

Full Length Research Paper

Use of plumules cryopreservation to save coconut germplasm in areas infected by lethal yellowing

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Plumules excised from zygotic embryos through the largest representative diversity of four of the five different areas of coconut cash and food crops were used in a cryopreservation process using encapsulation-dehydration technique. Five accessions of coconut trees were used [Panama Tall (PNT/GPA), Brazilian Green Tall (BGD/NVB), Cameroon Red Dwarf (CRD/NRC), Vanuatu Tall (VTT/VNT/GVT), and Tagnanan Tall (TAGT/GTN)] in addition to the accession model [Malayan Yellow Dwarf (MYD)] from which an optimal protocol was obtained. A great variability of response was observed depending on accessions with survival and growth recovery rates varying from 6 to 66% and 0 to 24% after 2 and 7 months of culture, respectively.

Key words: Coconut, accessions, germplasm, plumules, cryopreservation, encapsulation-dehydration.

INTRODUCTION

Coconut germplasm is subjected to an increasing genetic erosion based on its particular germplasm conservation. Its mode of conservation based on field collections, because of the characteristics of the seed (no dormancy and recalcitrant to storage), exposes collections to climatic adversity, pest and diseases. Among coconut diseases, lethal yellowing (LY) is actually the most dreadful (Dollet, 1999). It has devastated hundreds of thousand hectares throughout the world (Africa, Latin-America, and Caribbean). This disease is caused by the phytoplasma's presence in the phloem. The use of

zygotic embryo for exchanges and conservation of germplasm can be tricky because their tissues contain differentiated vascular system in which the pathogen can be maintained (Harrison et al., 1995; Cordova et al., 2003). In this context, the use of plumule, composed of the apical dome with three or four leaf primordia excised from coconut zygotic embryo, was presented as an attractive approach to coconut cryopreservation as it has only provascular strands without differentiated phloem (N'Nan et al., 2008). International germplasm exchange amplification between countries belonging to Cogent

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Table 1. List of coconut accessions used by culture area (French and English appellation).

“Tall” Accessions	Culture areas	“Dwarf” Accessions	Culture areas
Grand du Vanuatu (GVT) / Vanuatu Tall (VTT/VNT)	South Pacific, Vanuatu	Nain Rouge Cameroun (NRC) / Cameroon Red Dwarf (CRD)	Africa, Cameroon
Grand Panama (GPA) / Panama Tall (PNT)	Latin-America, Panama, Pacifique coast	Nain Vert Brésil (NVB) / Brazilian Green Dwarf (BGD)	Latin-America, Brazil
Grand Tagnanan (GTN) / Tagnanan Tall (TAGT)	South-East Asia, Philippines	Nain Jaune Malais (NJM) / Malayan Yellow Dwarf (MYD)	South-East Asia, Malaysia

(Coconut germplasm network) and laboratories from the South imply the use of disease-free planting material (Morel and Martin, 1952). The plumule is therefore expected to limit the risk of some disease transmission such as lethal yellowing (Frison et al., 1993; Malaurie, 2001; Hocher et al., 2004). The use of plumule as germplasm exchange material and its cryopreservation are an effective approach to preserve disease-free planting material, particularly when the material comes from infected areas

The recent works done by N'Nan et al. (2008) showed that cryopreservation of coconut plumules is possible after freezing in liquid nitrogen at -196°C . This article evaluates the plumule cryoconservation of «Talls» and «Dwarfs» coconut accessions. These accessions in addition to the Malayan Yellow Dwarf (MYD) are common in coconut culture areas.

MATERIALS AND METHODS

Plant material

Plumules tissues (caulinary meristem surrounded by three to four leaf primordia) were excised from mature zygotic embryos (10 to 12 months after pollination). The nuts in the form of endosperm cylinders or albumen cores were supplied by the Marc Delorme Research station of CNRA, Côte d'Ivoire. Six accessions were used (Table 1): three accessions “Dwarf” (Malayan Yellow Dwarf (MYD), Brazil Green Dwarf (BGD), and Cameroon Red Dwarf (CRD)), and three accessions “Tall” (Panama Tall (PNT), Vanuatu Tall (VTT), and Tagnanan Tall (TAGT)).

