Plant Cell, Tissue and Organ Culture **33**: 19–24, 1993.

Medium-term conservation of mature embryos of coconut

Béatrice Assy-Bah[1,2] & Florent Engelmann[2,*]
[1]*IDEFOR/DPO (Institut des Forêts, Département Plantes Oléagineuses), La Mé, 13 BP 989 Abidjan 13, Côte d'Ivoire*; [2]*ORSTOM (Institut Français de Recherche Scientifique pour le Développement en Coopération), Laboratoire de Ressources Génétiques et Amélioration des Plantes Tropicales, 911 av. Agropolis, BP 5045, 34032 Montpellier Cedex 01, France (* requests for offprints)*

Received 6 April 1992; accepted in revised form 29 September 1992

Key words: activated charcoal, disease control, germplasm storage, sucrose, zygotic embryos

Abstract

Optimal conditions for the medium-term conservation of zygotic embryos of coconut were developed. After 6 months of storage on a medium devoid of sucrose and containing $2\,g\,l^{-1}$ activated charcoal, 100% of the embryos developed into whole plantlets within 5 months after transfer to the recovery medium. After a 12-month storage period on medium containing $15\,g\,l^{-1}$ sucrose and devoid of activated charcoal, 51% of the embryos germinated within 2 months after transfer to the recovery medium.

Introduction

The use of *in vitro* culture for the conservation of plant genetic resources calls for different techniques, according to the required time scale of storage. For medium-term conservation (several months to 1–2 years), the aim is to reduce the growth rate so as to increase the interval between subcultures. This is achieved by modifying culture conditions, principally by lowering the temperature to 15–20°C in the case of tropical plants (Banerjee & De Langhe 1985), which are often cold sensitive. These slow growth techniques are now routinely employed for the storage of shoot cultures in many industrial laboratories as well as in regional and international germplasm conservation centers (Withers 1991).

Research on *in vitro* medium-term conservation mainly concerns shoot cultures but experiments have also been conducted with cell suspensions, calluses and embryos (Engelmann 1991). In this latter case, to our knowledge, only somatic embryos have been used and no work has been carried out yet with zygotic embryos. With somatic embryos, various techniques have been tried: encapsulation in alginate beads (Bapat & Rao 1988; Arumugam & Bhojwani 1990), partial desiccation (Gray 1989), use of controlled atmosphere in which the oxygen concentration is reduced (Engelmann 1990).

Research on the conservation of coconut genetic resources started eight years ago, in the framework of a joint program involving IBPGR (International Board for Plant Genetic Resources, Rome), IRHO (Institut de Recherche pour les Huiles et Oléagineux, Département Oléagineux du CIRAD) and ORSTOM. Routine techniques for the collection (Assy-Bah et al. 1987) and *in vitro* culture of zygotic embryos (Assy-Bah 1986; Assy-Bah et al. 1989) have been developed. Efficient cryopreservation techniques have been developed recently for immature (Assy-Bah & Engelmann 1992a) and mature embryos (Assy-Bah & Engelmann 1992b). These should allow long-term conservation of coconut genetic resources in the form of zygotic embryos. However, there is a need for a medium-term

conservation technique for embryos, because of problems and constraints linked to the occurrence of diseases of coconut in different coconut growing areas (Frison & Putter 1992). For example, for material originating from areas where lethal yellowing occurs, parent palms that have not shown disease symptoms should be observed for one year (the period of incubation of the disease), before embryos may be released and exported to a country free of coconut lethal yellowing. In the meantime, excised embryos need to be maintained *in vitro*, preferably under slow growth conditions.

In this paper, we describe the conservation of mature embryos of coconut under slow growth conditions for 6 and 12 months.

Materials and methods

Plant material

The plant material used in this study consisted of mature embryos (10–12 months after pollination) from seednuts of the variety Ghanaian Yellow Dwarf. Nuts were harvested from adult palms at the Port Bouet Research Station (IDEFOR, Ivory Coast).

