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Cryopreservation of zygotic embryos and kernels of oil palm (*Elaeis guineensis* Jacq.)

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Abstract

In the present study, the efficiency of two cryopreservation strategies for oil palm (*Elaeis guineensis* Jacq.) was compared. If extracted from rehydrated kernels, 65% of the embryos desiccated to around 0.3 g H_2O/g DW developed into plantlets after cryopreservation. In contrast, only 25% of embryos (0.12 g H_2O/g DW) extracted from cryopreserved dry kernels developed into plantlets. However, this value was increased to 63% if kernels were partially rehydrated before freezing until the water content of embryos reached 0.3 g H_2O/g DW. This study emphasizes the importance of partial rehydration of oil palm embryos before cryopreservation.

Keywords: Cryopreservation; desiccation; oil palm; rehydration; kernels; zygotic embryos.

Introduction

As regards conservation possibilities, oil palm was classified recently as being neither recalcitrant nor orthodox, but showing an intermediate seed storage behaviour (Ellis *et al.*, 1991). Indeed, these authors showed that viability of seeds of four oil-palm cultivars could be maintained after 12 months of storage at 15°C with a 10–12% moisture content. However, long-term storage of oil-palm seeds still remains impossible using conventional techniques. Cryopreservation (liquid nitrogen, –196°C) presently represents the only long-term conservation option for this species. Cryopreservation protocols have been set up for somatic and zygotic embryos (Engelmann, 1992) and for desiccated seeds of various species (Stanwood, 1985). After the storage period, embryos are extracted from the seed and grown *in vitro*, or seeds are germinated *in vivo* as in the case of coffee (Normah and Vengadasalam, 1992).

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An efficient cryopreservation technique has been developed for oil-palm somatic embryos (Dumet *et al.*, 1993a) which is now employed routinely for the storage of large numbers of elite clones (Dumet *et al.*, 1993b). Preliminary experiments performed with zygotic embryos showed that they could withstand freezing after partial desiccation (Grout *et al.*, 1983).

The present work aimed at investigating the efficiency of two cryopreservation strategies for oil palm, i.e. cryopreservation of desiccated embryos excised from rehydrated kernels, or cryopreservation of dry kernels followed by rehydration, excision and *in vitro* culture of the embryos. The effects of desiccation and of cryopreservation on the survival and developmental pattern of embryos were also observed.

Material and methods

Plant material

Dry seeds of hybrid oil palms (Deli × La Mé) were kindly provided by IDEFOR/DPO (Institut des Forêts/Département Plantes Oléagineuses) La Mé Station, Ivory Coast. The water content of dry kernels was 0.06 g/g dry weight (DW) and that of embryos 0.12 g H_2O/g DW.

Cryopreservation

For cryopreservation experiments using excised embryos, shells were broken and removed and kernels rehydrated in water at room temperature for 5–6 days, according to the method of Rabéchault *et al.* (1967). After rehydration, the water content of kernels was 0.29 g H_2O/g DW and that of embryos 3.06 g H_2O/g DW. Kernels were then disinfected with a solution of mercuric chloride (0.1%) for 20 min and rinsed three times with sterile water.

In experiment A, embryos were extracted from the kernels and dehydrated in the air current of a laminar flow cabinet for 0–6 h. They were then placed in sterile 2-ml polypropylene cryotubes and frozen by direct

immersion in liquid nitrogen. After a minimum of 1 h at –196°C, embryos were thawed rapidly by plunging the cryotubes for 1 min in a 40°C water-bath. Embryos were then transferred on to a modified Murashige and Skoog (1962) medium supplemented with 10 µg/l naphthalene acetic acid and 100 µg/l kinetin, solidified with 2 g/l gelrite. They were placed at 27°C in the dark until shoot emergence, then transferred to light conditions, under a photoperiod of 12 h light/12 h dark, with a light intensity of 40 µmol m^{-2} s^{-1}. Transfers on to new medium were performed monthly.

For cryopreservation experiments using kernels, seed shells were broken and removed. Kernels were frozen using a two-step method in order to avoid cracking during freezing and/or thawing, as previously observed with seeds of cassava (Marin *et al.*, 1990). Kernels were held for 30 min 1 cm over the surface liquid nitrogen contained in a Dewar vessel and then immersed in liquid nitrogen. After a minimum of 1 h at –196°C, they were rewarmed by placing them at room temperature for 1 h. Kernels were then rehydrated, embryos extracted and cultured *in vitro* as described previously.