Plant material extraction, disinfection and conditioning

The material originated from Côte d'Ivoire in the form of endosperm cylinders containing embryos. The extraction and disinfection of endosperm cylinders before dispatching was performed in Côte d'Ivoire as previously described by Assy Bah et al. (1987). For their conditioning before mail invoice, rinsed endosperm cylinders were transferred in cleaned, disinfected, plastic bags. They are then packaged in small plastic bags of 10 and sealed in a bigger plastic bag up to 100. Each transportation mailing may concern up to 4 big plastic bags, put in a polystyrene box filled with several plastic bags containing frozen water. The material was kept as long as possible under refrigeration before leaving it to the mailing post service companies (preferably DHL). After receiving the endosperm cylinders, their disinfection anew, the extraction of the embryo contained in these endosperm cylinders and the excision of

plumules from embryos were carried out following the protocol described by Malaurie et al. (2006) and N'Nan et al. (2008).

In vitro culture (medium and culture conditions)

The medium and the culture conditions applied to these accessions have been previously described by N'Nan et al. (2008). After different step of cryopreservation plumules were placed at 27°C in the dark, until the first 3 to 4 leaves emerged. Then, they were exposed to a daily photoperiod of 12 h with light intensity of $45\mu\text{E m}^{-2}\text{s}^{-1}$.

Cryopreservation (encapsulation, pre-treatment-dehydration and freezing)

For encapsulation, the plumules were suspended in standard medium solution containing 3% (v/v) Na-alginate and 0.15 M sucrose. The plumules-containing mixture was dispensed with a sterile pipette into 0.1 M calcium chloride (CaCl_2) solution containing 0.15M 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 two sucrose concentrations (0.75 M and 1 M). Up to 20 beads for each accession 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 placed to dehydrate for 8 h (1 M) or 16 h (0.75 M) on sterile filter paper over 40 g silica gel in 125 ml airtight boxes. Up to 20 beads were put in each airtight box. Following dehydration, half of the beads (ten) 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. Three replicates of each treatment have been done.

Evaluation of survival and recovery

The effect of pre-treatment and freezing on the plumule are evaluated by determining the percentage of survival and the percentage of recovery. Plumules were considered alive when they increased in size from 1 mm to about 3 mm and more after 1 to 3 months. Recovery is considered normal if plumules grew and produced shoots and leaves after at least eight months. Dehydrated and unfrozen (pre-treated) material is noted $-\text{LN}$ and the dehydrated and frozen material is noted $+\text{LN}$. Unfrozen material allowed seeing the effect of dehydration which is the most difficult step for recalcitrant seeds.

Table 2. Survival and recovery rate of plumules from different accessions in function of pretreatment and dehydration duration.

Pretreatment (d / M / h)	Accessions												
	MYD		BGD		CRD		PNT		VTT		TAGT		
	PT1	PT2	PT1	PT2	PT1	PT2	PT1	PT2	PT1	PT2	PT1	PT2	
Survival (%) $F=0.5809$; $P=0.799$													
Freezing	LN -	60 ± 15.3	32.8 ± 3.6	21 ± 9	31.1 ± 8.9	84.5 ± 4.4	61.1 ± 5.5	60	75 ± 25	0	36.3 ± 23.8	18.3 ± 4	0
	LN +	50 ± 5.8	40 ± 5.8	5.6 ± 5.5	0	67.5 ± 7.5	48.8 ± 12.3	12.5 ± 12.5	50 ± 20	0	25 ± 5	5 ± 5	0
Recovery (%) $F=0.4858$; $P=0.7439$													
Freezing	LN -	30 ± 9.5	21.4 ± 4.6	0	0	52.8 ± 2.8	10.6 ± 0.6	35 ± 5	30 ± 20	0	0	5.6 ± 5.5	0
	LN +	23.3 ± 6.5	20 ± 5.8	0	0	36.3 ± 26.3	10 ± 10	0	20 ± 10	0	0	5 ± 5	0

Malayan Yellow Dwarf (MYD), Brazilian Green Tall, Cameroon Red Dwarf (CRD), Panama Tall, Vanuatu Tall, Tagnanan Tall. The given values correspond to the mean of obtained measures after 3 replicates. Survival and recovery rate are measured from 2 months and 5 months of culture, respectively. One ANOVA factorial was used for the analysis. When the test is significant, $P < 0.05$, as the test was not significant, none classification was done. *PT1*: 2 days with 1 M and 8 h dehydration; *PT2*: 3 days with 0.75 M and 16 h dehydration; *LN-*: unfrozen plumules; *LN+*: frozen plumules, d / M / h: days/concentration of sucrose/hours.