Methods

Zygotic embryos were extracted from the nuts and inoculated *in vitro* according to the method of Assy-Bah et al. (1987). Embryos were cultured on the medium defined by Assy-Bah et al. (1989), containing Murashige & Skoog's (1962) macro- and microelements, Morel & Wetmore's (1951) vitamins, 41 mg l^{-1} NaFeEDTA, 100 mg l^{-1} sodium ascorbate and 8 g l^{-1} agar (Labosi AL 540). For storage experiments, treatments consisted of media with different sucrose concentrations (0, 5, 10, 15, 20, 60 g l^{-1}) and the presence or absence of activated charcoal (2 g l^{-1}, Sigma C 5386). For recovery after the storage period, embryos were transferred to the same basal medium containing 60 g l^{-1} sucrose and 2 g l^{-1} activated charcoal. Transfers were then performed monthly.

The embryos were cultured in 24 × 150 mm test tubes containing 20 ml of medium, sealed with plastic cling film. For storage experiments (6 and 12 months in storage), embryos were placed on the various media at 27 ± 1°C in the dark. For recovery after the storage period, the embryos on recovery medium were kept in the same conditions until the gemmule emerged. They were then exposed to a photoperiod of 12 h light/12 h dark at 35 μmol m^{-2} s^{-1}. Fourteen to 49 embryos per treatment were used.

During storage, all germinated embryos were eliminated. The number of living, nongerminated embryos was recorded at the end of each storage period and their fresh weight increase measured. The number of dead embryos was also recorded at the end of each storage period. Embryos which died during storage turned brown and necrosed progressively. The number of embryos that had developed a gemmule after 2 months on the recovery medium was noted. The number of plantlets sufficiently developed to be transferred *in vivo* after 5 months in culture on the recovery medium was recorded only in the case of embryos subjected to 6 months of storage.

Results

Embryos stored for 6 months

The frequency of germination during storage fell gradually from 94 to 0% in line with the reduction in sugar concentration on media containing charcoal (Table 1). All other treatments led to almost total inhibition of germination (only 0 to 6% germination) on media lacking charcoal, except with 60 g l^{-1} of sugar where half of the embryos germinated. Embryos that had not germinated increased in weight in line with the increase in sugar concentration and reached a large size at the end of the storage period only in the presence of charcoal (Fig. 1). Without charcoal, the increase in fresh weight was less marked (Fig. 2) and there were fewer differences between sucrose treatments. The death rate during storage was low (0 to 2%), except on a medium containing neither sucrose nor charcoal, where over 20% of the embryos died in storage.

Germination of embryos stored on a medium containing charcoal was satisfactory for the low-

Table 1. The effect of the presence of activated charcoal and concentration of sucrose in the medium on the germination, death, survival and fresh weight increase of embryos during a 6-month storage period and on their further development.

	With 2 g l^{-1} charcoal (sucrose g l^{-1})						Without charcoal (sucrose g l^{-1})					
	0	5	10	15	20	60	0	5	10	15	20	60
Number of embryos cultured	44	38	40	46	49	49	46	45	39	48	49	49
Germinated embryos (%)	0	10	40	73	65	93	0	0	2	4	4	40
Dead embryos (%)	0	0	2	2	2	0	21	0	0	2	0	0
Non germinated living embryos (%)	100	89	57	24	33	6	78	100	97	94	96	59
Fresh weight increase of living embryos (%)	155	221	278	311	290	331	126	93	70	107	110	170
Embryos with gemmule (2 months) (%)	77	60	42	42	20	4	30	44	36	44	47	49
Plantlets (5 months) (%)	63	63	50	24	26	4	4	49	23	29	35	51

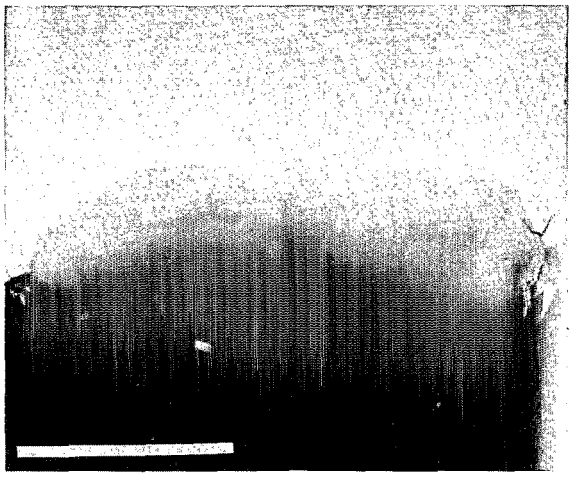

Fig. 1. Development of an embryo after a 6-month storage period on a medium containing 5 g l^{-1} sucrose and 2 g l^{-1} activated charcoal (Bar = 1 cm).

