II.7 Cryopreservation of Date Palm, Oil Palm, and Coconut

F. ENGELMANN[1], B. ASSY-BAH[2], S. BAGNIOL[3], D. DUMET[1], and N. MICHAUX-FERRIÈRE[4]

1 Introduction

In tropical and subtropical regions, palms are well represented among cultivated species. Date palm, coconut palm, and oil palm are among the most important crops in the economy of many developing countries around the world. The safe conservation of genetic resources of these species is faced with various problems. Indeed, germplasm collections of these species are conserved under field conditions since seeds of oil palm and coconut are recalcitrant and those of date palm have a germination rate which decreases rapidly even after short storage periods. In vitro culture techniques present interesting solutions to improve the conditions for their propagation and conservation. Indeed, tissue culture systems allow to multiply and store plant germplasm in aseptic conditions, with drastic reduction of space requirements and of labour costs.

The aim of this chapter is to review the literature and to present the most recent work carried out at ORSTOM/Montpellier in collaboration with various institutes for the development of cryopreservation processes for these species.

2 Date Palm (*Phoenix dactylifera* L.)

2.1 Plant Distribution and Importance

Date palm is present on all continents (Branton and Blake 1989): on the mediterranean borders of Europe and Africa, in west and east Africa. In Asia, the most important producing countries are located in the Middle East (Saudi Arabia, Iraq). Plantations also exist in India and China. Date palm was introduced in America in the 16th century and is now found in the USA and South America. Finally, it was introduced in Australia a century ago.

[1]ORSTOM, 911 av. Agropolis, BP 5045, 34032 Montpellier Cédex 1, France. (*Present address*: IPGRI, Via delle Sette Chiese 142, 00145 Rome, Italy)
[2]IDEFOR/DPO, Station Principale de La Mé, 13 BP 989, Abidjan 13, Ivory Coast
[3]GRFP, Conservatoire National Botanique dePorquerolles, 83400 Ile de Porquerolles, France
[4]CIRAD, BIOTROP, BP 5035, 34032 Montpellier Cédex 1, France

Biotechnology in Agriculture and Forestry, Vol. 32
Cryopreservation of Plant Germplasm I (ed. by Y.P.S. Bajaj)

About 5000 named varieties have been identified (Anonymous 1914); most cultivars originated from chance seedlings which were propagated vegetatively through offshoots. Also, a multitude of unnamed varieties occur, since each seedling may be considered a potential cultivar due to the high degree of heterozygosity in date palm (Tisserat et al. 1983b). Some offshoot varieties have been cloned for hundreds of years; isolated cases of offshoots cloned for up to 1300 years are known (Anonymous 1914).

Oases have a double economical importance, firstly for the production of dates, secondly by providing shed which allows associated cultures to grow (fruits, vegetables, cereals). World date production was estimated to 2883000 t in 1988 (Anonymous 1989). The main production areas are located in the Middle East (Iran, Iraq, Saudi Arabia) and in North Africa (Algeria, Morocco, Tunisia, Egypt).

2.2 Methods for Storage and Need for Cryopreservation

Date palm germplasm is traditionally conserved in the form of field collections of trees in its cultivation areas. Seeds are not recalcitrant, since they can remain viable for up to 15 years at room temperature. However, germination rate markedly decreases after 6 years of conservation (Munier 1973). Date palm is traditionally vegetatively propagated using sprouts. However, it produces a limited number of sprouts only, around 10 to 20 maximum during the whole life of the palm (Toutain and Rhiss 1973), which considerably slows down its propagation. Moreover, due to the mode of reproduction of this species, seed progenies are highly heterogenous. Therefore, various in vitro techniques including micropropagation through axillary budding and somatic embryogenesis have been developed to multiply genotypes (Tisserat 1987). In vitro collections of germplasm in the form of plantlets have been established, notably in France at GRFP (Groupement de Recherche Français sur le Palmier Dattier).

Date palm is threatened by various pests (acarians) and diseases (fungi, bacteria) (Munier 1973). The most important is a fungal disease induced by *Fusarium oxysporum* Schlect. f. sp. *albedinis* commonly termed bayoud, which occurs in North Africa. Preservation of tolerant or resistant genotypes is therefore essential in the frame of breeding programs against this disease. Maintenance of in vivo collections is costly due to the large size of palms, which induces low planting densities and to their slow growth habit. Thus, at the US Date Palm Repository (Indio, California), the in vivo collection which previously comprised numerous Old World and local varieties was restricted to those female varieties which were considered useful commercially and to five males showing interesting characters (Carpenter 1979). Moreover, plants conserved in natural conditions remain exposed to pests, pathogens, and natural disasters.

Management of large-scale in vitro collections poses numerous practical problems. Moreover, risks of contaminations and of somaclonal variation increase with time. It is therefore essential to develop long-term conservation techniques for the germplasm of numerous potentially useful varieties of date palm.

2.3 Cryopreservation Studies

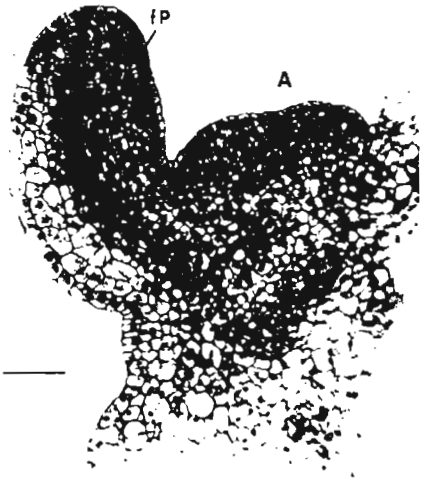
Only a limited amount of work has been carried out on cryopreservation of date palm, using in vivo (seeds, pollen, apices) or in vitro material (calli) (Table 1). The first experiments were performed in the 1980s with embryogenic calli of different varieties (Deglet Noor, Khadrawy, Medjool) (Tisserat et al. 1981; Finkle et al. 1982; Ulrich et al. 1982) using a classical freezing process. Plantlets could be regenerated from the cryopreserved calli. Pollen grains were successfully cryopreserved and germinated subsequently (Tisserat et al. 1983a). Fruits of the variety Deglet Noor obtained from flowers pollinated with cryopreserved pollen showed normal development (Tisserat et al. 1985). Seeds of the variety Zahidi could be cryopreserved, stored for up to 546 days in LN and thawed with a recovery rate between 85 and 100% (Al-Madeni and Tisserat 1986). Our work on the cryopreservation of date palm apices is discussed here. The successive steps of the cryopreservation process for apices were optimized (Bagniol and Engelmann 1991, 1992) and histological studies were performed after each step of the cryopreservation process and during the first days of recovery (Bagniol et al. 1992).

