CryoLetters **29(4),** 339-350 (2008)
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CRYOPRESERVATION BY ENCAPSULATION–DEHYDRATION OF PLUMULES OF COCONUT (*Cocos nucifera* L.)

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Abstract

This study describes the use of an encapsulation-dehydration cryopreservation technique on coconut plumules (apical dome with three or four leaf primordia) excised from embryos. In order to establish a reliable cryopreservation process for plumules, several different key factors were tested: pretreatment duration, sugar concentration, dehydration period and freezing. In parallel, histological studies were performed to describe the structural changes of tissues and plumule cells subjected to dehydration and freezing. A good survival level of around 60% was obtained. However, after 8 months culture regrowth, this level decreased to a maximum of 20% which was achieved using sucrose treatment. In this paper we report for the first time the regeneration of leafy shoots from coconut plumules after cryopreservation.

Keywords: *Cocos nucifera* L., cryopreservation, encapsulation-dehydration, histology, plumule, sucrose.

INTRODUCTION

Coconut is a perennial crop for which genetic resource conservation is mainly based on field collections because of its seed size (one of the largest of the plant kingdom) and physiology (no dormancy and recalcitrant to storage). However, field conservation of coconut genetic resources is faced with various problems, with losses caused by pests, diseases, and natural calamities (15). One solution could be the use of cryopreservation, which allows long term storage of material in liquid nitrogen (15, 16).

The first studies of coconut cryopreservation were reported in 1984 using whole zygotic embryos (5). To date, the most successful report in terms of post-thaw regrowth levels (30 to 90%) was that of Assy Bah and Engelmann with mature embryos (4). Such results were not the same when immature embryos were used (3). It was shown by Assy Bah and Engelmann (4), that during the cryopreservation of coconut zygotic embryos, most embryonic cells were destroyed after freezing except those of the plumule representing the apical dome and three or four leaf primordia. Plumules are very small (1 mm), with a similar size range (0.1-0.3 mm) to immature zygotic embryos. So the application of cryopreservation techniques such as encapsulation-dehydration, which is effective for small organs, could be possible also for this

tissue. However, immature zygotic embryos (about 8 mm in length) remain very hard to excise compared to mature zygotic embryos, and previous cryopreservation studies (3) gave unsuccessful results. The plumule is known to be a good explant for clonal propagation (8). It is also expected to limit the risk of some disease transmission such as lethal yellowing which can destroy many square kilometres of coconut fields in a short time (30). Lethal yellowing is caused by a phytoplasma, which is thought to be in embryos containing differentiated phloem (1). In these contexts, the plumule represents an attractive new approach to coconut cryopreservation as it has only provascular strands without differentiated phloem.

Encapsulation/dehydration cryopreservation methods are based on the embedding of plant material in alginate beads which is used to protect them from dehydration and freezing damage (17). This method has been successfully applied to several species (33, 13). Recent work focusing on coconut clonal propagation has reported that plumule cryopreservation may be a valuable method for germplasm conservation of clonal material (23). Cryopreservation by encapsulation/dehydration requires high concentrations of sugars as cryoprotectants for pretreatment before dehydration and freezing. Sugars are known to play an important role but their mode of action is not entirely understood and various theories have been put forward (12). Many sugars can be used, but notably sucrose is suggested to be one of the best cryoprotectants (37).

This study describes the use of an encapsulation/dehydration cryopreservation technique on coconut plumules using sucrose as the cryoprotectant. In order to establish a reliable cryopreservation process for coconut plumules, several important factors were tested: pretreatment duration, sugar concentration, dehydration period and mode of freezing. In parallel, histological studies were performed to characterize the structural changes in tissues and plumule cells induced by dehydration and freezing.

MATERIALS AND METHODS

Plant material

Plant material consisted of plumule tissues (shoot meristem surrounded by leaf primordia) excised from mature embryos (10 to 12 months after pollination). The nuts, from the autogamous ecotype Malayan Yellow Dwarf (MYD), were supplied by the Marc Delorme Research station in CNRA, Côte d'Ivoire, in the form of endosperm cylinders containing zygotic embryos. The extraction of endosperm cylinders was performed in Côte d'Ivoire as previously described by Assy Bah *et al* (1); following disinfection in sodium hypochlorite (8% active chlorine) for 20 min the cylinders were rinsed three times with sterile water. The cylinders were then sent by air to France. They were disinfected again with sodium hypochlorite (6% active chlorine) and rinsed in sterile water, before the extraction of embryos. Extracted embryos were disinfected for 5 min and rinsed 5 times with distilled sterile water. Plumules (1 mm^3 size) were then excised from embryos using a dissecting microscope under laminar airflow conditions, according to the process described by Chan *et al* (8).

