dry seeds or dehydrated to this level, was very low, whereas that of embryos extracted from partially rehydrated seeds was comparable to that of embryos extracted from hydrated seeds and desiccated to 0.3 g/g DW. This increased tolerance may be linked with metabolic changes occurring during imbibition of seeds. Indeed, imbibition very rapidly induces dramatic metabolic changes such as mobilization of stored carbohydrate and lipid reserves and protein synthesis (Bewley and Black 1983). Notably, the degradation of starch which is present in large quantities in oil palm embryos (Vallade 1965) may lead to a rapid increase in the concentration of soluble sugars, which play a crucial role in the acquisition of tolerance to desiccation, 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).

Cryopreservation of somatic embryos: oil palm

Classical cryopreservation protocols including cryoprotective treatment in liquid medium followed by slow cooling are generally employed for somatic embryos (Engelmann 1992). However, an original protocol has been developed recently for oil palm somatic embryos (Dumet *et al.* 1993a). It comprised a 7-day pregrowth treatment of embryos on solid medium followed by partial desiccation (16 hours with silica gel) before freezing. These experiments showed the importance of pretreatment with sucrose for the acquisition of resistance of embryos to desiccation and to cryopreservation (Dumet *et al.* 1993b). Indeed, survival of non-pregrown embryos decreased in line with increasing desiccation periods. Without pregrowth treatment, no survival was obtained after freezing in liquid nitrogen whatever the dehydration duration. With pregrown control embryos, 100% survival was obtained whatever the desiccation period. Survival after freezing in liquid nitrogen was possible at a low rate (40%) without desiccation but it was significantly improved (up to 80-90%) after extended desiccation. Thermal analysis using differential scanning calorimetry revealed that these differences in survival rate could be correlated with differences in the thermal events recorded in embryos during freezing. Non-pregrown embryos displayed crystallization peaks, indicating lethal ice formation, whatever the desiccation period. On the contrary, the increase in survival of pregrown embryos in line with increasing desiccation durations was correlated with the progressive disappearance of crystallization peaks and their replacement by glass transitions. The evolution of sugar concentration in embryos was followed during the pregrowth treatment on medium with high sucrose concentration (Dumet *et al.* 1994a). Sucrose was predominantly accumulated (10-fold increase), whereas glucose and fructose concentration remained constant. Arabinose was the only new sugar detected but its concentration remained very low. Starch accumulation (20-fold increase) was also noted.

Experiments were also performed in order to evaluate the specificity of sucrose in the acquisition of tolerance of oil palm somatic embryos to desiccation and freezing (Dumet *et al.* 1994a). Embryos were pregrown on media containing various sugars or polyols at the same osmolarity. Only sucrose allowed survival after freezing in liquid nitrogen when embryos had not been dehydrated. However, when embryos had been dehydrated, several compounds (galactose, fructose, raffinose) ensured survival rates comparable to that obtained with sucrose. Thus, sucrose seems to have a high specificity in the acquisition of tolerance to freezing of embryos with high water levels, whereas it has a low specificity in the acquisition of tolerance to freezing of embryos with a low water level.

Experiments concerning the effect of various storage temperatures (-12, -80 or -196°C) on the survival of embryos were performed (Dumet *et al.* 1994b). After pregrowth treatment on medium with high sucrose concentration, embryos were desiccated or not before storage. Control embryos were stored at -196°C. Embryos stored at -12 and -80°C were either placed directly at these temperatures or immersed for 5 minutes in liquid nitrogen and then transferred at -12 or -80°C. The evolution of survival was recorded over a 6-month period. Non-desiccated clumps of embryos did not withstand 1 month of storage at -12°C and only 6% of clumps placed at -80°C survived after 3 months if they had been briefly immersed in liquid nitrogen before the storage period. Survival of desiccated clumps stored at -12°C after 6 months decreased progressively down to 27% with and 3% without transitory immersion in liquid nitrogen before storage. Survival of desiccated clumps of embryos stored at -80°C did not vary during the experiment and was comparable to that of clumps cryopreserved in liquid nitrogen (87-100%).