2.3.1 Materials and Methods

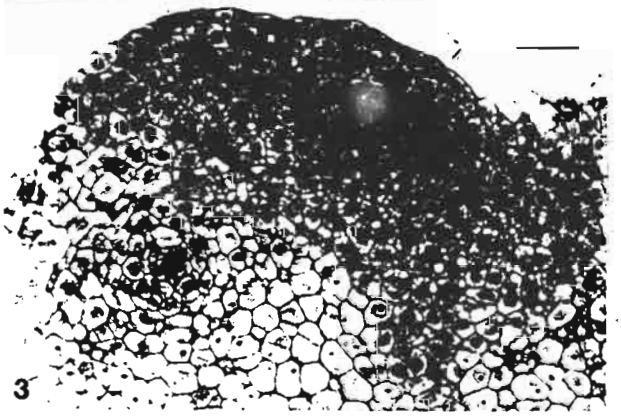
Apices (Fig. 1) were taken from in vitro plantlets of several varieties (Bou Sthammi noir, Zahidi, and Nabut Seif), at the end of a 6-week culture period on a multiplication medium containing 0.1 M sucrose (Ferry et al. 1987). They

Table 1. Summary of work done on cryopreservation of date palm, oil palm, and coconut

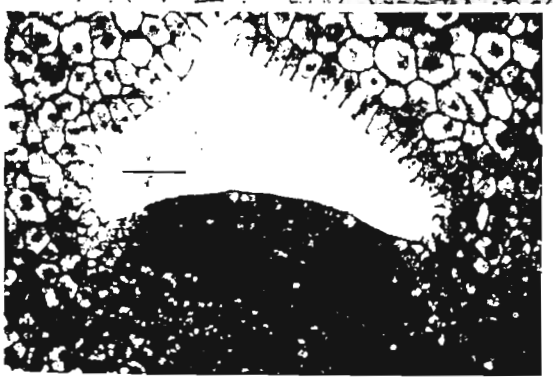
Plant species	Explant frozen	Results/remarks	Reference
Date palm	Embryogenic callus	Callus + plantlets	Tisserat et al. (1981), Finkle et al. (1982), Ulrich et al. (1982)
	Pollen grains	Germination + fruits	Tisserat et al. (1985)
	Seeds	85–100% germination	Al-Madeni and Tisserat (1986)
	Apices from in vitro plantlets	11.8–48.2% survival + plants	Bagniol and Engelmann (1991, 1992)
Oil palm	Zygotic embryos	Survival + plants in soil	Grout et al. (1983)
	Somatic embryos	31–55% survival	Engelmann et al. (1985)
		Normal plants in soil	Engelmann (1991b)
		13–100% survival	Dumet et al. (1993)
Coconut	Transverse halves of young embryos	Callus	Bajaj (1984)
	Mature embryos	Callus + 1 plantlet	Chin et al. (1989)
	Immature embryos	0–43% survival	Assy-Bah and Engelmann (1992a)
	Mature embyros	73–93% survival and plant production	Assay-Bah and Engelmann (1992b)



2



3



Figs. 1–12. Date palm

Fig. 1. Morphological aspect of an explant. Apical zone with two foliar primordia recently formed (*fp*) surrounded by a foliar sheath (*fs*). *Bar* 0.2 mm. (Bagniol 1992)

Fig. 2. Histological structure of a control sample. *A* Apical dome; *fp* foliar primordium. *Bar* 125 μM. (Bagniol et al. 1992)

Fig. 3. Longitudinal section of an apex after a 24-h culture on standard medium. Structure of the apical dome. *Arrows* indicate figures of mitosis. *Bar* 140 μM. (Bagniol et al. 1992)

Fig. 4. Sample after cryoprotective treatment. Note starch grains (*dark points*) in superficial cells. *Bar* 130 μm. (Bagniol et al. 1992)

consisted of the apical dome, foliar primordia, and a basal part (Fig. 2). Cells showed characteristics of meristematic cells: they had a high nucleoplasmic ratio, a dense cytoplasm, and small vacuoles. No polysaccharide reserves were present. A gradient of differentiation was visible in meristems, from the superficial layers to the central region, where cells had a lower nucleoplasmic ratio and a more accentuated vacuolization.

Before pretreatment, meristems could be maintained for 1 to 7 days, immediately after their dissection, on an induction medium, favoring growth of meristems (Ferry et al. 1987). For pregrowth, meristems were placed for 24h on media containing 0.3 or 0.5 M sucrose. For cryoprotective treatment, apices were placed at 0 °C for 2h in liquid sucrose-enriched medium supplemented with 0 to 15% DMSO.

Meristems, suspended in 1 ml of cryoprotective medium, were placed in 2 ml sterile polypropylene cryovials. Freezing was carried out at 0.5 to 5 °C/min to various prefreezing temperatures (end of programmed freezing). Once prefreezing temperature was reached, ampoules were either thawed immediately or immersed in LN, where they were kept for 1 h before being thawed. Meristems were thawed either rapidly by immersion of the cryotubes in a water bath thermostated at 40, 60, or 80 °C, or slowly by placing the cryotubes at room temperature in the air current of a laminar flow cabinet. Thawed meristems were transferred at 24-h intervals in Petri dishes containing 10 ml of solid media with progressively lower sucrose concentrations (0.5, 0.3 M) until standard concentration of 0.1 M was reached. These media were used with or without 1g/l activated charcoal. Cultures were placed either directly under standard lighting conditions (photoperiod of 16h/24, 42 $\mu E/m^2/s$) or in the dark for the first 10 days following thawing and then under attenuated light (7 $\mu E/m^2/s$, same photoperiod).

2.3.2 *Results and Discussion*

Effect of Culture on Induction Medium Prior to Pregrowth Treatment. In order to determine the survival rate of apices after freezing, it was necessary to submit them after dissection to a culture on induction medium for 1 to 3 days (Table 2). Extension of this culture duration to 5 or 7 days did not allow growth recovery.

Table 2. Effect of the duration of culture on induction medium on the survival rate of control (−LN) and frozen (+LN) meristems. Apices were pregrown for 24h on a medium with 0.5 M sucrose, cryoprotected with 0.5 M sucrose +15% DMSO and frozen to −30 °C at 1 °C min. (Bagniol and Engelmann 1991)

	Duration of culture (days)				
	0	1	3	5	7
−LN	57.1	79.1	96.6	96.6	87.0
+LN	0.0	22.0	15.1	0.0	0.0

Table 3. Effect of sucrose and DMSO concentration during the cryoprotective treatment on the survival rate of control (−LN) and frozen (+LN) meristems. (Bagniol and Engelmann 1991)

Sucrose (M)		DMSO (%)			
		0	5	10	15
0.3	−LN	62.5	62.9	62.9	61.5
	+LN	0.0	0.0	3.6	7.5
0.5	−LN	66.6	56.6	56.6	57.1
	+LN	0.0	18.9	14.0	17.1

Histological examination of apices after 24 h culturing on standard medium revealed that this culture led to recovery of cellular activity (Fig. 3): several mitotic figures as well as numerous divisions were visible. Starch grains were also present in some cells (not shown in Fig. 3). It seems that recovery of cellular activity is necessary for survival. Also, it could have the effect of healing meristems as well as synchronizing explants, thus reducing their physiological heterogeneity.

Effect of Pregrowth and Cryoprotective Treatment. Pregrowth of apices consisted of a 24-h culture on medium containing 0.3 or 0.5 M sucrose. For cryoprotective treatment, meristems were suspended in liquid medium with the same sucrose concentration and various concentrations of DMSO (0 to 15%) were added. For unfrozen control meristems, there was no effect of DMSO concentration, whatever the sucrose level (Table 3). For cryopreserved apices, no survival was obtained without DMSO; however, survival was optimal after a cryoprotective treatment with 0.5 M sucrose and 5 to 15% DMSO. During pregrowth and cryoprotective treatment, synthesis of starch increased in intensity in some of the samples (Fig. 4). Starch grains were located in the superficial zones of the meristem where cells retained a very meristematic appearance. Other samples showed no starch synthesis.

The increased starch synthesis occurring when meristems are placed on a sucrose-enriched medium indicates that, contrary to previous hypotheses (Finkle et al. 1985), sucrose does not act as an osmoticum only but enters cells in large quantities. According to Grout et al. (1988), sugars, dissoluted or attached to the membrane through covalent bounds stabilize the membrane bilayer, thus protecting integrity of cell membranes during dehydration and freezing. Therefore meristems which showed no starch synthesis may be those which were unable to survive after cryopreservation.

