Cryo-Letters 13, 117-126(1992),
published by Cryo-Letters, 7, Wootton Way, Cambridge CB3 9LX, U.K.

CRYOPRESERVATION OF MATURE EMBRYOS OF COCONUT (*COCOS NUCIFERA* L.) AND SUBSEQUENT REGENERATION OF PLANTLETS

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SUMMARY: Mature embryos (10 to 12 months after pollination) of four varieties of coconut (hybrid PB 121, Cameroon Red Dwarf, Indian Tall, Renell Tall) could withstand cryopreservation in liquid nitrogen and develop into plants. Pretreatment consisted of a 4-hour desiccation in the air current of a laminar flow cabinet followed by a 11 to 20-hour culture on a medium containing 600 $g.l^{-1}$ glucose and 15% glycerol. Freezing and thawing were carried out rapidly. Recovery rates varied between 33 and 93% of frozen embryos, depending on the variety.

KEY WORDS: Coconut, *Cocos nucifera*, mature embryos, cryopreservation, pretreatment, genetic resources conservation.

INTRODUCTION

The safe preservation of the genetic resources of coconut, which is a species with recalcitrant seeds (1), has been defined as a priority objective for the present and future development of coconut breeding programs (2). For eight years, ORSTOM and IRHO,

in connexion with IBPGR (International Board for Plant Genetic Resources, Rome) have developed a research programme which led to the establishment of routine techniques for field collecting (3) and *in vitro* culture of coconut embryos (4, 5). For long-term storage, a cryopreservation process has been developed recently using immature embryos (6). However, the drawback of the utilization of immature embryos is that, unlike mature ones, the conditions for their *in vitro* culture are not yet perfectly mastered (7). As a result, only a limited proportion of immature embryos germinate and give rise to plants. Therefore, we have sought to develop a cryopreservation technique for mature embryos adapted from that set up for immature ones. This article presents the first results obtained in the cryopreservation of mature embryos from four different varieties of coconut.

MATERIALS AND METHODS

Plant material

The plant material used consisted of mature embryos (10-12 months after pollination) from seednuts of four varieties: the hybrid PB 121 (Malayan Yellow Dwarf x West African Tall), Indian Tall (IT7), Cameroon Red Dwarf (CRD) and Renell Tall (RT). The embryos were sampled according to the method of Assy-Bah *et al.* (3). The average initial weight of the embryos was 98.6 ±13.8 mg for PB 121, 81.6 ±12.4 mg for CRD, 112.3 ±22.2 mg for IT7 and 146.5 ±35.4 mg for RT.

Methods

In vitro culture

The embryos were cultured on the medium defined by Assy-Bah *et al.* (5) containing Murashige and Skoog's macro and microelements (8), Morel and Wetmore's vitamins (9), 41 $mg.l^{-1}$ FeEDTA, 100 $mg.l^{-1}$ sodium ascorbate, 60 $g.l^{-1}$ sucrose, 2 $g.l^{-1}$ activated charcoal. The pH was adjusted to 5.5 before autoclaving. Transfer to new medium was carried out monthly.

The embryos were cultured in 24 x 150 mm test tubes containing 20 ml of medium. They were placed at 27 ±1°C in the dark, until the gemmule emerged. They were then exposed to a photoperiod of 12 hours on 24 at 35 $\mu E.m^{-2}.s^{-1}$.

Cryopreservation

For pretreatment, the embryos were placed on open Petri dishes without culture medium and dehydrated for 4 hours in the air current of a laminar flow cabinet at room temperature. They were then transferred in open Petri dishes on the medium developed

for the pretreatment of immature embryos of coconut (5) containing 600 $g.l^{-1}$ glucose and 15% glycerol, and dehydrated for an additional period of 11 to 20 hours. Thus, the total duration of the pretreatment ranged from 15 to 24 hours. The embryos were then placed in 2 ml sterile cryotubes and immersed rapidly in liquid nitrogen. After 24 hours at -196°C, they were thawed rapidly by plunging the ampoules into a 40°C water-bath for 2 min and then transferred on the standard medium. 13 to 25 embryos were used per treatment. Control treatments involved recovery on standard medium with and without desiccation and with and without exposure to liquid nitrogen.

Water content measurement

The water content of the embryos (expressed in % of their initial fresh weight) was monitored during pretreatment. Fresh weight measurement was performed on batches of 10 embryos before pretreatment and after dehydration for different periods of time. Dry weights were determined after 48 hours desiccation in an oven at 102°C.