After the different treatments, the embryos were grown in culture for 7 days and their developmental stage was noted, using the classification set up by Rabéchault (1968). Stage I, 'turgescence', corresponded to embryos which had rehydrated, increased in volume after transfer onto culture medium, thus becoming turgescent. Stage II, 'geotropic curvature', corresponded to embryos whose haustorium had started growing. At stage III, 'nail shape', the meristematic pole of embryos had started growing, reaching a subconical shape. Survival was noted after 21 days in culture and the fresh weight of living embryos was measured. Embryos were considered surviving at 21 days when any sign of regrowth was observed: normal development, development of the root pole, and/or shoot pole, and/or cotyledon, callusing, volume expansion. The development rate was estimated after 28 days by counting the number of embryos which had developed into a whole plantlet or produced a leaf (they could be subsequently submitted to a rooting treatment to give rise to a whole plant). Each treatment included control embryos, desiccated embryos and cryopreserved embryos. Twenty-one to 30 embryos were used per condition.

The water content of embryos and kernels (expressed on a dry weight basis) was determined by oven drying using two batches of 10 embryos or kernels. In experiment B, embryos were extracted from hydrated kernels and desiccated down to 0.12 g H_2O/g DW before cryopreservation, i.e. the water content of embryos measured in dry kernels. This was achieved by placing the embryos for 5.5 h in an air-tight box containing silica gel. Their survival, development and fresh weight were compared with that of embryos extracted from dry cryopreserved kernels.

In Experiment C, dry kernels were partially rehydrated for 5.25 h, until embryo water content reached 0.3 g H_2O/g DW, and frozen. After rewarming, embryos were extracted from the kernels and cultivated as described previously. Their survival, development and fresh weight were compared with that of embryos extracted from rehydrated kernels and desiccated under the laminar flow until their water content reached 0.3 g H_2O/g DW, before their cryopreservation.

Statistical analysis of the results

In Figures 1, 2 and 3, χ^2 test was used to test the effect of desiccation duration on survival and development of cryopreserved and non-cryopreserved embryos. In cases where the effect was significant, Ryan's test (1960) was used for multiple comparison of frequencies. In Figures 1, 2 and 3, frequencies followed by the same letter are not significantly different at the 0.05 probability level, as determined by Ryan's test. When the expected frequency of a treatment was lower than 3, the χ^2 test could not be used. Therefore, Fisher's exact test was applied.

In Figure 4, each point corresponds to the average value of four replicates randomly sampled in the set of surviving embryos. A two-way analysis of variance was applied to test the effects of desiccation duration, freezing and the interaction between desiccation duration and freezing on the fresh weight of embryos (Table 1). Desiccation duration and freezing were fixed-effect factors. Newman and Keuls' test was used for multiple comparison of categorial means (Newman, 1939; Keuls, 1952). In Figures 1–4, points followed by the same letter are not significantly different at the 0.05 probability level, as determined by Newman and Keuls' test. In Tables 2 and 3, Ryan's test was used for multiple comparison of frequencies for survival and development. Frequencies followed by the same letter are not significantly different at the 0.05 probability level, as determined by this test. When the expected frequency of a treatment was lower than 3, the χ^2 test could not be

Table 1. Experiment A: Effect of desiccation duration, freezing and interaction between both effects on the fresh weight of embryos

Effect		Fresh weight
Desiccation duration	F	2.73
	P	0.025
		*
Freezing	F	56.52
	P	0.000
		***
Desiccation duration-freezing interaction	F	5.92
	P	0.020
		*

Results of two-way analysis of variance. F (observed value of F-test) and *P* (observed probability) values are given for each effect (rows) studied.
*significant; ***very highly significant.

used. Therefore, Fisher's exact test was applied. Each fresh weight data corresponds to the average value of 17 (Table 2) or 22 (Table 4) replicates randomly sampled in the set of surviving embryos. A two-way analysis of variance was applied to test the effects of the desiccation treatment, freezing and the interaction between desiccation treatment and freezing on the fresh weight of embryos (Table 4). Desiccation treatment and freezing were fixed-effect factors. Newman and Keuls' test was used for multiple comparison of categorial means. In Tables 2 and 3, values followed by the same letter are not significantly different at the 0.05 probability level, as determined by Newman and Keuls' test.

