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CRYOPRESERVATION OF IMMATURE EMBRYOS OF COCONUT (*Cocos nucifera* L.)

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SUMMARY : Immature embryos of coconut (7 to 8 months after pollination) can withstand rapid freezing in liquid nitrogen after 4 hours of pregrowth on a semi-solid medium containing 600 $g.l^{-1}$ glucose and 10 or 15% glycerol or sorbitol. In these conditions, survival ranged from 10 to 43% and one embryo developed into a rooted plantlet, 2.5 months after freezing.

KEY-WORDS : coconut, *Cocos nucifera* L., immature embryos, cryopreservation, genetic resources conservation.

INTRODUCTION

The preservation of genetic resources of coconut is a prioritary objective for the development of present and future breeding programs (1). This task is made difficult due to the characteristics of the nut, which is recalcitrant and is very large (2). Efficient *in vitro* techniques have been developed for collecting (3) and culturing coconut embryos (4, 5). The conservation of coconut genetic resources is presently performed through field collections. However, the safe conservation of coconut germplasm is impossible to consider without a long-term storage technique. Only cryopreservation in liquid nitrogen (-196°C) presently offers a long-term conservation option (6).

Cryopreservation has already been applied to more than 70 different plant species (7). Freezing of zygotic embryos has been mentioned for around 10 tropical species (8). Among tropical oleaginous crops, oil palm embryos can survive freezing (9). Preliminary work has been carried out recently on the cryopreservation of coconut using mature embryos (10, 11). Bajaj (10) observed initial recovery through callusing followed by death on halves of transversely cut embryos. With whole embryos, Chin *et al.* (11) obtained callusing and the normal germination of one embryo, 15 months after freezing.

In this paper, we present the first report of the successful cryopreservation of immature embryos of coconut.

MATERIALS AND METHODS

Plant material

The planting material used consisted of immature embryos (7 to 8 months after pollination) from PB 121 seednuts (Malayan Yellow Dwarf x West African Tall hybrid). The embryos were sampled according to the method of Assy-Bah *et al.* (3). The embryos weighed 5 to 15 mg and measured 0.1 to 0.3 mm in diameter (Fig. 1).

Methods

In vitro culture

The embryos were cultured on a medium containing the macro- and microelements of Monnier (12), the vitamins of Morel and Wetmore (13), 1 $mg.l^{-1}$ Fe EDTA, 34 $g.l^{-1}$ sucrose and 45 $g.l^{-1}$ glucose, 0.01 $mg.l^{-1}$ biotin, 200 $mg.l^{-1}$ glutamine and caseine, 100 $mg.l^{-1}$ inositol and sodium ascorbate, 2 $g.l^{-1}$ activated charcoal, 7 $g.l^{-1}$ agar. The pH was adjusted to 4.6 before autoclaving.

The embryos were cultured in 24 x 150 mm test tubes containing 20 ml 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

The embryos were placed for 4 hours in Petri dishes on standard medium from which the mixture of sucrose and glucose was replaced with 600 $g.l^{-1}$ glucose and the

which the mixture of sucrose and glucose was replaced with 600 $g.l^{-1}$ glucose and the activated charcoal was omitted. Pregrowth on this medium was compared with pregrowth on medium supplemented with the cryoprotectants glycerol, sorbitol or polyethyleneglycol (PEG) 6000, at 5, 10 or 15 %. The embryos were then placed in sterile 2 ml cryotubes and immersed rapidly in liquid nitrogen. After 24 hours at -196°C, they were thawed rapidly by immersion of the ampoules for 30 s in a 40°C water-bath and transferred on the standard medium. Eighteen to twenty embryos were used per treatment. Control treatments involved recovery on standard medium with and without pregrowth/cryoprotection and with and without exposure to liquid nitrogen.

RESULTS

During the cryoprotective treatments, the water content of the embryos, expressed in % of their fresh weight, dropped from 82 to an average of 12%.

After one month, the survival of the control embryos was satisfactory for all the pregrowth conditions (Table 1), ranging from 73 to 100%. No difference was noted with control embryos which were not submitted to the cryoprotective treatment (-prg). After freezing in liquid nitrogen, survival was obtained in three conditions only : pregrowth with 10 and 15% glycerol (25 and 10% survival respectively) and 10% sorbitol (43% survival). PEG showed no cryoprotective effect at the concentrations employed.

After 2.5 months in culture, germination of up to 100% of the control embryos could be observed. The embryos first showed some browning (Fig. 2), then increased in size (Fig. 3) and eventually germinated (Fig. 4). Some of them showed abnormal morphology including hypertrophy of the gemmule (Fig. 5) or malformation of the haustorium (Fig. 6).

In the case of frozen embryos, only those treated with 15% glycerol have developed to date and one rooted plantlet has been obtained 2.5 months after freezing and thawing (Fig. 7). The germination of embryos treated and frozen in the same conditions was observed in two additional independent experiments. In the other conditions where survival was noted, no germination has yet been obtained, although the embryos increased in size.