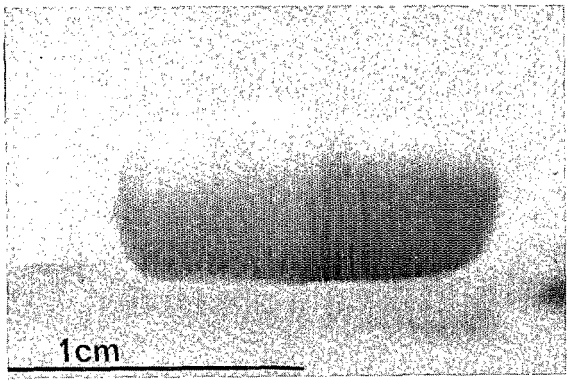

Fig. 2. Development of an embryo after a 6-month storage period on a medium containing 5 g l^{-1} sucrose, devoid of activated charcoal.

est sugar concentrations (60% with 5 g l^{-1} sucrose, 100% without sucrose) two months after transfer to the recovery medium. However, storage in the absence of charcoal led to a much lower germination rate (30 to 49%), except in the case of embryos stored on 60 g l^{-1} of sugar. Five months after transfer to the recovery medium, 63% of the embryos stored on medium with charcoal and 0 or 5 g l^{-1} sucrose gave rise to plantlets which were sufficiently developed to be transferred *in vivo* (Fig. 3). Results were lower for embryos stored under all other conditions.

Embryos stored for 12 months

The presence of activated charcoal during storage increased the germination rate of embryos subsequently transferred to the recovery medium (Table 2). Only 33 and 10%, respectively of living embryos failed to germinate after 12 months under the conditions optimal for 6 months of storage (0 and 5 g l^{-1} sucrose). Without charcoal, the number of embryos that germinated during storage was comparatively lower and decreased with decrease in the sugar concentration, from 82% with 60 g l^{-1} sucrose down to 0% with 5 g l^{-1} sucrose. Without sugar, no embryos could survive this storage period.