In vitro *culture*

Plumules were cultured on a standard medium using the main conditions of culture and components described by Sandoval *et al* (32) for shoot meristems. Modifications included the incorporation of 2 g l^{-1} Morel and Wetmore vitamins (28) modified with 1 mg l^{-1} of biotin and supplemented with 1 ml FeEDTA, as Jacobson (1951) (see 21), 100 mg l^{-1} ascorbic acid and 30 mg l^{-1} adenine sulphate. This medium was supplemented with 0.12 M of sucrose instead of glucose. Plumules were cultured in 24 x 150 mm test tubes containing 20 ml of medium. They

were placed at 27°C in the dark, until the first 3-4 leaves emerged, after which they were exposed to a daily photoperiod of 12 hours with light intensity of 45µE m^2 s^{-1}.

Cryopreservation by encapsulation-dehydration

Plumules excised from mature embryos were first precultured on solid standard medium with 0.12 M sucrose for 3 days to screen for contamination before cryopreservation treatment. For encapsulation, the precultured plumules were suspended in standard medium solution containing 3% (v/v) Na-alginate and 0.15 M sucrose. The plumule-containing mixture was dispensed with a sterile pipette into 0.1 M calcium chloride ($CaCl_2$) solution containing 0.15 M sucrose at room temperature to form beads (about 3 to 4 mm in diameter), with each bead containing one plumule. Thereafter, the beads were pretreated for 2 to 3 days sequentially in standard medium (without Gelrite and activated charcoal) containing various sucrose concentrations (0.5 M, 0.75 M, 1 M). Up to 22 beads were put in each 125 ml Erlenmeyer flask containing 30 ml medium, and shaken on a rotary shaker set at 90 to 100 rpm, at room temperature. After pretreatment with sucrose, the beads were dried to remove excess pretreatment medium. They were weighed and placed to dehydrate for 6 to 24 h on sterile filter paper over 40 g silica gel in 125 ml airtight boxes. Up to 22 beads were put in each airtight box. Following dehydration, half of the beads (i.e., eleven) were transferred to standard medium. The other half were transferred into a 2 ml cryotube and immersed directly in liquid nitrogen for at least 2 h. Thawing was performed by immersing the cryotubes in a water bath at 40°C for 3 min. Each cryopreserved bead was then transferred to a test tube filled with standard medium.

Water content determination, assessment of regrowth and statistical analyses

The water content of pretreated and dehydrated beads containing plumules was determined after the different treatments by drying them in an oven at 103°C for 24 h. Three samples of five beads were each weighed at different times during the dehydration treatment and placed at 103°C. Water contents were expressed on a dry mass basis as g H_2O g^{-1} dw (shortened hereafer to g g^{-1}).

Survival of the plumules was recorded after 4 months of culture. Plumules were considered alive when they increased in height (3 to 4 mm; Fig. 1). Further development of shoots was taken as an indication of regrowth following desiccation alone or desiccation and freezing, as judged after at least 8 months (e.g. Fig. 1d). Treatments were arranged in a randomized complete block and each treatment was replicated three times. ANOVA factorial or one way ANOVA were used to determine treatment effects. When significance was indicated ($P \leq 0.05$), the least significant difference was calculated at the 5% level of probability by a Newman-Keuls test (29, 24).

Tissue processing for light microscopy

Plumules at the different steps of the cryopreservation process were collected for fixation after three days of culture. Untreated plumules (control) and plumules sampled at different steps of the cryopreservation process were fixed for three days in 10% (v/v) phosphate-buffered paraformaldehyde containing 4 ml of glutaraldehyde solution at 25% (v/v) to a final volume of 100 ml; this also contained 1% caffeine. After fixation, samples were dehydrated through an ethanol series (30, 50, 60, 70, 80, 90, 100 and 100%) for one hour each. Samples were then embedded in resin (LKB). Polymerisation was carried out for 12 hours at room temperature. Sections (3 µm thick) were obtained using a microtome with steel blades and mounted on glass slides (35). Sections were double stained with Periodic Acid Schiff (PAS) reagent combined with protein specific Naphthol Blue-Black (NBB) (20). PAS stains starch

reserves and cell walls pink and NBB specifically stains soluble or stored proteins dark blue (7).

RESULTS

Effect of different sucrose concentrations on water content of embedded plumules after dehydration

Before dehydration, pretreatment over three days with different concentrations of sucrose was undertaken. Sucrose concentrations used for pretreatment had a significant influence on water content. The water content of beads before sucrose pre-treatment was 9.47 g g^{-1} and decreased to 8.7 g g^{-1}, 3.34 g g^{-1} and 2.47 g g^{-1}, with concentrations of 0.5, 0.75 and 1 M, respectively. After dehydration (6 h or 16 h), water content decreased to mean values of 0.78, 0.56 and 0.49 g g^{-1}. No significant differences between treatments in the water content were observed for 6 h to 16 h of dehydration performed with silica gel.