Assessment of recovery

Survival of embryos was recorded after 1 month of culture. Embryos were considered alive when regrowth had been observed. After 3 months in culture, the development of the surviving embryos was examined by assessing the number of embryos having developed a gemmule (the length of which was measured) and at least one root.

RESULTS

The initial water content of the embryos was very similar in all 4 varieties (Table 1). It decreased rapidly during the first 15 hours of pretreatment, from an average of 78.4% to an average of 11.4%. The largest embryos (RT and IT) dehydrated more slowly and the smallest ones (CRD) more rapidly. Between 15 and 24 hours of pretreatment, the rate of dehydration slowed down. Over this period, the water content was lowered by an average of only 1.8 (average water content of 6.4% after 24 hours).

Survival of unfrozen embryos was 100% without pretreatment (Table 1) and only slightly decreased after 24 hours of pretreatment. With frozen embryos, no survival was observed without pretreatment. Survival increased with increasing pretreatment durations for PB 121, RT and IT7 embryos. For 15 hours of pretreatment, survival ranged between 6.6% (RT and IT7) and 35.3% (PB 121) and reached 85.7 (RT) to 92.8% (PB 121) after 24 hours of pretreatment. In the case of CRD embryos, survival was greatest after 17 hours of pretreatment (73%) and decreased for longer pretreatment durations. The haustorium of frozen embryos browned rapidly after thawing and did not develop further, contrarily to unfrozen controls (Fig.1).

Table 1: Water content (± S.D.) and survival after 1 month culturing of control (-LN) and frozen (+LN) embryos of coconut as a function of the pretreatment duration.

			pretreatment duration (hrs)				
			0	15	17	19	24
PB 121	H2O (%)		77.5±6.1	9.2±5.7	8.4±4.6	7.9±3.6	6.4±4.3
	survival	-LN	100%	100%	100%	100%	87%
		+LN	0%	35.30%	53.30%	50.00%	92.80%
CRD	H2O(%)		79.3±6.7	7.9±4.1	7.6±5.5	5.9±5.3	5.7±4.2
	survival	-LN	100%	66.60%	76.00%	65.00%	77.70%
		+LN	0%	61.00%	73.00%	58.80%	56.00%
RT	H2O (%)		78.0±3.9	15.9±8.9	11.8±4.8	10.9±7.4	7.2±4.6
	survival	-LN	100%	100%	87.50%	85.70%	82.60%
		+LN	0%	6.60%	11.70%	38.00%	85.70%
IT7	H2O (%)		77.9±6.2	12.8±9.7	9.9±6.8	8.6±4.7	6.5±5.6
	survival	-LN	100%	100%	100%	82.30%	100%
		+LN	0%	6.60%	26.30%	23.50%	88.20%