Results

Experiment A

Dehydration of embryos under the laminar air flow was rapid since their water content dropped from an initial value of 3.1 g H_2O/g DW to 0.12 g H_2O/g DW within 6 h (Fig. 1). Desiccation of embryos in this way drastically reduced the rate of their subsequent development. Only embryos that were dried for less than 2 h developed to stage III after 7 days of culture (Fig. 2). When the desiccation period was 2–5 h, embryos were predominantly as stage II and after 6 h of dehydration 70% were still at stage I. Freezing in liquid nitrogen further reduced their rate of development since no embryos reached stage III and most of them were still at stage I after 7 days regardless of the desiccation period.

After 21 days in culture, survival of control embryos was between 81 and 100%, without significant effect of the dehydration duration (Fig. 1). In contrast, survival of cryopreserved embryos increased with increasing desiccation periods, from 13% without desiccation to nearly 90% after 4 h of dehydration. Survival rates of frozen embryos were not significantly different from those of unfrozen controls for desiccation periods over

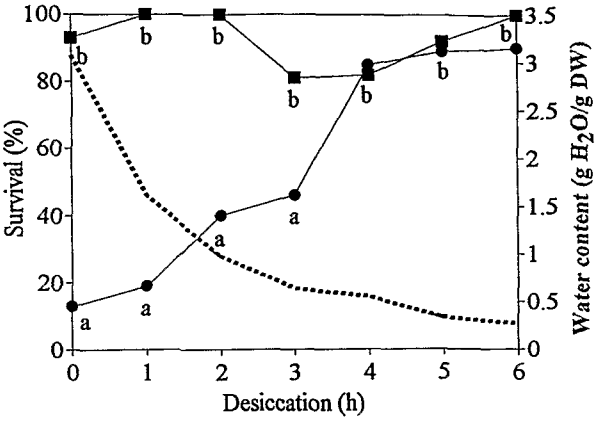

Figure 1. Effect of desiccation period on the water content (dry weight basis) (·····) and on survival rate (%) of control (■) and cryopreserved (●) oil-palm embryos extracted from rehydrated kernels. Embryos were cultured for 21 d. Points followed by the same letter are not significantly different at the 0.05 probability level as determined by Ryan's test.

3 h. Although increasing the desiccation duration had no significant effect on the survival of control embryos, it induced a progressive decrease in the percentage of embryos developing into whole plants or producing leaves, from 86% without desiccation to 55% after 6 h of dehydration (Fig. 3). After cryopreservation, though embryo survival was high the percentage of embryos able to grow and develop further was much lower, being nil or very low for dehydration durations shorter than 4 h; but it was equivalent to that of control embryos after 4–6 h of desiccation. Both desiccation duration and cryopreservation had a significant effect on the fresh weight increase of embryos (Table 1). Although the increase in fresh weight of control embryos was generally not significantly modified after extended desiccation periods, it was much lower with frozen embryos for dehydration durations shorter than 3 h (Fig. 4).

Table 2. Experiment B: Survival rates, development rates and fresh weights of isolated embryos following different desiccation and freezing treatments

Cryopreserved material	Treatment	Survival (%)	Development (%)	Fresh weight (mg)
Isolated embryos	rehydration + desiccation	–LN 92.3 ab	61.5 a	97.6 a
		+LN 96.2 a	26.9 b	42.6 b
Kernels	none	–LN 92.9 ab	85.7 c	184.7 c
		+LN 71.4 b	25.0 b	98.6 a

Embryos were removed from kernels rehydrated for 5 d and were then desiccated (5.5 h) to 0.12 g H_2O/g DW over silica gel. Some were cryopreserved with liquid nitrogen (+LN) or received no cryopreservation (–LN). Other embryos were isolated from cryopreserved (+LN) or non-cryopreserved (–LN) dry kernels (0.12 g H_2O/g DW). Frequencies followed by the same letter are not significantly different at the 0.05 probability level as determined by Ryan's test (survival and development) and Newman and Keuls' test (fresh weight).