Effect of sucrose concentrations on survival and regrowth of plumules after dehydration and freezing

As glucose has been reported to be a good cryoprotector for complete mature zygotic embryos (4) preliminary work was carried out using glucose or sucrose. Results obtained with glucose showed that regrowth was strongly inhibited (10% with 0.75 M) or zero for higher concentrations while the use of sucrose increased regrowth up to 27% with the same concentration (data not shown). Subsequent studies were carried out using sucrose, as described below (data presented in Table 1).

The survival (viability) of non-dehydrated plumules was 95% following treatment with culture medium containing 0.12 M sucrose (Table 1A). It decreased sharply to 29%, 55% and 50% after 6 hours of pretreatment with 0.5, 0.75 and 1 M concentrations, respectively. After freezing (+LN), the viability of plumules remained in the same range as unfrozen (-LN) plumules (20 to 40%) when 0.75 and 1 M concentrations were used for pretreatment. However, very low survival (0 to 4%) was obtained when 0.5 M sucrose was used for the pretreatment.

Significant differences in regrowth were observed between treatments (Table 1A). The regrowth of non-dehydrated plumules (control) was 85%. For unfrozen dehydrated plumules (-LN), it decreased very sharply after 6 h dehydration irrespective of the sucrose concentration: 29% when 0.75 M was used, 20% in the case of 1 M and to 0% when 0.5 M was used. The best regrowth (21 to 29%) was observed when 0.75 M was used for the pretreatment for all dehydration times and when 1 M sucrose was used (17 to 20%) for pretreatment during the first phase of dehydration (6 to 8 h). Very low regrowth was observed when 0.5 M sucrose was used for the pretreatment except when dehydration was performed over 14 h (21%).

After freezing (+LN), the best regrowth of plumules was obtained for plumules dehydrated for 14 h (21%) and 16 h (20%) when 0.75 M sucrose was used, and for 8 h dehydration (17%) in the case of 1 M sucrose. No significant difference was observed for these treatments between unfrozen and frozen plumules. The use of 0.5 M sucrose for the pretreatment led to the death of all the plumules after freezing.

Effects of pretreatment duration on plumule survival and regrowth

Survival and regrowth level of plumules were assessed after pretreatment with two concentrations of sucrose (0.75 M and 1 M) for two and three days. Data are presented in Table 1B for cryopreserved plumules. No significant difference was observed between treatments for survival and regrowth. However, after 2 days of pretreatment with 0.75 M

sucrose, the survival of plumules was reduced (20 to 17%) when dehydration was performed over 8 h and 14 h in comparison to the survival of plumules after 3 days pretreatment (31 to 33%) dehydrated for the same duration. The survival level after dehydration for 16 h was the same as that obtained after 3 days pretreatment with 0.75 M (40%). In contrast, when pretreatment was carried out over 2 days with 1 M sucrose, higher survival (50%) was observed after dehydration for 8 and 14 h. Approximately the same survival was obtained after 16 h dehydration over 2 days (27%) and 3 days pretreatment (21%).

Compared to survival, regrowth was very low (7 to 10%) when pretreatment was performed for 2 days with 0.75 M sucrose in comparison to the 3 day treatment (7 to 21%). Pretreatment with 1 M sucrose for 2 days and 8 h dehydration resulted in 23% regrowth, compared to 17% after 3 days.

The pretreatment duration giving the best result (values around 20%) varied with sucrose concentration and dehydration duration (Table 1B). In Table 2, where all data have been combined and analyzed in an ANOVA factorial, a significant difference was found between pretreatment duration for each sucrose concentration used, survival and regrowth. The use of 0.75 M sucrose gave better results after 3 days compared with 2 days, but in the light of combined data, the use of 1 M sucrose is recommended.

Whole leafy shoots mostly without roots were recovered after freezing. The different steps in development of plumule after freezing are shown in Fig. 1.