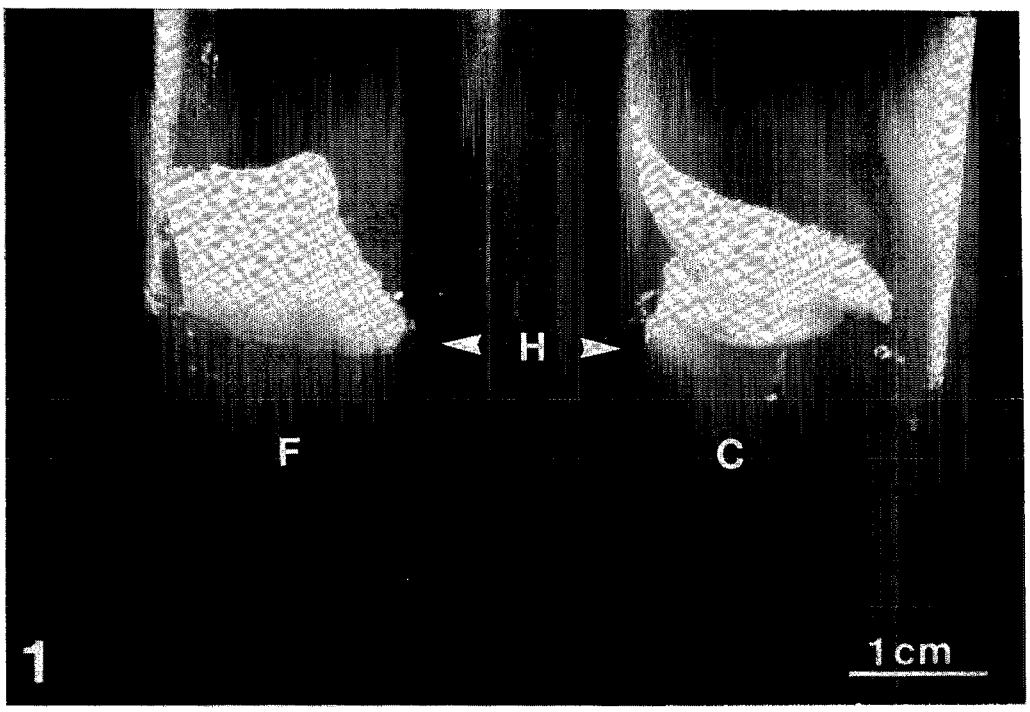

Fig. 1: Development of control (C) and frozen (F) embryos after 1 month culturing. H: haustorium.

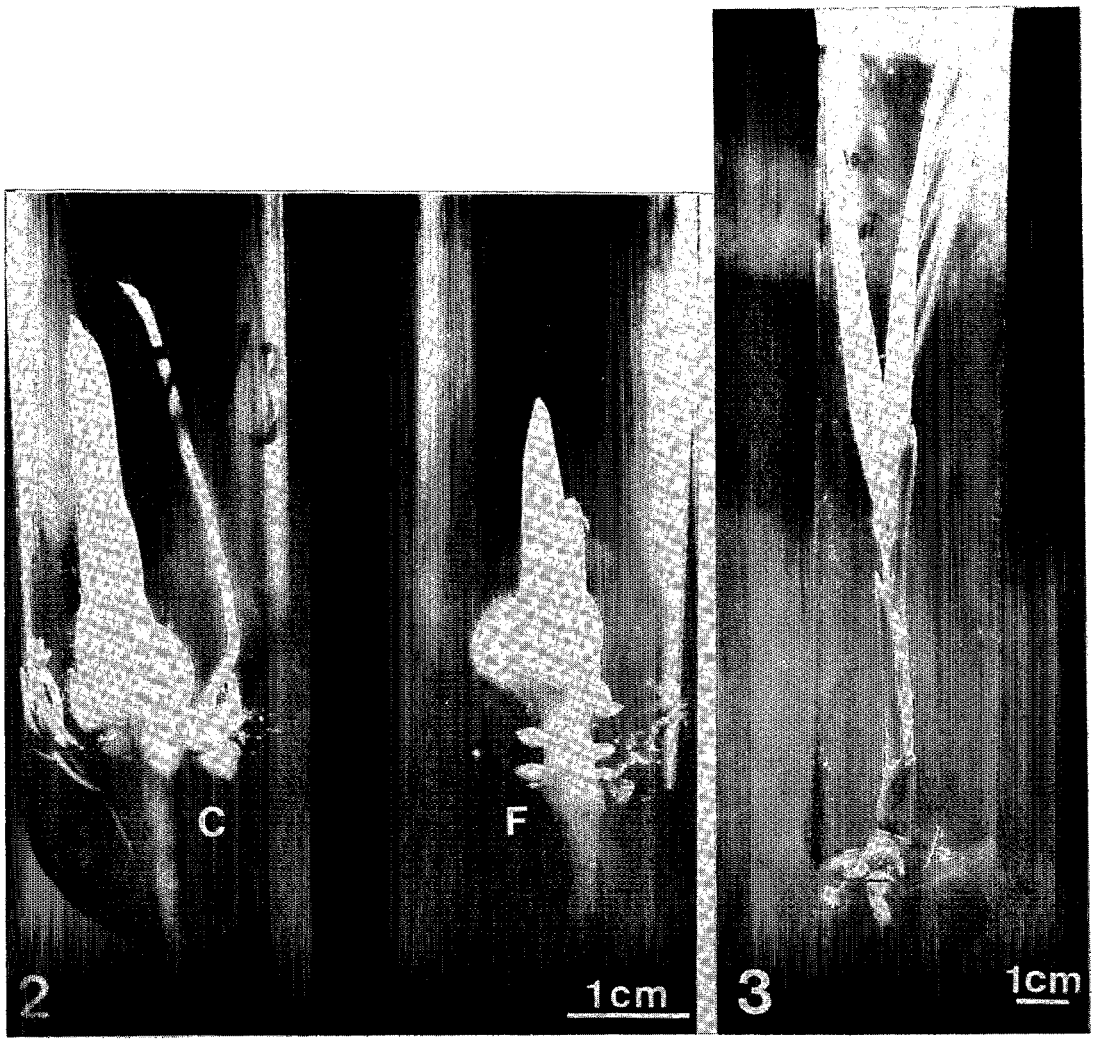

Fig. 2: Development of plantlets originating from control (C) and frozen (F) embryos after 3 months culturing.