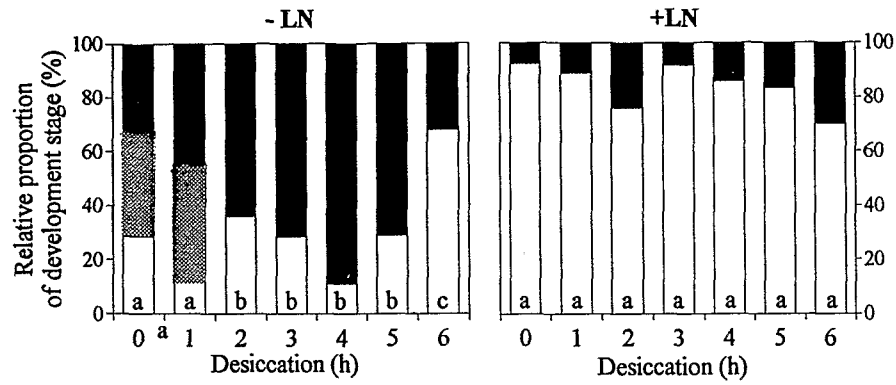

Figure 2. Effect of desiccation period on the relative proportion of control (–LN) and cryopreserved (+LN) oil palm embryos extracted from rehydrated kernels, at stage I, II and III after 7 days in culture. Stage I: □; Stage II: ▨; Stage III: ■. See text for description of stages. Columns having the same letter are not significantly different at the 0.05 probability level as determined by Ryan's test.

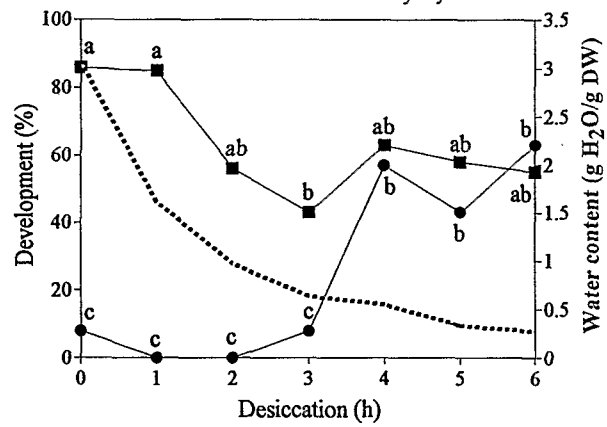

Figure 3. Effect of desiccation period on the water content (dry weight basis) (·····) and on development rate (% forming leaves or whole seedlings) of control (■) and cryopreserved (●) oil palm embryos. Points followed by the same letter are not significantly different at the 0.05 probability level as determined by Ryan's test.

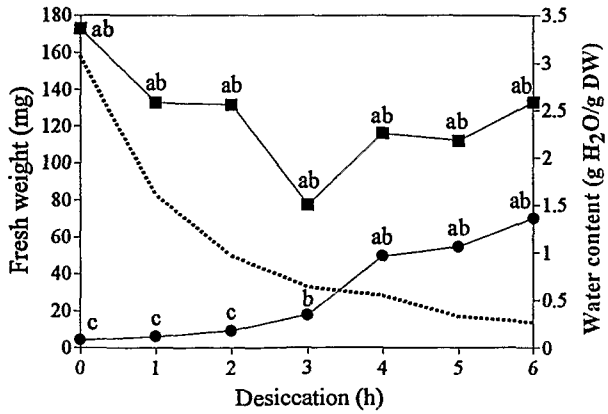

Figure 4. Effect of desiccation period on the water content (dry weight basis) (·····) and on the fresh weight increase (mg) of control (■) and cryopreserved (●) oil palm embryos after 21 days in culture. Points followed by the same letter are not significantly different at the 0.05 probability level as determined by Newman and Keuls' test.

Experiment B

Embryos isolated from rehydrated kernels were desiccated down to the level of embryos from dry kernels (0.12 g H_2O/g DW). Embryos removed from rehydrated and dry kernels displayed high survival rates after desiccation and cryopreservation (Table 2). Freezing had a significant effect on the further growth and development and fresh weight increase to a similar extent for both types of embryos (Tables 2 and 4).

Experiment C

Dry kernels were rehydrated until the water content of embryos reached 0.3 g H_2O/g DW, i.e. the optimal water content determined in Experiment A. Their survival and subsequent development rates were compared with those of embryos isolated from fully hydrated kernels desiccated down to 0.3 g H_2O/g DW. Both categories of embryos had similar survival and development rates after cryopreservation (Table 3). Desiccation had a positive effect on the development of control embryos removed from fully hydrated kernels (Tables 3 and 4).