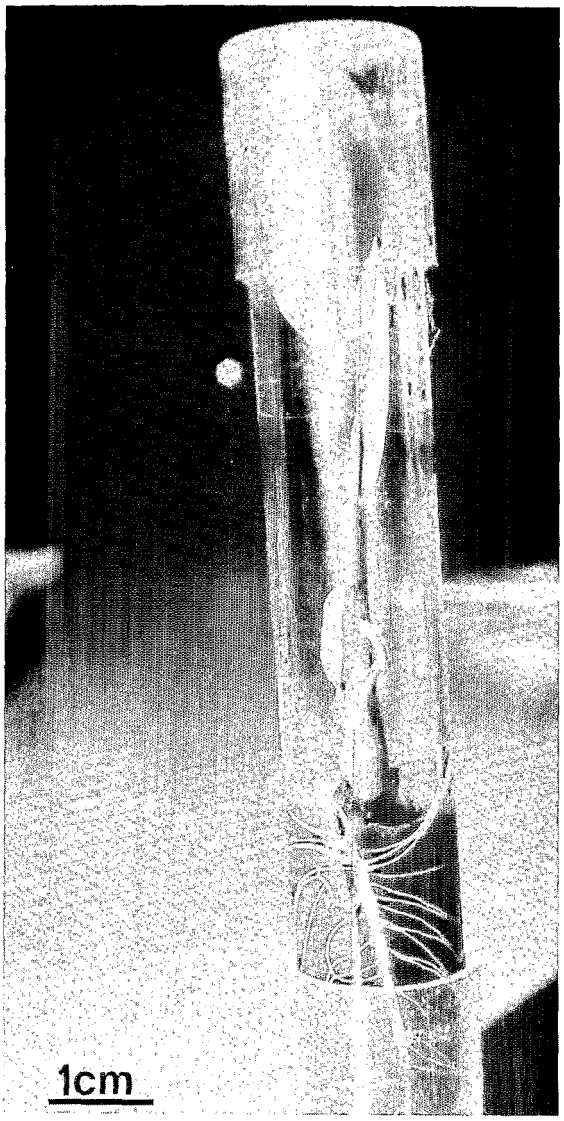

Fig. 3. Plantlet obtained from an embryo stored for 6 months on a medium devoid of sucrose and containing 2 g l^{-1} activated charcoal, after 5 months of culture on the recovery medium.

The percentage of germinated embryos was lower when storage had been performed in the presence of activated charcoal after 2 months in culture on the recovery medium. The highest percentages of germination (51 and 41%) were obtained with embryos kept on media containing 15 and 5 g l^{-1} sucrose, respectively, but no charcoal. Germination was delayed in comparison with control, non-stored embryos when embryos were kept on a charcoal-free medium. However, fully developed plantlets could finally be obtained from almost all embryos at the end of the storage period (data not shown).

Discussion

In this study, we have shown that mature embryos of one variety of coconut can germinate and give rise to fully developed plantlets after up to 12 months of storage under slow growth conditions.

Optimization of sucrose concentration and use of activated charcoal were shown to be critical to success. Sucrose is the carbon source in the medium. A previous report has shown that the optimal concentration of sucrose is 60 g l^{-1} for normal embryo development (Assy-Bah et al. 1989). When the sucrose concentration in the medium was lowered, growth decreased, leading to the death of embryos after 12 months in storage. Germination of the embryos and their further development depended greatly on the presence of activated charcoal. Growth of the embryos was increased and they germinated progressively, even with a low sucrose content and with charcoal in the storage medium. With charcoal, the results were optimal for a 6-month storage duration, but too much growth occurred over 12 months since all embryos germinated before the end of this period. On the contrary, without charcoal, growth was lowered and germination delayed for low sucrose concentrations. A large proportion of embryos remained ungerminated and alive, even during one year in storage.

Various authors have shown that activated charcoal is an essential medium additive for the germination and development of coconut embryos (De Guzman et al. 1971; Karunaratne et al. 1985; Assy-Bah et al. 1989; Rillo & Paloma 1990). Activated charcoal is widely used in tissue culture. Its beneficial effects are attributed to its property of adsorbing toxic substances produced by the tissues (Pierik 1987) as well as components of the medium, such as growth regulators (Ebert & Taylor 1990) and to the fact that it may contain elements which are necessary to the growth of the cultures (Pierik 1987). With coconut embryos, activated charcoal may adsorb

Table 2. The effect of the presence of activated charcoal and the concentration of sucrose in the culture medium on the germination, death, survival and fresh weight increase of embryos during a 12-month storage period and on their further development.

	With 2 g l^{-1} charcoal (sucrose g l^{-1})						Without charcoal (sucrose g l^{-1})					
	0	5	10	15	20	60	0	5	10	15	20	60
Number of embryos cultured	48	48	39	40	45	45	37	44	14	47	18	47
Germinated embryos (%)	29	72	87	95	84	88	0	0	14	19	38	82
Dead embryos (%)	37	16	0	5	11	0	100	0	0	0	0	0
Non germinated living embryos (%)	33	10	13	0	4	11	0	100	86	81	61	17
Fresh weight increase of living embryos (%)	99	204	190	0	288	245	0	126	131	137	124	121
Embryos with gemmule (2 months) (%)	19	10	13	0	4	11	0	41	14	51	39	4

a toxic substance produced by the embryos, which impedes their germination.