Effect of Freezing and Thawing Conditions. Among the various combinations of cooling rates (0.5 to 5 °C/min) and prefreezing temperatures experimented (−20 to −40 °C), survival after cryopreservation was achieved after prefreezing at 1 C/min down to −30 or −35 °C only and was optimal at −30 °C. Survival of prefrozen meristems decreased progressively in line with the lowering of prefreez-

Table 4. Effect of prefreezing temperature on the survival rate of prefrozen (−LN) and cryopreserved (+LN) meristems. (After Bagniol and Engelmann 1991)

Prefreezing temperature (°C)							
	−12	−15	−20	−25	−30	−35	−40
−LN	53±15	64±17	37±10	28±10	32±14	17±13	8±12
+LN	0	0	0	0	18± 8	10±12	0

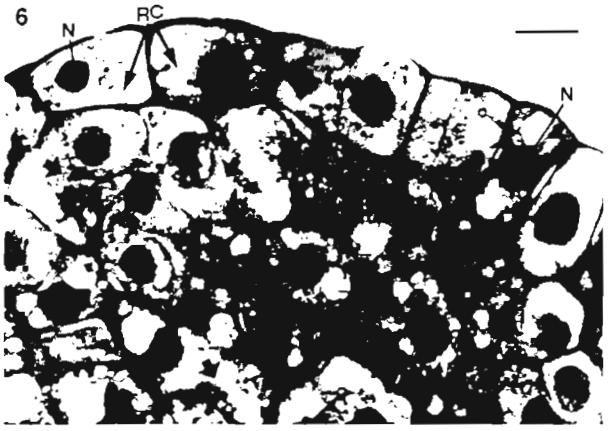
ing temperature (Table 4). No survival of cryopreserved meristems was noted after slow thawing at room temperature. Recovery of meristems was noted after thawing at 40, 60, and 80 °C, but was significantly higher at 40 °C.

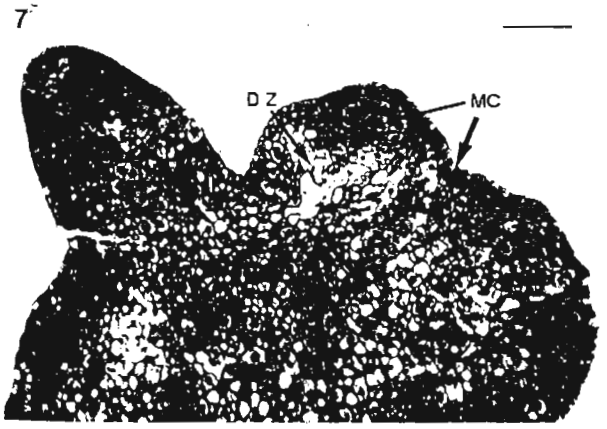
Insufficient dehydration during prefreezing down to −25 °C caused death of cryopreserved tissues, whereas from −40 °C onwards excessive dehydration was responsible for death of meristems. Only a very narrow range of temperatures (−30 to −35 °C) ensured survival after freezing in liquid nitrogen. With date palm meristems, rapid thawing in a water bath at 40 °C was preferable to slow rewarming and higher thawing temperatures. Indeed, rapid rewarming makes it possible to avoid recrystallization phenomena which induce lethal damages of the cells (Dereuddre 1985).

Histological examination revealed that after freezing, apices showed cellular heterogeneity (Fig. 5). Some cells conserved their meristematic characters with nuclei sometimes intensely stained (N). This was the case in cellular layers corresponding to the meristem itself, whereas in the underlying zone where cells were more vacuolated, some were damaged, showing broken cell walls. Finally, cells with cytoplasm contracted away from the cell wall were observed in some samples (Fig. 6). Many nuclei displayed a particular appearance, intensely and uniformly stained (Fig. 6). Starch grains were visible inside some cells, more often located in the superficial zone.

Date palm meristems did not withstand freezing in LN as intact structures. It seems that the more meristematic the cells were, the more they resisted. During the steps which precede freezing, gradients may be established both for outflow of water and penetration of cryoprotectants. In the case of date palm, as in that of other plants, the central cells as well as the ones located at the base of the explant are more vacuolated and their dehydration may be more difficult or they may be less tolerant to the necessary level of dehydration. This may explain their incapacity to withstand freezing. Superficial cells which are more meristematic are more likely to reversibly dehydrate since they have no or only a few vacuoles.

Effect of Recovery Conditions. Presence of activated charcoal in the recovery medium improved survival of frozen meristems (Table 5). Their response to lighting conditions depended on the composition of recovery medium, the presence of activated charcoal being the determining factor. Indeed, a minimal survival rate of 19.6% was obtained, whatever the lighting conditions if medium contained activated charcoal. Without charcoal, light totally inhibited recovery of frozen meristems.





Figs. 5,6. Samples after prefreezing and immersion in liquid nitrogen. *N* Intensely and uniformly stained nuclei; *RC* cells with retracted cytoplasm; *arrows* rupture and deformation of cell walls. *Bar* 125 μm (**Fig. 5**); 25 μm (**Fig. 6**). (Bagniol et al. 1992)

Fig. 7. Zonation in a sample 2 days after thawing showing meristematic cells (*MC*) and disrupted zones (*DZ*). *Bar* 200 μm. (Bagniol et al. 1992)

Table 5. Effect of lighting conditions and activated charcoal on the survival rate (±S.D.) of cryopreserved meristems after two weeks of culture. After thawing, meristems were exposed directly to a photon dose of 42 μE/m²/s or placed in the dark for 10 days and then exposed to a photon dose of 7 μE/m²/s. (Bagniol and Engelmann 1992)

	Activated charcoal (g/l)	
	0	1
42 μE/m²/s	0.0	19.6 ± 5.3
7 μE/m²/s	9.1 ± 0.0	25.9 ± 11.6

At the histological level, samples showed a cellular heterogeneity 2 days after thawing (Fig. 7): zones with meristematic cells and differentiated or harmed cells. The first mitoses occurred in the superficial layer 4 days after thawing (Fig. 8). However, not all apices observed at this stage showed mitotic figures. From this moment on, thickening of the cell wall could be observed in some cells of various areas. From the 6th day onwards, walls of meristematic or nonmeristematic cells had a sinuous aspect (Fig. 9). Up to the 7th day, the outline of the original meristem remained recognizable. Afterwards, in most cases, the initial apex was no longer distinguishable, hidden by proliferation of meristematic cells of the apical dome and of the foliar primordia (Fig. 10). Intense synthesis of starch occurred inside cells of superficial and underlying zones (Fig. 11). In samples which had shown no morphological sign of regrowth for 6 weeks and were thus considered dead, cells were poorly stained and had a retracted cytoplasm (Fig. 12).

Modifications of recovery conditions had a very positive effect on the growth pattern of cryopreserved meristems, as assessed by observation of their macroscopic evolution. Regrowth of apices which had survived after cryopreservation was visible 7 days after thawing. Proliferation occurred from the basal part of explants and led to the formation of a callus which had a limited development. Each sample which had formed a callus gave rise during the following weeks to a single shoot.