Discussion

The present study defined the optimal conditions for the cryopreservation of oil-palm zygotic embryos, i.e. embryos should be frozen after reaching a water content of 0.3 g H_2O/g DW. Similar results were obtained with embryos isolated from fully hydrated kernels, then desiccated down to the optimal water content, or from dry kernels partially rehydrated until embryos reached this optimal value. It also demonstrated that dry, rehydrated kernels could be successfully frozen in liquid nitrogen and embryos cultivated *in vitro* after excision.

Table 3. Experiment C: Survival rates, development rates and fresh weights of embryos at a relatively high water content, with or without freezing

Treatment	Preservation	Survival (%)	Development (%)	Fresh weight (mg)
Short hydration in the kernel	–LN	82.1 a	53.6 a	61.7 a
	+LN	76.7 a	63.3 a	49.9 a
Long hydration in the kernel, then desiccation	–LN	82.1 a	64.3 a	136.7 b
	+LN	85.0 a	65.0 a	57.2 a

Embryos at a water content of 0.3 g H_2O/g DW were removed from kernels rehydrated for 5.25 h. Some were cryopreserved with liquid nitrogen (+LN) or received no cryopreservation (–LN). Other embryos were isolated from kernels hydrated for 5 d and were then desiccated in a laminar flow cabinet to 0.3 g H_2O/g DW: these were cryopreserved (+LN) or non-cryopreserved (–LN). Frequencies followed by the same letter are not significantly different at the 0.05 probability level as determined by Ryan's test (survival and development) and Newman and Keuls' test (fresh weight).

The water content of embryos ensuring the highest survival and development rates was 0.3 g H_2O/g DW, which is comparable to the optimal values generally observed when freezing embryos of various species with recalcitrant seeds (Engelmann, 1992).

Damage caused by desiccation and/or freezing in liquid nitrogen reduced the survival rate (i.e. including all forms of regrowth) and the development rate of embryos (i.e. taking in account only the embryos developing normally or producing leaves). Differences in the development of zygotic embryos after desiccation or cryopreservation have already been mentioned in the cases of *Howea, Veitchia* (Chin *et al.*, 1988) and coconut (Assy-Bah and Engelmann, 1992). However, in these latter cases, the only difference was the lack of development of the cotyledon which was destroyed by desiccation and freezing but all embryos gave rise to plantlets. With oil palm, the damage was more severe since callusing or development of root pole or haustorium were observed in some cases. A histological study performed on desiccated and cryopreserved embryos would help to characterize more precisely the extent and localization of damage. Another hypothesis could be that the recovery medium was not optimal for regrowth of cryopreserved embryos. Indeed, Abdelnour *et al.* (1992) and Normah and Vengadasalam (1992) observed that modifications of the recovery medium could greatly improve the development rate of coffee embryos after cryopreservation. An additional pregrowth treatment of embryos on a medium with high sugar concentration may also prove beneficial to increase the resistance of embryos to desiccation and freezing, as noted in the case of zygotic embryos of *Capsella bursa-pastoris* (Monnier and Leddet, 1980) and coconut (Assy-Bah and Engelmann, 1992), and somatic embryos of oil palm (Dumet *et al.*, 1993a). This latter study also emphasized the paramount importance of rehydration of oil-palm kernels and embryos in order to increase their tolerance to dehydration and cryopreservation. Indeed, imbibition very rapidly induces substantial metabolic modifications in embryos such as mobilization of stored carbohydrate and lipid reserves, and protein synthesis (Bewley and Black, 1983). Notably, the degradation of starch which is present in great quantities in oil-palm embryos (Vallade, 1965) may lead to a rapid increase in the concentration of soluble sugars which play a crucial role in the acquisition of tolerance to desiccation, by substituting for water in stabilizing membranes in the dry state (Crowe and Crowe, 1986) and/or by inducing intracellular vitrification at ambient temperature (Williams and Leopold, 1989). Complementary research will be undertaken in order to describe the metabolic changes which take place during imbibition of oil-palm kernels and embryos, and during desiccation of the embryos.

Table 4. Experiment B and C: effect of desiccation process, freezing and interaction between both effects on the fresh weight of embryos

Effect		Fresh weight Experiment B	Fresh weight Experiment C
Desiccation treatment	F	30.3	15.9
	P	0.000 ***	0.000 ***
Freezing	F	29.5	19.6
	P	0.000 ***	0.000 ***
Desiccation-freezing	F	1.5	10.8
	P	0.234 NS	0.000 ***

For desiccation treatments see Tables 2 and 3. Results of two-way analysis of variance. F (observed value of F-test) and *P* (observed probability) values are given for each effect (rows) and experiment (columns) studied. NS: non significant; ***very highly significant

In conclusion, oil palm appears a good system for studying the mechanisms of tolerance to desiccation and cryopreservation of species with recalcitrant or intermediate seed storage behaviour.