DISCUSSION/CONCLUSION

During this work, the survival and germination of immature embryos were obtained after a 4-hour pregrowth on a medium containing a very high concentration of

The technique described differs from that generally employed for zygotic embryos of several tropical species. Indeed, in the case of oil palm (9), rubber (14), cassava (15), *Howea* and *Veitchia* (16) and tea (17), embryos were simply dehydrated for up to 5 hours in the air current of a laminar flow cabinet before freezing. With coconut, Bajaj (10) and Chin *et al.* (11) submitted the embryos to pregrowth in liquid medium containing cryoprotective substances (7% sucrose and dimethylsulfoxide in the first case, 10% dimethylsulfoxide in the second). Chin *et al.* (11) utilized a technique more similar to that usually employed in standard cryopreservation protocols, since after the cryoprotective treatment, the embryos were partially dehydrated for one hour in the laminar flow before freezing. In our work, the embryos were too small for a precise control of their dehydration in air. Therefore we used pregrowth on semi-solid medium enriched with cryoprotectants. The very high concentrations of cryoprotectants employed were close to those used during the vitrification process (18, 19) and induced a drastic dehydration of the embryos. It would be interesting to conduct thermal analysis during the freezing of the embryos in order to know if a vitrous transition or crystallization is taking place.

The advantage of using immature embryos instead of mature ones is that they are much smaller, less differentiated and may be more likely to survive as a whole. However the drawback of utilizing such young embryos is that the conditions of their *in vitro* culture are not yet perfectly mastered. Unlike mature embryos, not all immature ones will survive, develop and germinate *in vitro*.

In conclusion, this work demonstrated that it is possible to obtain the survival and the germination of immature embryos of coconut after freezing in liquid nitrogen. Trials are under way in order to improve the efficiency of the process and to try to adapt it to mature embryos. The long-term conservation of the genetic resources of coconut should thus be possible in the near future.

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Table 1 : Survival of control (-LN) and frozen (+LN) embryos after one month in culture. -pgr : no cryoprotective treatment ; +pgr : pregrowth with 600 $g.l^{-1}$ glucose only ; * : contaminated.

	-pgr	+pgr	GLYCEROL (%)				SORBITOL (%)				PEG (%)			
			2	5	10	15	2	5	10	15	2	5	10	15
-LN	17/20	20/20	18/19	19/20	17/18	11/15	19/19	19/19	17/18	*	19/19	14/18	17/20	*
	85%	100%	95%	95%	94%	73%	100%	100%	94%		100%	78%	85%	
+LN	0/20	0/20	0/19	0/20	**5/20**	**2/20**	0/20	0/20	**3/7**	0/20	0/20	0/20	0/20	*
	0%	0%	0%	0%	**25%**	**10%**	0%	0%	**43%**	0%	0%	0%	0%	

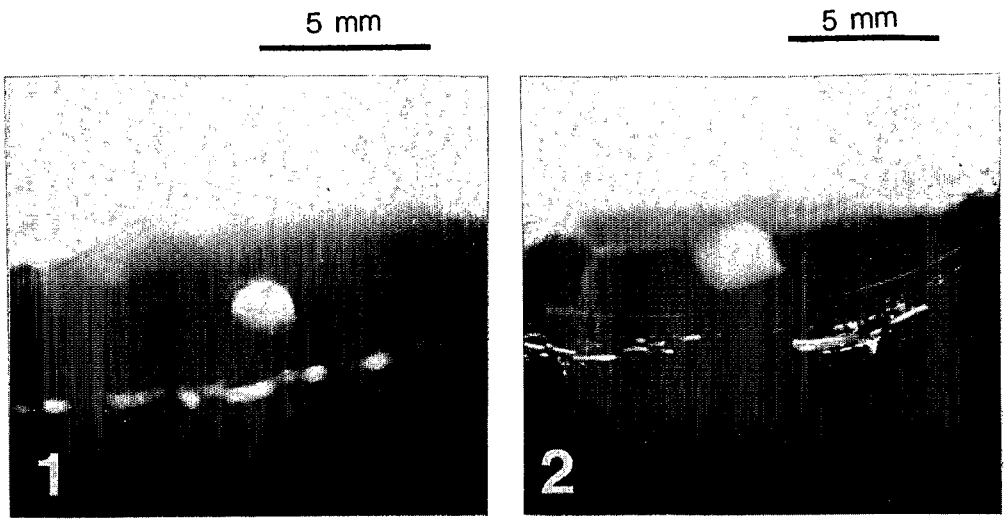


Figure 1 : Freshly dissected embryo.

Figure 2 : Browning of a control embryo after 15 days in culture.