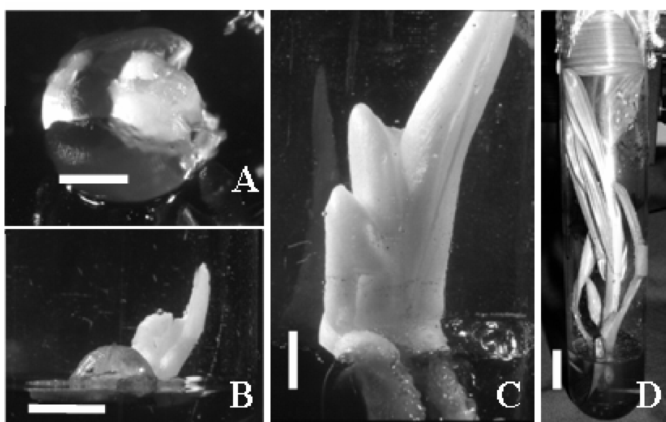


Figure 1. Different steps of plumule development after freezing.

A: encapsulated plumule by swelling (bar 2.5 mm); **B:** start of plumule germination, emergence of the first leaf from alginate bead (bar 4.5 mm); **C:** shoot development after 12 months of culture (bar 5 mm); **D:** leafy shoot after 18 months of culture, in a well developed chlorophyllous leafy shoot, after exposure to light (bar 2 cm).

Effect of dehydration duration and sucrose concentration on plumules structure

Structural analyses were performed using light microscopy. These characterize the effects of sucrose, dehydration and freezing on the structure of the plumule cells (Figs 2 and 3).

Plumules included the apical dome, which contains active meristematic cells. The apical dome is surrounded by two to four leaf primordia (Figs 2A-D). Untreated plumules (control) displayed good structural preservation and showed no sign of plasmolysis, the cytoplasm was dense with protein bodies (staining in blue by NBB), with small vacuoles and with a high nucleus-cytoplasm ratio. Meristematic cells were isodiametric, with voluminous and spherical nuclei in a central position, where heterochromatin is seen pressed against the nuclear membrane. Some mitotic figures were seen (Fig. 2D).

The effect of dehydration (for 6, 8 and 16 h) on plumule cellular structure was observed for plumules pretreated with 1 M sucrose and subjected to freezing and rewarming (Fig. 3A-F).

Table 1. **A:** Effect of sucrose concentration in pretreatment medium, on survival and regrowth of dehydrated plumules without freezing (-LN) and dehydrated and frozen (cryopreserved) (+LN) ones of coconut; **B:** Effect of pretreatment duration with sucrose, on plumule survival and regrowth after dehydration and freezing.

	Freezing	Sucrose pretreatment (days)	Sucrose concentration (M)					Sucrose concentration (M)				Sucrose concentration (M)			
			0.12 M	0.5				0.75				1.0			
				Dehydration duration (h)				Dehydration duration (h)				Dehydration duration (h)			
			Standard medium	6	8	14	16	6	8	14	16	6	8	14	16
Survival (%)	-LN	3 (**A**)	95±5	29±16	25±8	27±6	14±8	55±10	48±10	48±6	33±4	50±12	52±5	27±12	21±1
	+LN	3 (**A,B**)	-	4±4	0	4±4	0	30±12	31±5	33±42	40±6	38±9	43±9	38±4	21±1
	+LN	2 (**B**)	-	-	-	-	-	-	20	17±6	40±6	-	50±6	50±10	27±8
Regrowth (%)	-LN	3 (**A**)	85±7	0^{c}	$9±5^{c}$	$21±1^{b}$	0 c	$29±4^{a}$	$24±3^{b}$	$21±1^{b}$	$21±5^{b}$	$20±5^{b}$	$17±4^{b}$	10^{bc}	$3±3^{c}$
	+LN	3 (**A,B**)	-	0^{c}	0^{c}	0^{c}	0^{c}	$3±3^{c}$	$7±4^{bc}$	$21±5^{b}$	$20±6^{b}$	$3±3^{c}$	$17±9^{bc}$	10	0^{c}
	+LN	2 (**B**)	-	-	-	-	-	-	10±5	7±3	7±7	-	23±7	10	7±3

Encapsulated plumules were pretreated with several concentrations of sucrose in two ranges of pre-treatment duration: 3 days (0.5, 0.75 and 1 M) and 2 days (0.75 and 1 M). In the first case, the plumules were dehydrated for 6, 8, 14 and 16 h, whereas in the second case 6 h was not used. Twenty plumules were treated in each of three replicates. They were divided in two groups of ten plumules after dehydration. Ten were used for the dehydrated sample without freezing (-LN) and the other ten were frozen (+LN) after each dehydration duration. Values correspond to the means ± SEM obtained for the three replicates of (-LN) and (+LN). **A:** ANOVA values were F=0.638, P=0.837 and F=1.953, P=0.027 for survival and regrowth, respectively. **B:** In the context of pre-treatment duration (2 or 3 days), comparisons were made for 8, 14, 16 h dehydration with frozen plumules (+LN) and two sucrose molarities (0.75 and 1 M), with ANOVA values of F=1.926, P=0.158 and F=0.004, P=0.996 for survival and regrowth, respectively. Values, in the same line, when followed by the same letter are not significantly different according to a Newman and Keuls test at P< 0.05 (29, 24).