Statistical analyses

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 level of 5% by Newman-Keuls test (Newman, 1939; Keuls, 1952).

RESULTS

The effects of two treatments (3 days with 0.75 M sucrose/ 16 h dehydration, and 2 days with 1 M sucrose/ 8 h dehydration) are indicated in Table 2. Although these results do not show significant differences, some observations can be pointed out.

The survival and growth recovery rates vary in function of accessions and treatment used. With Panama Tall (PNT), the plumules pre-treatment with 0.75 M sucrose followed by 16 h dehydration seems to allow more interesting results for

dehydrated unfrozen (–LN) and dehydrated frozen (+LN) plumules with 75 and 50% survival rates, respectively. The growth recovery rate, as far as it is concerned, is of 30% for (–LN) and 20% for (+LN). More interesting results were obtained with Cameroon Red Dwarf (CRD), when pre-treatment is done with 1 M sucrose followed by 8 h dehydration. For this accession, survival rates reached 84.5 and 67.5% for (–LN) and (+LN), respectively; this was also observed for the growth recovery with 52.8 and 36.3 % for (–LN) and (+LN), respectively. With this accession, higher survival and growth recovery rates have been obtained than previously observed with the MYD accession. The 3 other accessions react to the 2 treatments with survival and growth recovery rates, lowest than those observed with MYD. No survival and growth recovery were obtained with VTT plumules when treatment is done with 1 M sucrose. When pre-treatment is done with 0.75 M sucrose, the plumule survival rate vary from 25 to 35% for (–LN) and (+LN) plumules, respectively.

On the other hand, no growth recovery was observed. Similar results were obtained with Brazil Red Dwarf (BRD). With regards to the survival response of this accession, it still remains low overall for frozen plumules (0% for 16 h and 5.56% for 8 h). For Tagnanan Tall (TAGT), no survival and growth recovery was observed when 0.75 M sucrose concentration is used for pretreatment followed by 16 h dehydration. Very low survival and growth recovery rates of about 5% were obtained when 1 M sucrose is used.

Highly significant differences ($P < 0.001$) were obtained between accessions when factor accession is only considered (Table 3). On the level of survival rate, the CRD is the accession which presents the best rate, followed by the PNT. In other group, the BGD, TAGT, and VTT accessions present lower survival rates ($P = P < 0.001$). A quite equivalent distribution is observed for the growth recovery rate, except for CRD and PNT which have the highest growth recovery rates.

Table 3. survival and recovery rate of plumules in function of accessions.

Accessions	Survival (%)	Recovery (%)
MYD	40.7 ± 7.6 ^b	23.7 ± 6.6 ^a
PNT	49.4 ± 10.8 ^b	21.3 ± 6.6 ^a
BGD	14.4 ± 5.3 ^c	0 ^b
CRD	65.5 ± 5.6 ^a	27.4 ± 8.6 ^a
VVT	15.3 ± 7.5 ^c	0 ^b
TAGT	5.8 ± 3 ^c	2.6 ± 1.7 ^b
	<i>F</i> = 23.9407	<i>F</i> = 9.91395
	<i>P</i> = 0.000000	<i>P</i> = 0.000137

The given values correspond to the mean of all the treatments done for a given accession. It combines the entire mean obtained at all the dehydration durations, the mean obtained with all the frozen and unfrozen plumules, knowing that all treatment are the mean obtained over 3 replicates. ANOVA factorial was used for the analysis. Values, in the same column, when followed by the same letter are not significantly different according to a Newman and Keuls test at $P < 0.05$ Newman (1939); Keuls (1952). Legend: MYD: Malayan Yellow Dwarf; BGD: Brazilian Green Dwarf; CRD: Cameroon Red Dwarf; PNT: Panama Tall; VTT: Vanuatu Tall; TAGT: Tagnanan Tall.