Activated charcoal has an effect of adsorbing growth regulators, thus decreasing the level of hormones available to the cultures (Weatherhead et al. 1978; Ebert and Taylor 1990). After freezing in LN, only groups of cells remain alive but not the whole meristems. When date palm meristems are transferred on a medium without activated charcoal, the quantity of hormones available in the medium may be too high compared with the number of living cells and induces intense callusing. With activated charcoal, the level of free hormones may become sufficiently low to be compatible with the number of living cells and allow regrowth of frozen meristems with limited callus formation. We observed with date palm that growth recovery in the dark improved survival of cryopreserved meristems. Chemiluminescence analyses showed an increase of oxygen singlets in cryopreserved tissues submitted to light immediately after thawing (Benson and Noronha-Dutra 1988). Light has an oxidative effect which induces browning and

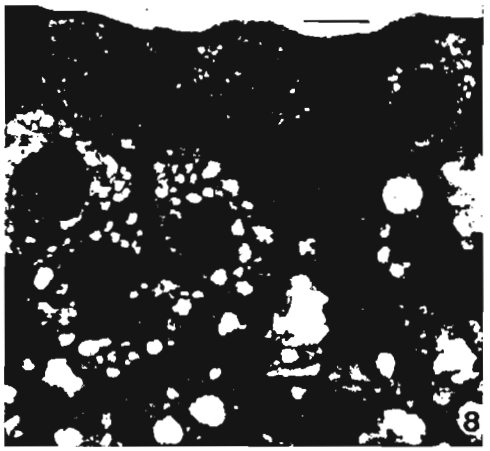


Fig. 8. First mitosis (*arrow*) observed in a cryopreserved sample 4 days after thawing. *Bar* 30 μm. (Bagniol et al. 1992)

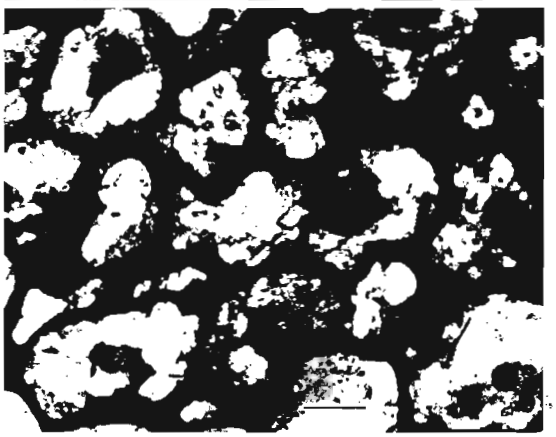


Fig. 9. Thickening of cell walls in a cryopreserved sample 6 days after thawing. *Bar* 35 μm. (Bagniol et al. 1992)

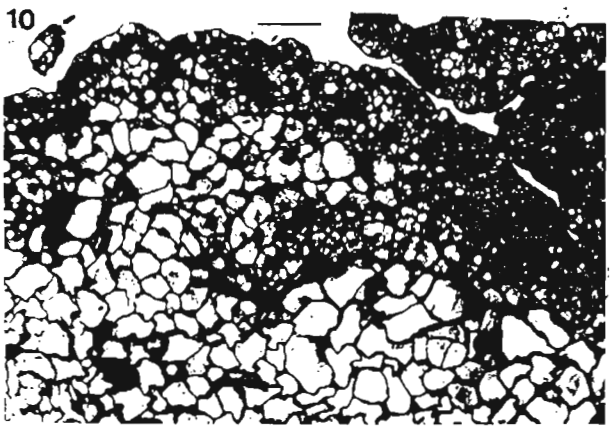


Fig. 10. Sample 15 days after thawing. Cell proliferation inside meristem and foliar primordia. *Bar* 150 μm. (Bagniol et al. 1992)

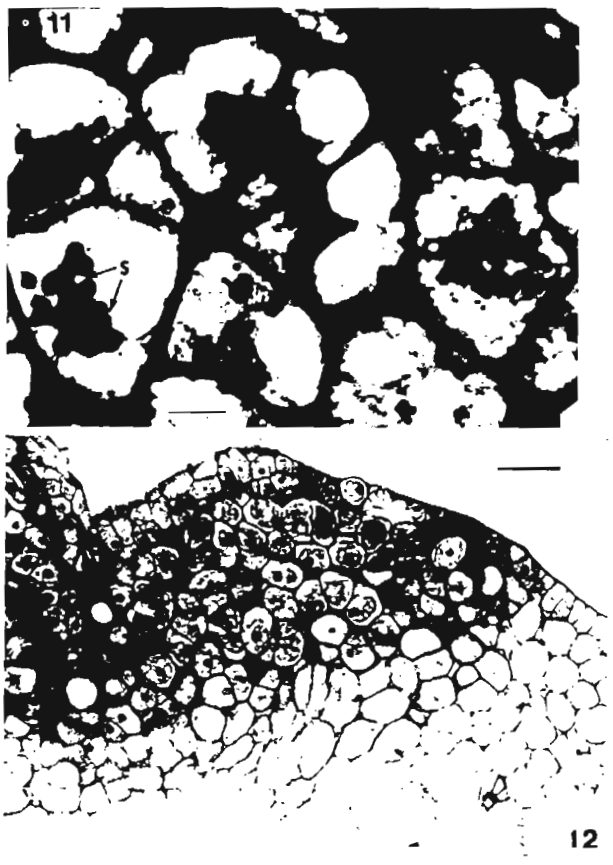


Fig. 11. Richness in starch content (*s*) in the underlying zone of the meristem, 15 days after thawing. *Bar* 40 μm. (Bagniol et al. 1992)

Fig. 12. Sample showing no morphological sign of regrowth 6 weeks after thawing. Note that cells have a retracted cytoplasm. *Bar* 115 μm. (Bagniol et al. 1992)

necroses which can lead to death of cells (Benson et al. 1989). This was observed in the case of date palm meristems. Presence of activated charcoal in recovery medium could also allow adsorption of oxidated substances which are toxic to cells, and thus increase the recovery rate, even under lighted conditions.

2.3.3 Conclusion

The present study allowed a protocol ensuring the cryopreservation of date palm apices, with satisfactory survival rates for two varieties out of the three experimented: 30.3% for Bou Sthammi noir and 48.2% for Nabut Seif, but 11.8% only for Zahidi (Bagniol and Engelmann 1992). However, these values are compar-

able to those mentioned in the literature dealing with cryopreservation of meristems (Kartha 1985; Bagniol 1992). These results may be related to the behavior of these varieties under in vitro conditions: Zahidi has a relatively low growth rate in comparison with that of the two other varieties. The cryopreservation protocol developed is a classical one, using cryoprotective treatment and programmed freezing. Importance of various parameters such as that of freezing rate could be underlined. The originality of the present work is linked with the observations made concerning the importance of culture conditions prerequisite to cryoprotective treatment and of recovery conditions. The extensive histological study performed also allowed the demonstration of the importance of sucrose (assessed by starch accumulation) during pregrowth treatment in order to ensure high survival rates. More detailed studies on the role of this compound should be performed, since it appears as a key substance for acquisition of tolerance to freezing at the temperature of liquid nitrogen.

3 Oil Palm (*Elaeis guineensis* Jacq.)

3.1 General Account

Oil palm is mainly cultivated in industrial plantations between 7 °S and 7 °N, at an altitude lower than 400 m in areas with high pluviometry. Plantations now exist on all continents, South East Asia, Africa, South America in Brazil and the Amazon basin (Equator, Columbia, Peru), and the Pacific Coast (Costa Rica). The second important species is *Elaeis oleifera* which is cultivated in South America. It is employed for interspecific crosses with *E. guineensis* in order to increase tolerance to pests and pathogens existing in this region.

Oil palm is after soybean, the second source of vegetable edible oil. Annual production was over 11 millions tons in 1991 and is still increasing due to a continuous rise in planted surfaces which reach today 3.5 million ha (Anonymous 1991).

Genetic resources of oil palm are conserved in the form of field collections. Seeds are not truly recalcitrant, but retain a good viability for short periods only, 1–2 years under optimal conditions according to Hartley (1988). In vitro propagation techniques based on somatic embryogenesis have been developed on a large scale in order to clonally propagate elite trees (see Duval et al. 1995 for a review). These elite genotypes are conserved as cultures of somatic embryos under standard conditions. Experiments using controlled atmospheres have also been performed (Engelmann 1990).