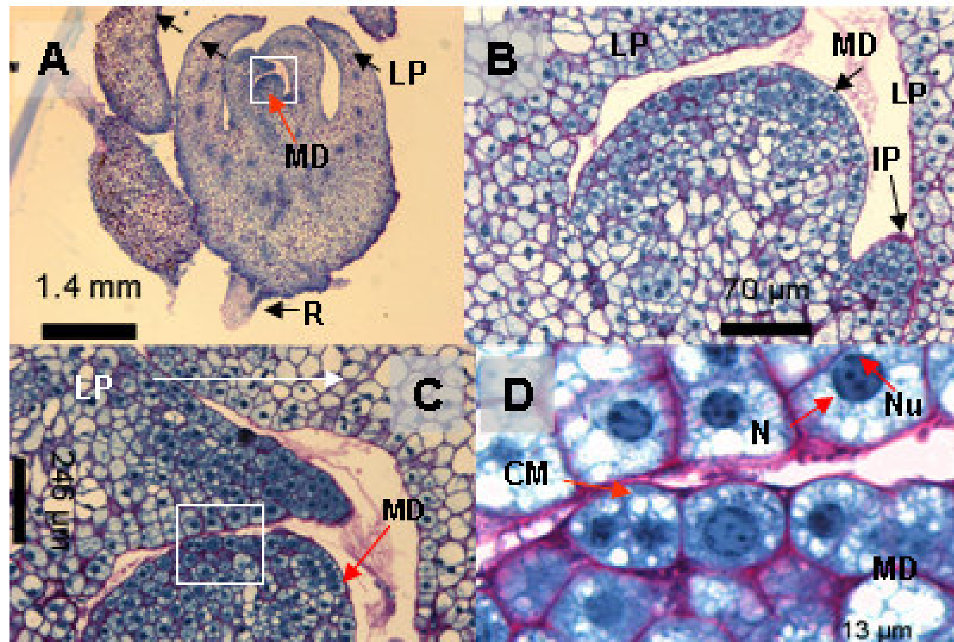


Figure 2. Histological sections of plumules used as controls as viewed by light microscopy.
A: Complete plumule with apical dome (*MD*) and leaf primordia (*LP*), with root extrusion (*R*); **B:** enlarged view shows the first cell layer of the meristem and an initium of primordia (*IP*); **C-D:** higher magnifications showing cells in mitosis (*CM*) and highly stained spherical nuclei *(N)* located in central position inside the cell compartment, with its nucleoli *(Nu)*.

Table 2 Effect of pretreatment duration with sucrose, on plumule survival and regrowth.

Sucrose concentrations (M)	Pretreatment duration (days)	Survival (%)	Regrowth (%)
0.75 M	2	33 ± 7[b]	9 ± 3[b]
	3	39 ± 3[a]	16 ± 2[a]
1 M	2	43 ± 6[a]	18 ± 4[a]
	3	34 ± 4[b]	10 ± 5[b]
		$F = 5.160$ $P = 0.028$	$F = 8.854$ $P = 0.005$

Encapsulated plumules were pretreated with two concentrations of sucrose (0.75 and 1 M) for 2 or 3 days. Twenty plumules were treated in each of three replicates. They were divided in two groups of ten plumules after dehydration. The values correspond to the means ± SE of all the treatments performed for a given concentration (dehydration and freezing); they combine all the means obtained at all dehydration durations, including frozen and unfrozen plumules. Values when followed by the same letter are not significantly different according to Newman and Keuls test at $P < 0.05$ (24, 29). Data were collected after 4 months for survival levels and after 8 months for regrowth levels.

For the shorter dehydration duration (6 h) (Fig. 3A), we observed that without LN cells of the first level of the meristematic zone showed a retracted cytoplasm with a significant periplasmic space (PPS). The cytoplasm was condensed and the nuclei shortened in size. They exhibited increased staining in blue with NBB, which corresponded to the presence of nuclear protein, suspected to correspond to condensed chromatin and more precisely histones. After freezing and rewarming, the nucleus was observed to recover its shape for cells belonging to the first layer (Fig. 3B), whereas the underlying layers showed a high plasmolysis with PPS and high staining nuclei.

For the intermediate dehydration duration (8 h), we observed without LN (Fig. 3C) the same type of cellular structure as in control. After freezing and rewarming, cells display a retracted cytoplasm and nucleus. The presence of starch grains coloured in pink was also noted (Fig. 3D).