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References

Abdelnour, A., Villalobos, V. and Engelmann, F. (1992) Cryopreservation of zygotic embryos of *Coffea* spp. *Cryo Letters* **13**, 297–302.

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

Bewley, J.D. and Black, M. (1983) *Physiology and biochemistry of seeds in relation to germination. Vol. 1, Development, germination and growth.* Heidelberg, Springer Verlag.

Chin, H.F., Krishnappillay, B. and Alang, Z.C. (1988) Cryopreservation of *Veitchia* and *Howea* palm embryos: non-development of the haustorium. *Cryo Letters* **9**, 372–379.

Crowe, J.H. and Crowe, L.M. (1986) Stabilisation of membranes in anhydrobiotic organisms. pp 188–209 *in* Leopold A.E. (Ed.) *Membranes, metabolism and dry organisms.* Ithaca, New York, Comstock Publishing Associates.

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

Dumet, D., Engelmann, F., Chabrillange, N. and Duval, Y. (1993b) Development of cryopreservation for oil palm somatic embryos using an improved process. *Oléagineux* **48**, 273–278.

Ellis, R.H., Hong, T.D., Roberts, E.H. and Soetisna, U. (1991) Seed storage behaviour in *Elaeis guineensis*. *Seed Science Research* **1**, 99–104.

Engelmann, F. (1992) Cryopreservation of embryos. pp 281–290 *in* Dattée Y., Dumas, C. and Gallais, A. (Eds) *Reproductive biology and plant breeding.* Berlin, Springer Verlag.

Grout, B.W.W., Wescott, R.J. and Pritchard, H.W. (1983) Orthodox behaviour of oil palm seeds and cryopreservation of the excised embryo for genetic conservation. *Annals of Botany* **52**, 381–384.

Keuls, M. (1952) The use of studentised range in connexion with analysis of variance. *Euphytica* **1**, 112–122.

Marin M.L., Mafla, G., Roca, W.M. and Withers, L.A. (1990) Cryopreservation of cassava zygotic embryos and whole seeds in liquid nitrogen. *Cryo Letters* **11**, 257–264

Monnier, M. and Leddet, C. (1980) Action du saccharose sur la résistance au gel des embryons immatures de capselle. *Bulletin de la Société Botanique de France* **127**, 71–77.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473–497.

Newman, D. (1939) The distribution of ranges in samples from a normal distribution expressed in terms of an independent estimate of standard deviation. *Biometrika* **31**, 20–30.

Normah, M.N. and Vengadasalam, M. (1992) Effects of moisture content on cryopreservation of *Coffea* and *Vigna* seeds and embryos. *Cryo Letters* **13**, 199–208.

Rabéchault, H. (1968) Recherches sur la culture *in vitro* des embryons de palmier à huile (*Elaeis guineensis* Jacq.) I Effets de l'acide β-indolyl-acétique. *Oléagineux* **17**, 757–764.

Rabéchault, H., Guénin, G. and Ahée, J. (1967) Absorption de l'eau par les noix de palme (*Elaeis guineensis* Jacq. var. *Dura* Becc.) I. Hydratation des différentes parties de graines amenées à des teneurs globales en eau déterminées. *Cahiers ORSTOM, Série Biologie* **4**, 31–41.

Ryan, T.A. (1960) Significance tests for multiple comparison of proportions, variances and other statistics. *Psychological Bulletin*, **57**, 318–328.

Stanwood, P.C. (1985) Cryopreservation of seed germplasm for genetic conservation. pp 199–226 *in* Kartha, K.K. (Ed.) *Cryopreservation of plant cells and organs.* Boca Raton, Florida, CRC Press.

Vallade, J. (1965) Recherches morphologiques et cytologiques sur l'embryon d'*Elaeis guineensis* Jacq. quiescent et en cours de germination. *Mémoire d'Etudes Supérieures*, Université de Dijon, 71 p.

Williams, R.J. and Leopold, A.C. (1989) The glassy state in corn embryos. *Plant Physiology* **89**, 977–981.

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