3.2 Cryopreservation

Before the research program developed by ORSTOM/CIRAD was initiated, cryopreservation experiments had been carried out with oil palm zygotic embryos only (Grout et al. 1983). Zygotic embryos extracted from seeds were

partially desiccated under the laminar flow and successfully frozen in liquid nitrogen.

3.2.1 *Methodology/Protocol*

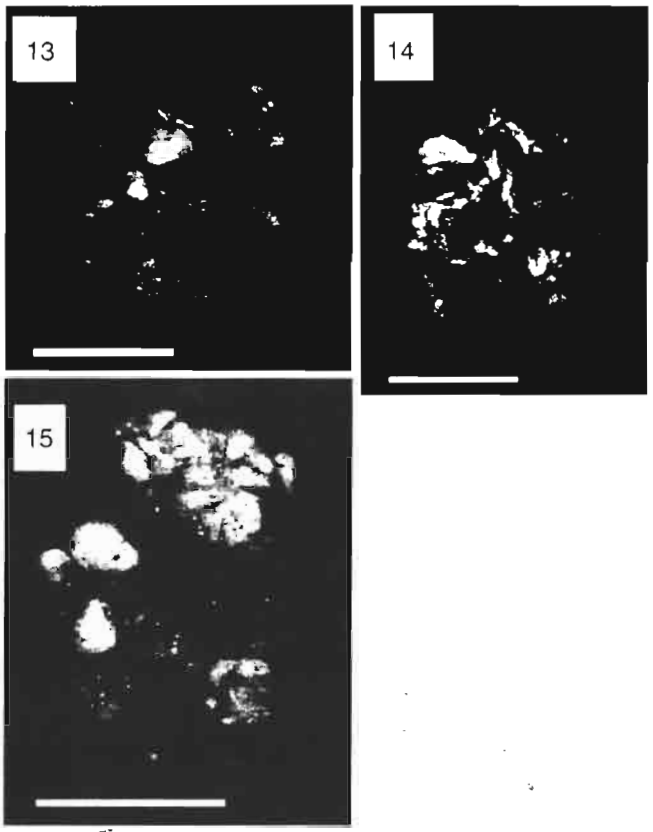
Original Protocol. The original protocol set up by ORSTOM/CIRAD (Engelmann et al. 1985) comprised the following steps: (1) production of a particular type of material, finger-shaped embryos by a 2-month culture on a medium supplemented with 0.3 M sucrose (Engelmann and Dereuddre 1988); (2) pretreatment for 7 days on a medium containing 0.75 M sucrose; (3) rapid freezing and rapid thawing; (4) recovery on media with progressively lowered sucrose concentrations and supplemented with 2,4 dichlorophenoxyacetic acid (2,4-D) for 3 weeks; (5) transfer onto standard medium devoid of growth regulators for proliferation recovery.

This process was developed on a large scale, since more than 150 different clones were frozen in five different units in Indonesia, Malaysia, the Ivory Coast, and France (Engelmann 1991b). Embryos could be stored for up to 52 months without any change in recovery rate (Engelmann 1992). Moreover, palms regenerated from cryopreserved material and planted in the field were phenotypically comparable to controls as regards their vegetative and floral development (Durand-Gasselin, pers. comm.). However, routine development of this technique was drastically slowed down by the low and erratic production of finger-shaped embryos which were at that time the only type likely to withstand freezing.

Improved Process. Dramatic improvement was made recently to the original technique by adding a dehydration step after treatment on high sucrose medium, before freezing in liquid nitrogen. Indeed, this allowed the use of standard somatic embryos (Fig. 13). Dehydration could be performed either by placing embryos for 0 to 10 h under the laminar flow, or in air-tight boxes containing silica gel for 0 to 18 h (Dumet et al. 1993).

3.2.2 *Results and Discussion*

Survival of unfrozen controls was affected by dehydration periods under the laminar flow longer than 4 or 6 h for clones A, D and B, C respectively (Table 6). However, this dehydration was indispensable in most cases to achieve survival after cryopreservation. A 6-h dehydration seemed optimal since it allowed to obtain survival with three out of the four clones tested. No survival was noted with clone B. When dehydration was performed with silica gel, survival of controls decreased from 16-h dehydration onwards (Table 7). With cryopreserved clumps, survival was sometimes higher than that of controls for the longest dehydration periods (16 to 18 h). Dehydration treatment increased the resistance of embryos to freezing in liquid nitrogen. Survival of clone B, which was previously 0%, was maximal after a 16-h dehydration. Survival of clone C was



Figs. 13–15. Oil palm

Fig. 13. Clumps of somatic embryos after 1 week pregrowth on a medium containing 0.75 M sucrose. *Bar* 5 mm. (Dumet et al. 1993)

Fig. 14. Clumps of somatic embryos after 1 week pregrowth and 16 h of dehydration with silica gel. *Bar* 5 mm. (Dumet et al. 1993)

Fig. 15. Proliferation recovery of somatic embryos 3 weeks after cryopreservation. *Bar* 1 cm. (Dumet et al. 1993)

improved, since nine clumps out of ten recovered after 18 h of dehydration. However, survival of clone D was not improved but remained high. These pretreatment conditions (16 h of dehydration with silica gel) were applied to seven additional clones. Survival rates of clumps after freezing in liquid nitrogen varied between 13 and 53% (Table 8), depending on the clone. Even though clumps of embryos seemed to be severely damaged by pregrowth and desiccation treatments (Fig. 14) proliferation recovery of clumps surviving after cryopreservation was very rapid and intense (Fig. 15). The dehydration period chosen was not optimal for all clones, since for some of them unfrozen controls had a low survival rate.

Table 6. Survival of unfrozen control (−LN) and cryopreserved (+LN) oil palm somatic embryos of four clones, as a function of the desiccation duration (DD) performed under a laminar flow cabinet. (After Dumet et al. 1993)

DD (h)	Clone A		Clone B		Clone C		Clone D	
	−LN	+LN	−LN	+LN	−LN	+LN	−LN	+LN
0	10/10	0/10	10/10	0/10	10/10	2/10	10/10	0/10
4	10/10	0/10	10/10	0/10	10/10	4/10	10/10	0/10
6	7/10	2/10	10/10	0/10	10/10	7/9	8/10	7/10
8	5/10	2/10	8/10	0/10	9/10	4/9	9/10	3/10
10	6/10	0/10	4/10	0/10	7/10	5/9	7/10*	1/9

Table 7. Survival of unfrozen control (−LN) and cryopreserved (+LN) oil palm somatic embryos of three clones, as a function of the desiccation duration (DD) using silica gel. -: not measured. (After Dumet et al. 1993)

DD (h)	Clone B		Clone C		Clone D	
	−LN	+LN	−LN	+LN	−LN	+LN
0	10/10	1/7	10/10	6/10	10/10	6/10
7	10/10	0/10	10/10	7/10	10/10	5/10
16	6/10	10/10	-	7/10	8/10	5/10
18	5/10	8/10	4/10	9/10	4/10	7/10

Table 8. Survival of unfrozen control (−LN) and cryopreserved (+LN) oil palm somatic embryos of seven clones. Pregrowth consisted of a 7-day culture on a medium containing 0.75 M sucrose followed by 16 h of dehydration with silica gel. (After Dumet et al. 1993)

	Clone						
	E	F	G	H	I	J	K
−LN	50	66	13	70	50	20	40
+LN	16	13	16	53	46	37	13

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

4 Coconut Palm (*Cocos nucifera* L.)

Coconut is widely spread in all tropical regions. Planted areas are estimated to around 10 million ha in three main culture zones: Asia-Oceania, Africa, Central and South America, and the Caribbean. It is a very important crop for many tropical countries. Even though industrial plantations exist, it is mainly cultivated by smallholders. Around 50% of the production is consumed as food and 50% used for oil. The annual world production of oil is estimated to 2.7 million tons, which represents 5.1% of the total vegetable oil (Anonymous 1991).