Fig. 3: Development of a plantlet originating from a cryopreserved embryo after 4.5 months culturing.

Table 2: Development of control embryos after 3 months culturing as a function of the pretreatment duration.

		pretreatment duration (hrs)				
		0	15	17	19	24
PB 121	Number of embryos	18	13	15	15	15
	Gemmules developed	18 (100%)	13 (100%)	15 (100%)	15 (100%)	11 (73%)
	Length of gemmule (mm)	42.9	22.0	24.0	17.7	13.7
	Rooted embryos	18 (100%)	11	12	15	6
CRD	Number of embryos	15	18	17	23	18
	Gemmules developed	14 (93%)	11 (61%)	10 (59%)	15 (65%)	6 (33%)
	Length of gemmule (mm)	32.0	14.6	13.7	13.5	12.4
	Rooted embryos	15	11	10	15	6
RT	Number of embryos	20	18	16	21	23
	Gemmules developed	20 (100%)	17 (94%)	14 (87%)	17 (81%)	18 (78%)
	Length of gemmule (mm)	36.0	15.8	23.8	25.3	18.7
	Rooted embryos	20 (100%)	18 (100%)	12	14	4 (23%)
IT7	Number of embryos	13	15	18	13	17
	Gemmules developed	9 (69%)	5 (33%)	6 (33%)	7 (54%)	5 (29%)
	Length of gemmule (mm)	22.3	17.7	18.0	20.5	24.5
	Rooted embryos	8	5	6	7	5

Table 3: Development of cryopreserved embryos after 3 months culturing as a function of the pretreatment duration. *: contaminated.

		pretreatment duration (hrs)			
		15	17	19	24
PB 121	Number of embryos	17	15	14	14
	Gemmules developed	3 (18%)	1 (7%)	2 (14%)	13 (93%)
	Length of gemmule (mm)	13.7	15.0	10.0	21.3
	Rooted embryos	3	2	3	13
CRD	Number of embryos	18	15	17	25
	Gemmules developed	7 (39%)	9 (60%)	7 (41%)	9 (36%)
	Length of gemmule (mm)	4.5	4.1	8.7	12.1
	Rooted embryos	7	9	7	7
RT	Number of embryos	15	17	21	21
	Gemmules developed	0	0	*	7 (33%)
	Length of gemmule (mm)	0	0	*	19.0
	Rooted embryos	0	0	*	8
IT7	Number of embryos	15	19	17	17
	Gemmules developed	0	2 (10%)	0	7 (48%)
	Length of gemmule (mm)	0	15.0	0	13.2
	Rooted embryos	0	2	0	6

Without pretreatment treatment, the development of unfrozen embryos was generally satisfactory (Table 2). 100% rooted plantlets were obtained with PB 121 and RT, 93% with CRD, but only 69% with IT7 embryos. With unfrozen pretreated embryos, the delay in their development was different among the varieties. With PB 121 and RT, the number of developing embryos was slightly lowered only after a 24-hour pretreatment (73% for PB121, 78% for RT). Rooting was also lowered and the average size of the gemmule drastically reduced. With CRD and IT7, the percentage of developing embryos was reduced much more for the same pretreatment duration (33% for CRD and 29% for IT7). The percentage of rooted embryos was not modified compared to untreated controls. However, the development of the gemmule was different between those two varieties: a similar decrease was observed with PB 121, RT and RT, whereas no change was observed with IT7 compared with untreated controls.

With frozen embryos (Table 3) the number of developing plants increased with the pretreatment duration in the case of PB 121 and IT7. With CRD, the optimal pretreatment duration was 17 hours. The highest percentage of developed embryos varied greatly between varieties, ranging from 33% with RT to 93% with PB 121. Under optimal pretreatment conditions, no decrease was observed in the recovery rate between unfrozen and frozen embryos of all varieties except RT. The average size of the gemmule of frozen embryos was lower than that of control embryos for non optimal pretreatment durations. It increased progressively with the pretreatment duration. The number of rooted plantlets was higher than that of unfrozen controls with PB 121, RT, IT7 and identical with CRD. The further development of the plantlets originating from cryopreserved embryos was comparable to that of the unfrozen controls (Fig. 2) and plantlets with fully expended leaves could be obtained after 4.5 months culturing (Fig. 3).