This experiment indicated the importance of dehydration of oil palm somatic embryos before storage, since desiccated clumps could be conserved for 6 months at -80°C without any viability loss in comparison with control embryos stored at -196°C. These results should be due to the fact that all free water has been removed from embryos during desiccation, thus allowing vitrification of intracellular solutes to take place during freezing, as was observed with thermal analysis (Dumet *et al.* 1993b). Moreover, glass transitions were recorded at temperatures between -50 and -60°C. Therefore, embryos stored at -80°C

are in a stable state and may be conserved at this temperature for extended periods without decrease in survival. These results may be of great interest for short-term storage of embryos since freezing in liquid nitrogen appears not to be required for storage periods up to 6 months.

Finally, this cryopreservation protocol was applied to 39 different clones of oil palm somatic embryos (Dumet *et al.* 1993c). This underlined the effect of the physiological state of the cultures on their resistance to cryopreservation. The average survival rate of clones in a good physiological state (i.e. displaying a normal aspect and growth characteristics) was 31% and 12% only for clones in a poor physiological state.

Conclusion

The experiments performed with zygotic and somatic embryos of these various species indicate the importance of several parameters, which should be taken into account when developing cryopreservation protocols for embryos.

Embryos should be used for cryopreservation only when they are in an optimal physiological state since this can greatly influence survival after freezing, as already showed notably in the case of cell suspensions (Withers 1985): zygotic embryos should be selected at a developmental stage at which their *in vitro* culture is fully operational and at which they display a high metabolic activity. Moreover, attention should be paid to the recovery conditions, since slight modifications can significantly enhance recovery rates. The experiments performed with oil palm somatic embryos underlined the important role of sugars, particularly sucrose, for the acquisition of tolerance to desiccation and cryopreservation. Pregrowth treatments on media with high sucrose concentration should be tried with zygotic embryos. These treatments may increase their tolerance to desiccation, thus reducing the extent of damage generally observed.

In conclusion, more fundamental research is needed to understand the mechanisms involved in the acquisition of tolerance to these stresses. In this aim, zygotic and somatic embryos from recalcitrant-seed species should be excellent materials to study desiccation tolerance/sensitivity, since desiccation appears as the key step in most cryopreservation protocols developed for embryos.

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Résumé

Facteurs influant sur la cryoconservation des embryons de caféier, cocotier et palmier à huile

Cet article décrit l'importance de divers paramètres qui peuvent avoir une influence significative sur les possibilités de cryoconservation des embryons zygotiques et somatiques de plusieurs espèces à semences récalcitrantes et intermédiaires (caféier, cocotier, palmier à huile). Les embryons ne doivent être utilisés que si leur état physiologique est optimal, notamment en ce qui concerne leur degré de maturité et leur état métabolique. La modification des conditions de reprise peut grandement améliorer le taux de survie des embryons zygotiques. Dans le cas des embryons somatiques de palmier à huile, une préculture sur un milieu à forte concentration en saccharose est nécessaire pour induire la tolérance à la dessiccation et à la cryoconservation.

Resumen

Factores que afectan la crioconservación de embriones del café, el coco y la palma de aceite

En este artículo se describe la importancia de diferentes parámetros que pueden influir considerablemente en la crioconservabilidad de embriones cigóticos y somáticos de varias especies de semillas recalcitrantes e intermedias (café, coco, palma de aceite). Los embriones deben utilizarse sólo cuando se encuentren en óptimas condiciones fisiológicas, sobre todo por lo que se refiere al grado de madurez y el estado metabólico. Las modificaciones de las condiciones de regeneración pueden aumentar enormemente la tasa de sobrevivencia de los embriones cigóticos. En el caso de los embriones somáticos de palma de aceite, se necesita el precultivo sobre un medio con una elevada concentración de sacarosa para inducir la tolerancia a la desecación y la crioconservación.

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