Coconut is a species with truly recalcitrant seeds which have no dormancy (nuts even germinate on the mother palm). There is no possibility of storing them even for short periods of time. Therefore, germplasm is conserved as field collections in national or regional centers. In vitro culture techniques have been developed for zygotic embryos which facilitate the collection and exchange of germplasm (Assay-Bah et al. 1987, 1989). Also, in vitro medium-term storage techniques are now available (Assay-Bah and Engelmann 1993) which make it possible to preserve zygotic embryos for 1 year under standard conditions with a slight viability loss.

Only a small proportion of the variability that exists within the species is currently available in existing field collections, and many sources of material are still almost unexploited (de Nucé de Lamothe 1992). The situation is all the more serious in that certain accessions are now in danger of dying out, due to the extension of the most widely grown varieties of hybrids and to urbanization. Their introduction and conservation in vitro as embryos is therefore of prior importance. There is also a need for conservation techniques because of problems and constraints linked to occurrence of diseases of coconut in different coconut-growing areas (IBPGR 1992). For example, for material originating from areas where lethal yellowing occurs, parent palms that have not shown disease symptoms should be observed for at least 1 year (the incubation period of the disease) before embryos may be released and exported to a country free of lethal yellowing. In the meantime, excised embryos need to be maintained in vitro, under slow growth or cryopreservation.

In the literature, only two papers report attempts to cryopreserve coconut embryos (Table 1). Bajaj (1984) observed callusing on transversely cut halves of embryos after a freeze-thaw cycle. With whole embryos, Chin et al. (1989) obtained callusing and normal germination of one embryo, 15 months after freezing.

4.1 Freezing Procedure

Experiments were performed with immature and mature embryos (Assy-Bah and Engelmann 1992a,b) of different varieties. Immature embryos (7–8 months after pollination) were placed for 4 h in Petri dishes on standard medium defined by Assy-Bah (1992) supplemented with 600 g/l glucose and 0 to 15% glycerol, sorbitol or polyethyleneglycol (PEG 6000). After rapid freezing and thawing, they were transferred onto standard medium for regrowth.

Table 9. Survival of control (−LN) and cryopreserved (+LN) immature coconut embryos after 7 month in culture. −pgr: no cryoprotective treatment; +pgr: pregrowth with 600 g/l glucose only; *: contaminated. (Assy-Bah and Engelmann 1992a)

			Glycerol (%)				Sorbitol (%)				PEG (%)			
	−prg	+prg	1	5	10	15	2	5	10	15	2	5	10	15
−LN	85	100	95	95	94	73	100	100	94	-	100	78	85	-
+LN	0	0	0	0	25	10	0	0	43	0	0	0	0	-

Mature embryos (11–12 months after pollination) were placed under the laminar flow for 4 h in empty Petri dishes and then transferred onto a culture medium containing 600 g/l glucose and 15% glycerol where they were kept for an additional 11 to 20 h. They were then frozen and thawed rapidly and transferred for regrowth on standard medium (Assay-Bah et al. 1989).

4.2 Results/Discussion

With immature embryos, survival of unfrozen controls after 1 month's culturing was satisfactory for all pregrowth conditions, ranging from 73 to 100% (Table 9). No difference was noted with control embryos not submitted to 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 had no cryoprotective effect at the concentrations studied. After 2.5 months culturing, 100% of control embryos germinated but many of them displayed abnormal development. With cryopreserved ones, surviving embryos increased in size but a limited number of them only could give rise to whole plantlets.

Pretreatment consisting of a desiccation which is usually employed with embryos (Engelmann 1991a) was impossible to use since immature embryos were too small for a precise control of their dehydration in air. Therefore, we used a classical pregrowth treatment on semisolid medium with cryoprotectants. As regards cryopreservation, immature embryos initially appeared as interesting material in that they were smaller and less differentiated than mature ones, and may be more likely to resist as a whole. However, the drawback of utilizing such young embryos was that the conditions for their in vitro culture are not yet perfectly mastered. Unlike mature ones, not all immature embryos could develop into whole plantlets. This considerably reduced the interest of using such material for cryopreservation for this crop.

With mature embryos, survival of controls was 100% without pretreatment (Table 10) and only slightly decreased after 19 or 24 h of pretreatment. With frozen embryos, no survival was observed without pretreatment. Survival increased with increasing pretreatment durations for PB 121, Renell Tall (RT) and Indian Tall (IT7) embryos. For 15 h of pretreatment, survival ranged between 7 (RT and IT7) and 35% (PB 121) and reached 86% (RT) to 93% (PB 121) after

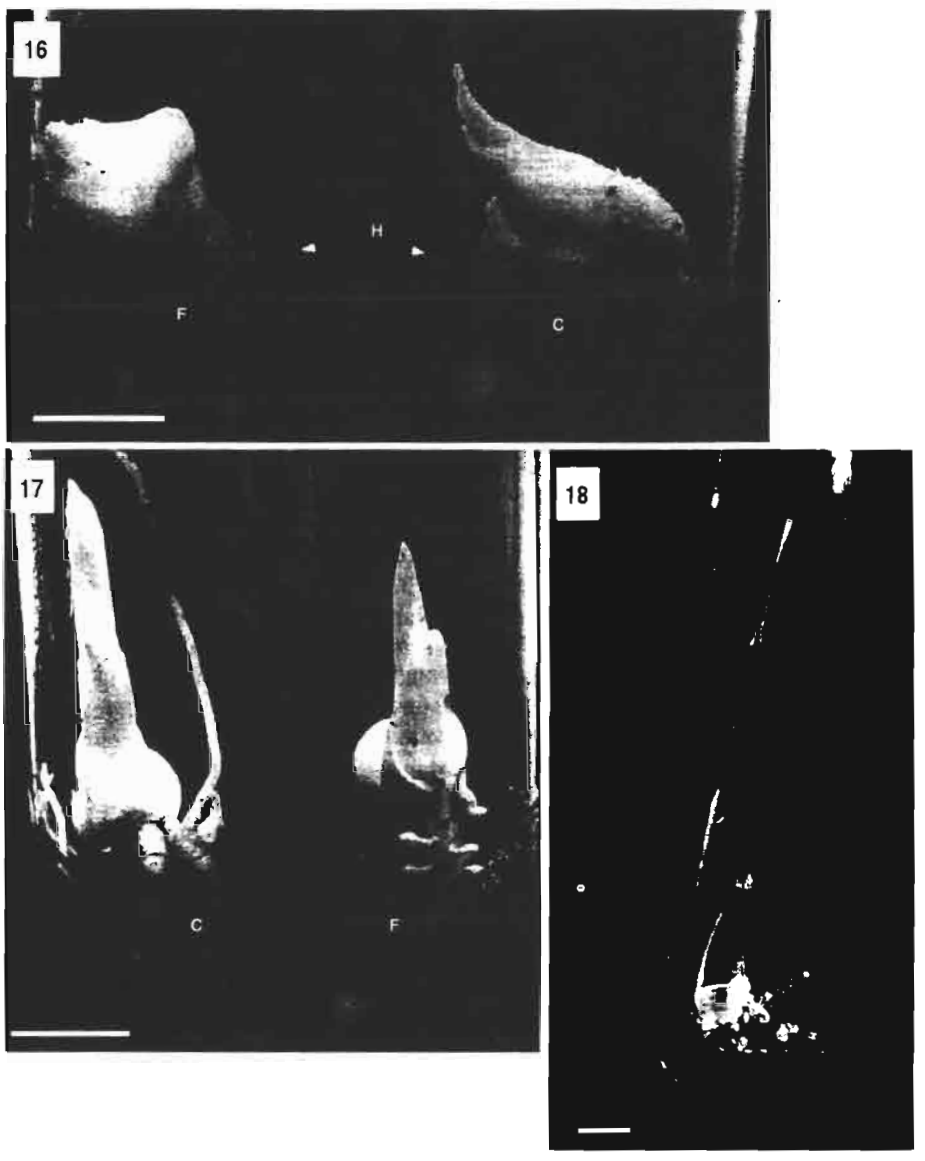
Table 10. Survival after 1 month culturing of control (−LN) and cryopreserved (+LN) mature embryos of four varieties of coconut as a function of pretreatment duration. (After Assy-Bah and Engelmann 1992)