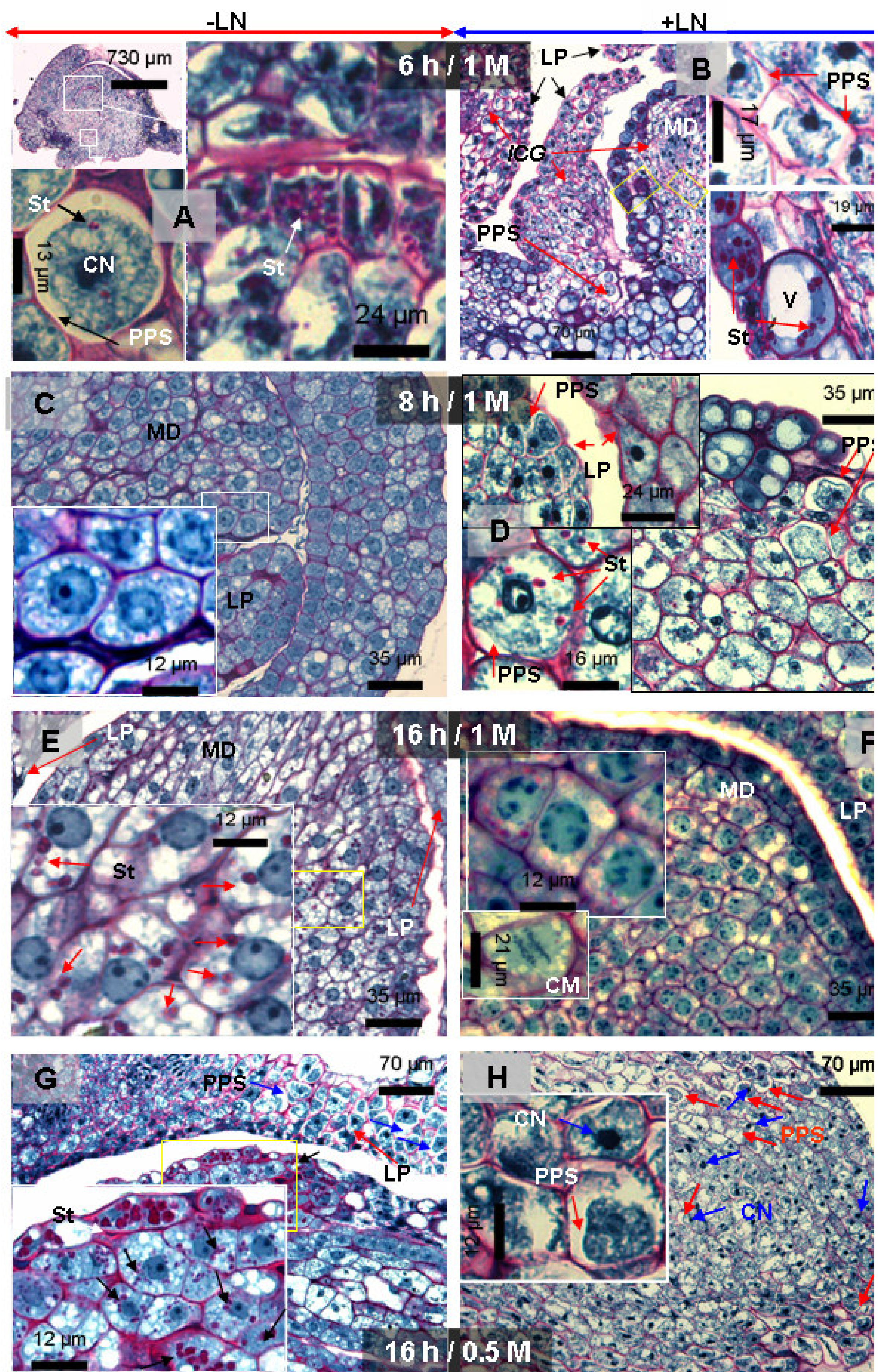
When long dehydration durations (16 h) were used, we observed an increase of starch storage in both conditions -LN (Fig. 3E) and +LN (Fig. 3F). This phenomenon was stronger in -LN conditions. We observed that in both conditions, cells appeared the same as in the control, i.e. with isodiametric shape, spherical nuclei in a central position with heterochromatin appressed against the nuclear membrane, and with cytoplasm with a high nucleus-cytoplasm ratio. Even if the cytoplasm appeared to contain fewer protein bodies, we observed some division figures (Fig. 3F).

The effect of increasing sucrose concentration was observed on plumules along three sucrose concentrations, 0.5 M, 0.75 M (view of histological section not shown) and 1 M, before and after freezing and rewarming for dehydration duration of 16 h (Fig. 3G-H).