DISCUSSION

Two treatments have been proposed to evaluate the plumule cryoconservation of coconut "Talls" and "Dwarfs" accessions. The first work on coconut plumule cryopreservation was reported in 2001 (Hornung et al., 2001). The authors obtained embryogenic callus after cryopreservation and post culture in media containing growth regulators such as 2,4 D. Our present work indicates that the application of these treatments to five accessions (BGD, TAGT VTT, CRD, PNT) give underwhelming results. While these treatments seem to be inappropriate to BGD, TAGT and VTT, they give good result with CRD and PNT where similar or higher growth recovery and survival rates compare to these obtained with MYD are observed. These results can be explained by the heterogeneity of the material used (seeds from open pollination), and the conditions of cryopreservation in general. Indeed cryopreservation requires a relatively large number of materials and a lot of repetitions that cannot be performed for materials such as coconut (slow development, difficulty in plumules excision). As for recalcitrant material in general, the dehydration of the plumule tissues is the most difficult step of cryopreservation (Chandel et al., 1995; N'nan et al., 2008; 2012). Freezing cannot be done without dehydration and dehydration of recalcitrant material without any protection (pre-treatment) leads to a loss of viability (N'nan et al. 2008). Pre-treatment is essential for allowing the material to withstand water loss (Ref). In this study, the use of two sucrose concentrations at different times (pre-treatment) help the plumules of some accessions to support dehydration and freezing.

A high concentration of sugar which causes rapid dehydration by osmotic dehydration must be performed for a short time inversely. Indeed further dehydration

cause the loss of bound water and damage of the material. Sugar used played a cryoprotectant role to offset the loss of water (free water) essential for freezing. In this study, no survival was obtained with slow freezing. This confirms the work of Berjak et al. (2000); Dussert et al. (2001) who indicated that for recalcitrant material, rapid freezing cause water vitrification by contrast to slow freezing which resulted in the formation of ice crystals that damage cells. The lack of survival without recovery for the plumules of some accessions suggested irreversible damage caused by dehydration as shown by several authors through structural studies (Pammenter et al., 1999; N'nan et al., 2008). According to Wilkinson et al. (2003) regenerating a plant came from several areas which are located between the dome and leaf primordia. Depending on the damage to these areas after dehydration and freezing, and the origin of regeneration, the material will undergo different damages in its development.

The accessions used in this study have been demonstrated to differ genetically (Perera et al., 2000; Dasanayaka et al., 2009). Such differences may contribute to the variable recovery properties of distinct accessions. 'Dwarf' accessions, notably MYD and CRD, are more tolerant to cryopreservation than 'Tall' accessions, principally West African Tall (WAT) Sri Lnaka Tall (SLT). Tall accessions are generally considered more recalcitrant to *in vitro* culture and cryopreservation notably WAT and SLT (Assy Bah and Engelmann, 1992, N'Nan et al., 2012). Malaurie et al. (2006) with SLT obtained 9% of recovery after addition of abscisic acid (ABA), showing the recalcitrance of this accession to cryopreservation. In contrast Bandupriya et al. (2007; 2010) obtained a better recovery on the same variety testing the effect of storage and the effect of the concentration of ABA on cryopreservation (30%). While

the genotype of some accessions can tolerate the condition of cryopreservation other genotype does not. Heterogeneity of material is a limiting factor for the cryopreservation of seeds species (Pammetier and Berjak, 1999). The same observation was made with coconut zygotic embryo. Despite the availability of a protocol that gives interesting results with some accessions, others accessions seem to have lower results (N'nan et al., 2012). Cryopreservation of the plumules is a recent study, the improvement of the protocol for its adaptation to a large number of accessions is essential. The plumule is an ideal material for the conservation of germplasm through cryopreservation especially for areas affected by lethal yellowing. Although improvements are still to be done, the present treatment applied to plumules excised from embryos provided from recalcitrant seeds is encouraging and allows starting a cryobank. This could help to preserve a large portion of germplasm in affected areas.

Conclusion

This study indicates that accessions react differentially to cryopreservation process. For some accessions a complete revision of the protocol is needed, while for others, only some improvements are needed. Our results show that cryopreservation of plumules is possible. Works should be continued to define all the difficulties and to know how to resolve them.

However, the use of complete zygotic embryo is still essential in the regions that are not infected by lethal yellowing disease. As long as embryos will be suspected for the transmission of lethal yellowing disease, plumules with their caulinary meristems will still constitute an ideal plant material, free of virus, ideal for phythosanitary exchange and cryopreservation.

Conflict of interests

The author(s) have not declared any conflict of interests.

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