		Pretreatment duration (h)				
		0	15	17	19	24
PB 121	−LN	100	100	100	100	87
	+LN	0	35	53	50	93
CRD	−LN	100	67	76	65	78
	+LN	0	61	73	59	56
RT	−LN	100	100	86	86	83
	+LN	0	7	12	38	86
IT7	−LN	100	100	100	82	100
	+LN	0	7	27	24	88

24h pregrowth. In the case of Cameroon Red Dwarf (CRD) embryos, survival was greatest after 17h of pretreatment (73%) and decreased afterwards. The haustorium of frozen embryos browned rapidly and did not develop further, contrarily to unfrozen controls (Fig. 16). Further development of cryopreserved embryos into plantlets was comparable to that of unfrozen controls (Fig. 17) and plantlets with fully expanded leaves could be obtained after 4.5 months culturing (Fig. 18).

The cryopreservation technique established with mature embryos was very efficient since high survival rates and rapid regrowth and development could be obtained with a large number of cryopreserved embryos. Considerable improvements have been made in comparison with previous work on the same material (Bajaj 1984; Chin et al. 1989). This technique is original since pretreatment combines desiccation of embryos in air and culture on medium with high levels of cryoprotectants. Such a combination has been employed up to now only with oil palm somatic embryos (Dumet et al. 1993). Differences observed between varieties concerning the optimal pretreatment period were related with the initial weight of embryos. For CRD, which has the smallest embryos, only 17h were necessary in comparison with 24h with other varieties which have larger embryos. The only difference in regrowth pattern between control and cryopreserved embryos concerned the nondevelopment of haustorium of frozen material. Haustorium is formed of highly vacualoted cells which are completely destroyed during freezing. This is in accordance with previous observations of Chin et al. (1988) on embryos of *Veitchia* and *Howea*.

This process is already very efficient in its present form but still has to be improved in several ways. Pretreatment duration has to be adapted to the different varieties and its conditions standardized.



Figs. 16–18. Coconut

Fig. 16. Development of control (*C*) and frozen (*F*) mature embryos after 1 month's culturing. *H* haustorium. *Bar* 1 cm. (Assy-Bah and Engelmann 1992b)

Fig. 17. Development of plantlets originating from control (*C*) and frozen (*F*) mature embryos after 3 months' culturing. *Bar* 1 cm. (Assy-Bah and Engelmann 1992b)

Fig. 18. Development of a plantlet originating from a cryopreserved mature embryo after 4.5 months' culturing. *Bar* 1 cm. (Assy-Bah and Engelmann 1992b)

5 Summary and Conclusions

Significant progress has been made for the cryopreservation of date palm, oil palm, and coconut. The present chapter illustrates the various approaches undertaken for freezing meristems, somatic or zygotic embryos. Date palm apices were frozen using a classical process involving cryoprotection in liquid medium and slow controlled freezing. Survival varied between 11.8 and 48.2%, depending on the variety. Growth recovery was generally slow and transitory callusing was often noted. Indeed, as revealed by the histological study performed, apices did not withstand freezing as a whole but only groups of cells remained alive. However, growth recovery pattern could be improved by placing apices in the dark on a medium supplemented with activated charcoal.

Standard oil palm somatic embryos could withstand rapid freezing after pregrowth on semisolid medium with high sucrose concentration and partial desiccation in air or using silica gel. Survival was generally high and proliferation recovery was very rapid and intense. Results may be improved by modifying the dehydration period which may differ between clones.

Immature coconut embryos could withstand cryopreservation, but only a limited number of them could give rise to plantlets due to their relatively low survival rate and possibly to inadequate in vitro culture conditions. On the contrary, a high percentage (73 to 93%) of mature coconut embryos survived to cryopreservation after pretreatment on semisolid medium with high concentration of glucose and glycerol followed by partial desiccation in air. All surviving cryopreserved embryos of the four varieties tested developed rapidly into whole plantlets.

In conclusion, the experiments described here show that even if the process set up for oil palm somatic embryos is now tested on a large scale and is routinely employed very soon, additional research is still needed for date palm and coconut.

Acknowledgments. Research on the development of cryopreservation techniques for oil palm and coconut is performed in the framework of a joint program between ORSTOM (Institut Français de Recherche Scientifique pour le Développement en Coopération), CIRAD/CP (Centre de Coopération Internationale en Recherche Agronomique pour le Développement/Département Cultures Pérennes), and IDEFOR/DPO (Institut des Forêts/Département Plantes Oléagineuses, Ivory Coast). The research project on coconut is supported by IBPGR (International Board for Plant Genetic Resources, Rome).

References

Al-Madeni MA, Tisserat B (1986) Survival of palm seeds under cryogenic conditions. Seed Sci Technol 14: 79–85

Anonymous (1914) Origin of the date palm. J Hered 5: 498–508

Anonymous (1989) FAO. Quartely Bulletin of Statistics, vol 2. FAO, Rome 147 pp

Anonymous (1991) Oil World Annual, ISTA Mielke GmbH, Hamburg, 108 pp

Assay-Bah B (1992) Utilisation de la culture in vitro pour la collecte et la conservation des ressources génétiques du cocotier (*Cocos nucifera* L.). Thèse Univ, Paris 6, 146 pp

Assy-Bah B, Engelmann F (1992a) Cryopreservation of immature embryos of coconut (*Cocos nucifera* L.) Cryo Lett 13: 67–74

Assy-Bah B, Engelmann F (1992b) Cryopreservation of mature embryos of coconut (*Cocos nucifera* L.) and subsequent regeneration of plantlets. Cryo Lett 13: 117–126

Assy-Bah B, Engelmann F (1993) Medium-term conservation of mature embryos of coconut (*Cocos nucifera* L.). Plant Cell Tissue Organ Cult 33: 19–24

Assy-Bah B, Durand-Gasselin T, Pannetier C (1987) Use of zygotic embryo culture to collect germplasm of cocònut (*Cocos nucifera* L.). FAO/IBPGR Plant Genet Res Newsl 71: 4–10

Assay-Bah, Durand-Gasselin T, Engelmann F, Pannetier C (1989) Culture in vitro d'embryons zygotiques de cocotier (*Cocos nucifera* L.). Méthode révisée et simplifiée d'obtention de plants de cocotiers transférables aux champs. Oléagineux 44: 515–523

Bagniol S (1992) Cryoconservation d'apex prélevés sur des vitroplants de palmier dattier (*Phoenix dactylifera* L.). Mise au point du protocole et étude histo-cytologique des échantillons au cours de la congélation et de la remise en culture. Thèse Univ. Paris 6, 125 pp

Bagniol S, Engelmann F (1991) Effects of pregrowth and freezing conditions on the resistance of meristems of date palm (*Phoenix dactylifera* L. var Bou Sthammi noir) to low temperatures and to freezing in liquid nitrogen. Cryo Lett 12: 279–286