DISCUSSION/CONCLUSION

The technique established gave high survival rates and rapid regrowth and development in a large number of mature cryopreserved embryos of four varieties of coconut. Considerable improvements have been made in comparison with previous work on the same material. Bajaj (10) observed only transient callusing on halves of transversally cut embryos. Chin *et al.* (11) could regenerate only one whole coconut palm, 15 months after the cryopreservation of the embryo. More recently, Assy-Bah and Engelmann (6) obtained up to 43% survival after freezing of immature embryos, but only a limited number of plants were regenerated.

This technique is original since pretreatment combines desiccation of the embryos in the air current of a laminar flow cabinet and the culture on a medium with high levels of cryoprotective substances. Such a combination has been employed up to now only with embryonic axes of pea (12). Indeed, pretreatment of zygotic embryos generally uses desiccation in an air flow (13) and pregrowth in the presence of

cryoprotective medium only in a limited number of cases, such as *Capsella bursa pastoris* (14), *Juglans* (15), *Prunus* (16) and immature embryos of coconut (6).

The pretreatment conditions set up for mature embryos of coconut were very drastic compared with those used for all other species: dehydration was very intense since the water content of the embryos was lowered from a high initial level (78.4%) to only 6.4%, which gave maximal survival in three of the varieties after cryopreservation. The duration of the pretreatment was very long (15 to 24 hours) certainly necessitated by the large size of the embryos. A recent review on cryopreservation of embryos (13) indicated that the initial water content of embryos is generally between 50 and 60% and maximal survival is observed with a higher water content (10 to 16%) reached after only 2 to 4 hours dehydration. Further desiccation generally leads to a dramatic loss of viability probably caused by severe dehydration injury. However, coconut embryos are formed of two distinct parts, the haustorium and the meristematic pole, which probably have different water contents. Indeed, the tissues of the haustorium comprize mainly highly vacuolated cells. It would be interesting to measure their dehydration separately. Indeed, the final water level of the meristematic pole may be closer to that generally observed with embryos of other species. The optimal dehydration duration varied between the coconut varieties tested in relation with the initial weight of the embryos: for the smallest ones (CRD) the maximal survival was obtained after 17 hours pretreatment, whereas it was observed after 24 hours with the other varieties which have larger embryos.

The pretreatment and freezing conditions used in this study (high levels of cryoprotective substances causing a low water content compatible with freezing at a rapid rate) were similar to that used during a vitrification process (17, 18). Vitrification may take place during freezing of coconut embryos and this could explain the high survival rates obtained. Thermal analysis conducted during the freezing of the embryos would show if a vitrous transition or crystallization is taking place.

The detrimental effects of the preculture were more apparent after 3 months in culture than 1 month. With control embryos, the rate of development was mainly affected and not the survival. The effects of preculture were different between varieties: the delay was more pronounced with rapidly growing varieties like PB 121. On the contrary, with IT7, which grows very slowly, no difference was observed between untreated controls and pretreated embryos.

With frozen embryos, similar delays were observed in comparison with pretreated controls, particularly with embryos which had not been pretreated in the optimal conditions, i.e. not enough or too much dehydrated. Differences appeared also between varieties, in relation with the size of the embryos The main difference in the development of frozen embryos compared with unfrozen controls was that growth of the haustorium was suppressed. Its tissues which are formed of highly vacuolated cells are completely destroyed during freezing. This is in accordance with the previous

observations of Chin *et al.* (19) in the case of *Howea* and *Veitchia* zygotic embryos.

The technique described in this paper is efficient since the percentage of embryos which are capable of developing into whole plants after cryopreservation is high with the 4 varieties tested. Moreover the development of cryopreserved embryos is normal. It is only delayed of one to two months, compared with control untreated embryos.

However, this process has to be improved in several ways. The pretreatment duration has to be adapted to the different varieties and its conditions standardized. More research is needed to check to which extent the successive use of desiccation and pretreatment on cryoprotective medium is necessary. Finally, additional trials should be performed with larger numbers and new varieties of coconut.

In conclusion, these preliminary experiments indicate that the safe long-term conservation of the genetic resources of coconut should be possible in the near future using cryopreservation.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to the personnel of the Marc Delorme Research Station (IRHO, Côte d'Ivoire) for kindly supplying the plant material. These experiments were performed in the tissue culture laboratory of the La Mé Research Station (IRHO, Côte d'Ivoire). The *in vitro* culture of the embryos was supervised by Dr. T. Durand-Gasselin, head of the laboratory. The help of Dr. L.A. Withers for her comments on the manuscript is gratefully acknowledged. This work is supported by IBPGR.

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