We note that there was little difference between the effects of 0.5 M, 0.75 M and 1 M concentrations in –LN condition. The parameters observed to change were the increase in starch grains from 0.5 M (Fig. 3G) to 1 M sucrose (Fig. 3E). Although we could see that with 0.5 M some cells of one of the leaf primordia showed signs of plasmolysis with marked PPS (Fig. 3G), most cells showed characteristics similar to those observed in the control (Fig. 2) and for higher sucrose concentrations (Figs. 3C, 3E). These observations make it possible to conclude that for dehydration duration of 16 h, sucrose concentrations do not have important impact on cell dehydration effects when evaluated using cytological criteria.

Conversely, with +LN condition, we see a decreasing degree of damage from 0.5 M to 1 M. Some 0.5 M cells displayed significant PPS and a certain degree of damage (nucleus completely compacted) which make further development irreversible (Fig. 3H). With 0.75 M (view of histological section not shown) it appears that, depending on the tissue in the plumule, different degrees of starch accumulation occur in cells. On greater magnification, cells still appeared mostly as in the control. For the 1 M treatment, descriptions were given previously in the paragraph on 16 h dehydration. In the context of our experiments, 1 M sucrose and 16 h dehydration led to the best results.

Figure 3. Histological sections of plumules after dehydration and freezing, in light microscopy. Dh (Dehydration), Su (Sucrose). **A-B:** 6 h Dh and 1 M Su. **A (-LN):** cells had some St and were in plasmolysis with a significant PPS between membrane and cell wall. **B (+LN):** cells with PPS increased in number, whereas important areas, mostly in the LP, were in an ICG with lysis of the cell; some St were observed. **C-D:** 8 h Dh and 1 M Su. **C (-LN):** characteristics of cells in LP or in MD were close to those in control conditions. **D (+LN):** many cells displayed PPS, whereas St were often observed. **E-F:** 16 h Dh and 1 M Su. Cells were in a similar condition for **(-LN; E)** and **(+LN; F)**, and CM were observed after **(+LN)**. **G-H:** 16 h Dh and 0.5 M Su**. G (-LN):** lots of starch grains (arrows) evident. **H (+LN):** CN (blue arrows) and PPS (red arrows) evident**. Key:** CM: cell in mitosis; CN: compact nucleus; ICG: irreversible cell growth with lysis of the cells; LP: leaf primordia; MD: apical meristem dome; N: nucleus; PPS: periplasmic space; St: starch grains; V: vacuole.


-LN
+LN
730 µm
6 h / 1 M
LP
B
PPS
MD
ICG
17 µm
St
A
CN
13 µm
PPS
St
24 µm
PPS
70 µm
19 µm
St
V
C
MD
8 h / 1 M
PPS
LP
24 µm
35 µm
PPS
D
St
LP
12 µm
35 µm
PPS
16 µm
E
LP
MD
16 h / 1 M
F
12 µm
St
MD
LP
LP
12 µm
21 µm
CM
35 µm
G
PPS
70 µm
H
70 µm
CN
PPS
LP
PPS
St
CN
12 µm
16 h / 0.5 M

DISCUSSION

This is the first report of coconut plumule cryopreservation allowing the recovery of leafy shoots after liquid nitrogen immersion without callus formation. This result is important because this is a novel organ for cryopreservation, the only shoot meristem of a monocotyledonous woody perennial species. It raises possibilities for the conservation of coconut germplasm allowing the maintenance of genetic diversity through a coconut germplasm cryobank. Because of the small size and the phloem-free nature of plumule tissues, it can be expected to be disease-free, so facilitating germplasm exchange.

The first work on coconut plumule cryopreservation was reported in 2001 (23). The latter authors obtained embryogenic callus after cryopreservation and post culture in media containing growth regulators such as 2,4 D. This embryogenic callus represented the basis for cloning of elite coconut genotypes. However, as plumules are excised from zygotic embryos, they can only produce clones with unknown performance. This handicap can be overcome using embryos obtained by controlled pollination of selected parents which could allow production of clones showing many of the desired characteristics of the parent palms (8).

The aim of our studies was to show that genetic conservation was feasible through plumule cryopreservation, and we succeeded with 20% leafy shoot development from cryopreserved explants, avoiding the callus pathway. In our study, no growth regulator was added in the medium. Callus formation is less desirable since somaclonal variation may constitute a real risk to genetic integrity (25), also direct regeneration without callogenesis is much quicker.