Bagniol S, Engelmann F (1992) Effect of thawing and recovery conditions on the regrowth of meristems of date palm (*Phoenix dactylifera* L.) after cryopreservation in liquid nitrogen. Cryo Lett 13: 253–260

Bagniol S, Engelmann F, Michaux-Ferrière N (1992) Histo-cytological study of apices from in vitro plantlets of date palm (*Phoenix dactylifera* L.) during a cryopreservation process. Cryo Lett 13: 405–412

Bajaj YPS (1984) Induction of growth in frozen embryos of coconut and ovules of *Citrus*. Curr Sci 53: 1215–1216

Benson EE, Noronha-Dutra AA (1988) Chemiluminescence in cryopreserved plant tissue culture: the possible involvement of singlet oxygen in cryoinjury. Cryo Lett 7: 120–131

Benson EE, Harding K, Smith H (1989) Variation in recovery of cryopreserved shoot tips of *Solanum tuberosum* exposed to different pre- and post-freeze light regimes. Cryo Lett 10: 323–344

Branton RL, Blake J (1989) Date palm (*Phoenix dactylifera* L.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol. 5. Trees II. Springer, Berlin Heidelberg New York, pp 161–175

Carpenter JB (1979) The national date palm germplasm repository. Date Growers' Inst Rep 54: 29–32

Chin HF, Krishnapillay B, Alang ZC (1988) Cryopreservation of *Veitchia* and *Howea* palm embryos: non-development of the haustorium. Cryo Lett 9: 372–379

Chin HF, Krishnapillay B, Hor YL (1989) A note on the cryopreservation of embryos from young plants of coconuts (*Cocos nucifera* var. Mawa). Pertanika 12: 183–186

Crowe JH, Crowe LM, Carpenter JF, Rudolph AS, Wistrom CA, Spargo BJ, Anchordoguy TJ (1988) Interactions of sugars with membranes. Biochim Biophys Acta 947: 367–384

De Nucé de Lamothe M (1992) Coconut improvement – needs and opportunities. In: IBPGR (ed) Coconut genetic resources. IBPGR, Rome. Int Crop Network Ser 8: 31–34

Dereuddre J (1985) Problèmes posés par la conservation des souches cellulaires et des organes cultivés in vitro. Bull Soc Bot Fr Actual Bot 314: 123

Dumet D, Engelmann F, Chabrillange N, Duval Y (1993) Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) somatic embryos involving a desiccation step. Plant Cell Rep 12: 352–355

Duval Y, Engelmann F, Durand-Gasselin T (1995) Somatic embryogenesis in oil palm (*Elaeis guineensis* Jacq.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 30. Somatic embryogenesis and synthetic seed I. Springer, Berlin Heidelberg New York, pp 335–352

Ebert A, Taylor HF (1990) Assessment of the changes of 2,4-dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. Plant Cell Tissue Organ Cult 20: 165–172

Engelmann F (1990) Utilisation d'atmosphères à teneur en oxygène réduite pour la conservation de cultures d'embryons somatiques de palmier à huile (*Elaeis guineensis* Jacq.). CR Acad Sci Paris 310 Sér III: 679–684

Engelmann F (1991a) In vitro conservation of tropical plant germplasm – a review. Euphytica 57: 227–243

Engelmann F (1991b) Le développement actuel de la cryoconservation des embryons somatiques de palmier à huile. In: IIR (ed) Proc XVIIIth Refrigeration Congr, Montréal, 10–17 Aug, vol IV, pp 1676–1680

Engelmann F (1992) Cryopreservation of embryos. In: Dattée Y, Dumas C, Gallais A (eds) Reproductive biology and plant breeding. Springer, Berlin Heidelberg New York, pp 281–290

Engelmann F, Dereuddre J (1988) Effect du milieu de culture sur la production d'embryoïdes destinés à la cryoconservation chez le palmier à huile (*Elaeis guineensis* Jacq.). CR Acad Sci Paris 306 Sér III: 515–520

Engelmann F, Duval Y, Dereuddre J (1985) Survie et prolifération d'embryons somatiques de palmier à huile (*Elaeis guineensis* Jacq.) après congélation dans l'azote liquide. CR Acad Sci Paris 301 Sér III: 111–116

Ferry M, Louvet J, Louvet JM, Monfort S, Toutain G (1987) The specific character of the research into in vitro propagation and mass production of the date palm. Acta Hortic 212: 576

Finkle BJ, Ulrich JM, Tisserat B (1982) Responses of several lines of rice and date palm callus to freezing at −196 °C. In: Li PH, Sakai A (eds) Plant cold hardiness and freezing stress. Academic Press, New York, pp 643–660

Finkle BJ, Zavala ME, Ulrich JM (1985) Cryoprotective compounds in the viable freezing of plant tissues. In: Kartha KK (ed) Cryopreservation of plant cells and organs. CRC Press, Boca Raton, Florida, pp 75–113

IBPGR (1992) Coconut genetic resources. Papers of an IBPGR Workshop, Cipanas, Indonesia, 8–11 Oct, 1991. International Crop Network Series No 8. IBPGR, Rome, 78 pp

Grout BWW, Shelton K, Pritchard HW (1983) Orthodox behaviour of oil palm seed and cryopreservation of the excised embryo for genetic conservation. Ann Bot 52: 381–384

Hartley CWS (1988) Germination and the preparation and storage of seed. In: Hartley CWS (ed) The oil palm, 3rd edn. Tropical Agric Ser, Longman, London, pp 304–328

Kartha KK (1985) Meristem culture and germplasm preservation. In: Kartha KK (ed) Cryopreservation of plant cells and organs. CRC Press, Boca Raton, Florida, pp 115–134

Munier P (1973) Le Palmier Dattier. Maisonneuve and Larose, Paris, 221 pp

Tisserat B (1987) Palms. In: Bonga JM, Durzan DJ (eds) Cell and tissue culture in forestry. Martinus Nijhoff, Dordrecht, pp 338–356

Tisserat B, Ulrich JM, Finkle BJ (1981) Cryogenic preservation and regeneration of date palm tissue. Hortic Sci 16: 47–48

Tisserat B, Ulrich JM, Finkle BJ (1983a) Survival of *Phoenix* pollen grains under cryogenic conditions. Crop Sci 23: 254–256

Tisserat BJ, Nelson MD, Ulrich JM, Finkle BJ (1983b) Cryostorage techniques to preserve date palm germplasm. Proc 1st Symp on the Date Palm in Saudi Arabia, Al-Hassa, King Faisal Univ, March 23–25, 1982, pp 108–119

Tisserat B, Gabr MF, Sabour MT (1985) Viability of cryogenically treated date palm pollen. Date Palm J 4: 25–32

Toutain G, Rhiss A (1973) Production du palmier dattier. II. Formation de rejets sur de jeunes palmiers dattiers. Al Awamia 48: 79–89

Ulrich JM, Finkle BJ, Tisserat B (1982) Effects of cryogenic treatment on plantlet production from frozen and unfrozen date palm callus. Plant Physiol 69: 624–627

Weatherhead MA, Burdon J, Henshaw GG (1978) Some effects of activated charcoal as an additive to plant tissue culture media. Z Pflanzenphysiol 89: 141–147

Engelmann Florent, Assy-Bah B., Bagniol S., Dumet Dominique, Michaux-Ferrière N. (1995)

Cryopreservation of date palm, oil palm, and coconut

In: Bajaj Y.P.S (ed.). Cryopreservation of plant germplasm 1. Berlin: Springer, 32, 148-169. (Biotechnology in Agriculture and Forestry)

ISBN 9780387574516