A delay in embryo development was not observed in comparing those stored in the presence of activated charcoal with non-stored control embryos. Indeed, all embryos living after 6 months in storage in the presence of charcoal developed into a whole plant within an average of 5 months after placement on the recovery medium (Assy-Bah et al. 1989) or developed a gemmule after 2 months, for embryos stored for 12 months. Without charcoal in the storage medium, germination and development were delayed. However, almost all living embryos gave rise to fully developed plantlets, with a delay of 2 to 3 months in comparison with non stored embryos.

Additional work is needed to confirm these preliminary results. From a more fundamental point of view, the exact role of activated charcoal should be more extensively studied. For wider application, the technique should be tested with other varieties of coconut. Also a two-year storage duration should be tested, which would have useful applications in indexing for diseases of the coconut, such as those induced by mycoplasma-like organisms (Frison & Putter 1992), since they have a two-year incubation period. Finally, palms originating from control and stored embryos should be observed for their genetic stability.

In conclusion, this study has shown that the medium-term storage of coconut embryos is feasible. The safe preservation and international movement of coconut germplasm may therefore be foreseen in the coming years.

Acknowledgements

The authors wish to express their thanks to the personnel of the Port Bouët Coconut Research Station (IDEFOR, Ivory Coast) for kindly supplying plant material. This experiment was conducted in the tissue culture laboratory of the La Mé Research Station (IDEFOR, Ivory Coast) under the supervision of Dr. T. Durand-Gasselin, head of the laboratory. The help of Dr. L.A. Withers (IBPGR, Rome) is kindly acknowledged for her comments on the original manuscript. This work is supported by IBPGR.

References

Arumugam N & Bhojwani SS (1990) *In vitro* propagation of *Podophyllum hexandrum* Boyle via somatic embryogenesis. In: Abst. VIIth Int. Cong. Plant Tissue and Cell Culture, Amsterdam (p 243)

Assy-Bah B (1986) Culture *in vitro* d'embryons zygotiques de cocotiers. Oléagineux 41: 321–328

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

Assy-Bah B, 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 transférables aux champs. Oléagineux 44: 515–523

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

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

Banerjee N & De Langhe EA (1985) A tissue culture technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (banana and plantain). Plant Cell Rep. 4: 351–354

Bapat VA & Rao PS (1988) Sandalwood plantlets from 'synthetic seeds'. Plant Cell Rep. 7: 434–436

De Guzman EV, Del Rosario AG & Eusebio EC (1971) The growth and development of coconut 'Makapuno' embryo *in vitro*. Resumption of growth in high sugar media. Philipp. Agri. 53: 566–579

Ebert A & Taylor HF (1990) Assessment of the changes of 2,4-dichlorophenoxyacetic acid concentration in plant tissue culture media in the presence of activated charcoal. Plant Cell Tiss. Org. 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.). C.R. Acad. Sci. Paris 310, Sér. III: 679–684

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

Frison EA & Putter CAJ (1992) FAO/IBPGR Technical Guidelines for the Safe Movement of Germplasm. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome (in press)

Gray DJ (1989) Effects of dehydration and exogenous growth regulators on dormancy, quiescence and germination of grape somatic embryos. *In Vitro* Cell Dev. Biol. 25: 1173–1178

Karunaratne S, Kurukulaarachchi C & Gamage C (1985) A report on the culture of dwarf coconut, *Cocos nucifera* L. var. *nana in vitro*. Cocos 3: 1–8

Morel G & Wetmore RH (1951) Tissue culture of monocotyledons. Am. J. Bot. 38: 138–140

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Pierik RLM (1987) *In Vitro* Culture of Higher Plants. Martinus Nijhoff, Dordrecht

Rillo EP & Paloma BF (1990) Comparison of three media formulations for *in vitro* culture of coconut embryos. Oléagineux 45: 319–323

Withers LA (1991) Tissue culture in the conservation of plant genetic resources. In: Zakri AH, Normah MN, Senawi MT & Abdul Karim AG (Eds) Conservation of plant genetic resources through *in vitro* methods (pp 1–18). Forest Research Institute Malaysia/Malaysian National Committee on Plant Genetic Resources, Kuala Lumpur, Malaysia