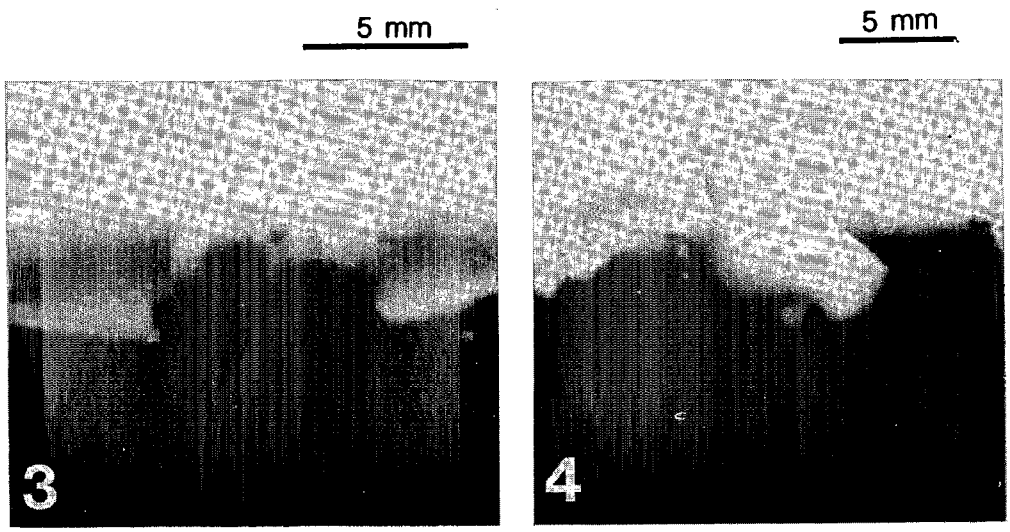
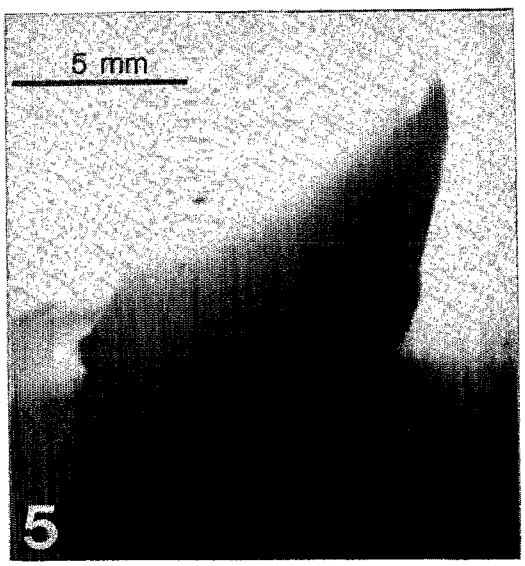


Figure 3 : Increase in size of a control embryo after 3 weeks in culture.

Figure 4 : Germination of a control embryo, after 8 weeks in culture.



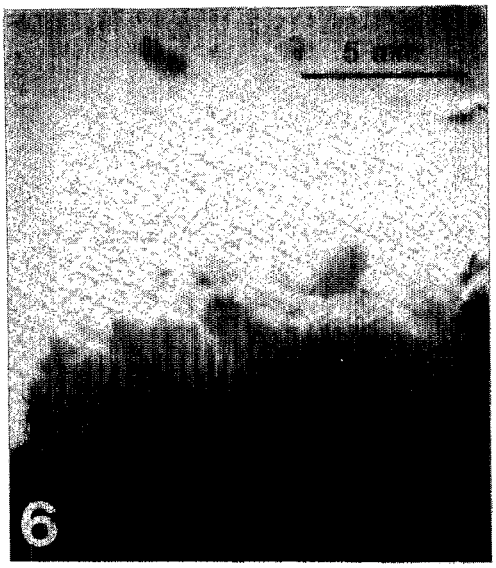


Figure 5 : Abnormal development of a control embryo : hypertrophy of the gemmule.

Figure 6 : Abnormal development of a control embryo : hypertrophy of the haustorium.

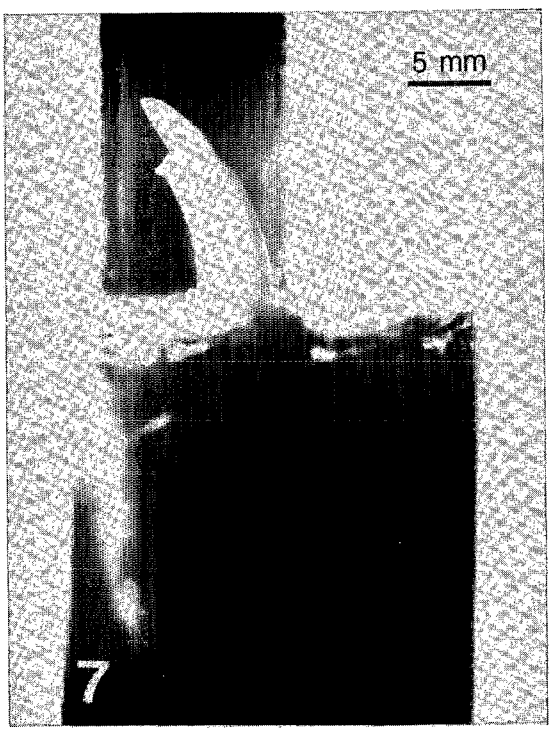


Figure 7 : Plantlet obtained from a frozen embryo after 2.5 months in culture.