The first work on coconut cryopreservation was initiated with whole zygotic embryos by Chin and Krishnapilly (10) and Bajaj (5). But recovery was obtained only eight years later by Assy Bah and Engelmann (4), with a level of 30 to 90% completely rooted plantlets, and the observation that the number of rooted plantlets was higher than that of unfrozen controls for three of the four varieties.

Achieving 20% regrowth of plumules after dehydration and freezing in liquid nitrogen is very satisfactory for a recalcitrant seed such as coconut where the zygotic embryo is maintained in liquid endosperm environment throughout its development. Moreover, this result can be considered as good in the context of genetic resource conservation (14). In this connection, with 20% estimated survival from 50 plumules we need to cryopreserve 30 plumules to regenerate at least one plant. These results represent a new basis for coconut tissue conservation in liquid nitrogen. Whole leafy shoots were obtained after 8 months of culture of coconut plumules after freezing. Roots were sometimes observed but, compared to zygotic (1) or somatic (34) embryo-generated plants, the root system developed is considered to be insufficient to ensure the survival of plants. In our case, we focused more on the regrowth of plumules under leafy shoots, as the rooting step was already mastered. Different studies have been carried out on this aspect and processes are available. Assy-Bah *et al* (1, 2) applied their rooting process for zygotic embryos and concluded that the excision of the haustorium part and supply of 60 g l^{-1} sucrose was sufficient to increase the rooting level of zygotic embryos from 70 to 90%. From zygotic embryos it was possible to master this step with the addition of indoleacetic acid (IAA) and also to optimize the formation of secondary roots by culturing on medium containing 60 g l^{-1} glucose (18). In the case of shoots regenerated from somatic embryos induced on plumules, rooting induction was obtained by pulse treatment of IAA and resulted in successful rhizogenesis in more than 90% of the treated shoots (19).

As for recalcitrant material in general, the dehydration of the plumule tissues was the most difficult step of cryopreservation (9, 16). After dehydration for 6 h to 16 h, a better frequency of regrowth (29%) was obtained, whereas after freezing regrowth reached only

20%. The present work has shown that for coconut plumule cryopreservation, two concentrations of sucrose can be used. When high sucrose concentration (1 M) is used, the pretreatment duration has to be reduced (2 days) followed by 8 h dehydration and when an intermediate concentration (0.75 M) is used, the pretreatment can be carried out for 3 days with 16 h dehydration. Water content is important, but can apparently not be dissociated from the other parameters (31). So it is not possible to identify a specific water content corresponding to desiccation damage. This depends on many factors such as the material used (36), the cryoprotectant and its concentration, the duration of pretreatment and the mode of further dehydration.

Several published works refer to histology studies, using light microscopy, to explain or characterise the impact of cryopreservation treatments on cells (6, 22, 26, 38). Light microscopy provides a better understanding of the effects of dehydration and freezing (6, 22). The observed change in the morphology of the nucleus, which became compacted and deeply stained, suggests that chromatin remains condensed and probably no transcription or protein synthesis for mitosis would be possible (6, 27). The cells which remained alive appeared to be grouped at the periphery of the meristem dome (22). Moreover, we observed in our sections that living cells were more extensive in the plumular tissues, and at different cell layer levels in the dome, even with long dehydration durations (up to 16 h) and high sucrose concentration up to 1 M.

Our histological studies have shown that dehydration duration, for the optimal sucrose concentration, have a stronger effect on cells than with increasing sucrose concentrations at the optimal dehydration duration. We have shown that using optimal conditions (1 M and 16 h), dividing cells could be observed after freezing (+LN), in accordance with observations made of morphogenetic development.

The slow development of the plumular tissues into shoots after the cryopreservation treatments could be due to different pockets of cells present in the meristem at the dome or in the leaf primordia that survive the freezing process (38), but these events were observed more frequently in untreated samples in our case.

Despite the fact that we observed cells in division after 16 h dehydration, this step remains the most difficult of plumule cryopreservation, and much work needs to be done to increase plumule regrowth. Regarding whole zygotic embryos, recent work using *in situ* PCR (11) has revealed that they may contain the phytoplasma of lethal yellowing disease. In view of its higher plant recovery frequency (4), the present work illustrates the potential of using plumules in a new and complementary approach to coconut germplasm conservation by cryopreservation.

Acknowledgements: The authors thank Dr. F. Bonnot for help with the statistical analysis. We gratefully acknowledge financial support from BRG (Bureau des Ressources Génétiques, Paris, France) and IRD for an IRD-DSF grant to O. N'Nan. The histological studies were carried out in part in the cell imaging laboratory at the Montpellier Rio-Imaging site. We thank Dr. J. Tregear for critical reading of the manuscript.

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Accepted for publication 21/3/08