Status of cryopreservation research in coconut

F Engelmann[1,2], B Malaurie[3], O N'Nan[4] and M Borges[5]

[1]*Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, Cedex 5, Montpellier, France*

[2]*Honorary Research Fellow, International Plant Genetic Resources Institute (IPGRI), Via dei Tre Denari 472/a, Maccarese, Rome, Italy*

[3]*Senior Scientist, Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, Cedex 5, Montpellier, France*

[4]*Researcher, Centre National de Recherche Agronomique (CNRA), Station de Recherche Marc Delorme, Port Bouët - 07 BP 13 Abidjan 07, Côte d'Ivoire*

[5]*Senior Research Officer, Instituto de Investigaciones Agropecuarias "Jorge Dimitrov" (IIA-JD), Bayamo, Granma, Cuba*

Introduction

Seeds cannot be used for coconut germplasm conservation owing to their large size and their highly recalcitrant storage behaviour (Chin and Roberts 1980), which renders their storage under conventional dry and low-temperature conditions impossible. Genetic resources of coconut are thus traditionally maintained in field genebanks. There are many field collections of coconuts in various countries, usually connected with coconut research institutes, which conserve a total of 1416 accessions. This number is projected to increase over the next few years, with the establishment of the multi-site International Coconut Genebank or ICG (see preceding article) under the coordination of the International Coconut Genetic Resources Network (COGENT).

In some ways, field genebanks offer a satisfactory approach to conservation. The genetic resources under conservation can be readily accessed and observed, permitting detailed evaluation. However, there are certain drawbacks that limit their efficiency and threaten their security (Withers and Engels 1990). The genetic resources are exposed to pests, diseases and other natural hazards such as drought, weather damage, human error and vandalism. Nor are they in a condition that is readily conducive to germplasm exchange. Field genebanks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance and even their very survival in times of economic stringency. Even under the best circumstances, field genebanks require considerable inputs in the form of land, labour, management and materials (see article on 'Coconut field genebank' in this chapter).

It is now well recognized that the efficient and cost-effective conservation of any given genepool can be achieved only through the

implementation of a complementary conservation strategy integrating *in situ* and *ex situ* approaches and utilizing relevant storage methods (Maxted *et al*. 1997). In this context, *in vitro* culture techniques have great potential for the collecting, exchange and conservation of plant germplasm, especially for problem plant species, i.e. those with recalcitrant seeds and those that are propagated vegetatively (Engelmann 1997; 2000). Tissue culture systems allow propagation of plants with high multiplication rates in an aseptic environment. Virus-free plants can be obtained through meristem culture and thermotherapy, thus ensuring the production of disease-free stocks and simplifying quarantine procedures for the international exchange of germplasm. The miniaturization of explants allows genebank managers to reduce space requirements and consequently, the labour costs for the maintenance of germplasm collections. For long-term conservation, cryopreservation, i.e. conservation at ultra-low temperature, usually immersion in liquid nitrogen (-196°C), is the only method currently available for problem species. Cryopreservation protocols have been developed for a wide range of plant species, an increasing number of which are from the tropics (Engelmann and Takagi 2000).

In the case of coconut, in *vitro* culture of zygotic embryos is now routinely applied in numerous laboratories in the framework of germplasm collecting and exchange activities (Batugal and Engelmann 1998; Engelmann *et al*. 2002). This paper summarizes the present status of coconut cryopreservation research.

Status of cryopreservation research

In the case of coconut, only a limited amount of research has been conducted towards the development of cryopreservation protocols, involving research teams in Malaysia, Côte d'Ivoire, France and the UK. Cryopreservation experiments have been performed with zygotic embryos, plumules and pollen. Chin *et al*. (1989) first reported the survival of one single coconut embryo 15 months after freezing using a classical protocol (cryoprotection with DMSO + slow freezing). Assy-Bah *et al*. (1992a,b) reported high recovery of frozen embryos using the pregrowth/desiccation technique. In the UK, Hornung *et al*. (2001) obtained callus growth from plumules of one coconut variety after cryopreservation using an encapsulation/dehydration protocol, including preculture of encapsulated plumules for 72-96 h in medium with 0.75 M sucrose followed by desiccation with silica gel to around 30% moisture content and rapid freezing. Research on cryopreservation of plumules using the encapsulation-dehydration and vitrification techniques has also been initiated in France (Malaurie *et al*. 2002b). Finally, preliminary

unpublished experiments performed in Côte d'Ivoire by the late Béatrice Assy-Bah showed that coconut pollen is amenable to cryopreservation after partial desiccation.

Immature embryos

Experiments were performed with immature embryos sampled from seednuts of the hybrid PB121 (Malaysian Yellow Dwarf x West African Tall) 7 to 8 months after pollination (Assy-Bah and Engelmann 1992a). It was decided to start working with immature embryos, on the assumption that they would be more likely to withstand cryopreservation than mature ones, owing to their smaller size and lower degree of differentiation (Engelmann 1992).

For cryopreservation, embryos were placed for 4 h on pretreatment medium containing 600 g L^{-1} glucose and glycerol, sorbitol or polyethyleneglycol (PEG) 6000 at various concentrations and then immersed directly in liquid nitrogen. After rapid thawing in a water-bath at 40°C, embryos were cultured on standard medium (Assy-Bah *et al*. 1989) for recovery.

After one month, the survival of non-cryopreserved embryos was high for all preculture conditions tested, ranging from 73 to 100%. In the case of cryopreserved embryos, lower survival, ranging between 10 and 43%, was obtained when glycerol at 5 or 10% or sorbitol at 10% was used in the preculture medium. However, numerous abnormalities were observed in the further development of non-cryopserved and cryopreserved embryos, and only few fully developed, normal plantlets could be obtained. This was because conditions for their *in vitro* culture were not mastered (Engelmann and Assy-Bah 1992).

Mature embryos

Experiments were performed with mature embryos sampled from the seednuts of the hybrid PB121; Cameroon Red Dwarf (CRD); Rennell Tall (RT); and Indian Tall (IT), 10 to 12 months after pollination (Assy-Bah and Engelmann 1992b).

For cryopreservation, embryos were placed in open Petri dishes without culture medium and dehydrated for 4 h in the air current of the laminar flow cabinet at room temperature. They were then transferred to open Petri dishes with the medium employed for pretreatment of immature embryos containing 600 g L^{-1} glucose and 15% glycerol (Assy-Bah and Engelmann 1992a), and dehydrated for an additional period of 11 to 20 h. Hence, the total duration of the pretreatment ranged from 15 to 24 h. Embryos were then placed in 2 ml cryotubes and immersed directly in liquid nitrogen. After rapid thawing in a 40°C water-bath, embryos

were cultured on standard medium (Assy-Bah *et al*. 1989) for recovery.

The initial moisture content of embryos, which was very similar in all four varieties, averaged 78.4%. It decreased rapidly during the first 15 h of pretreatment to an average of 11.4% and then more slowly, reaching 6.4 % after 24 h. Larger embryos (RT and IT) dehydrated more slowly than smaller ones (PB 121 and CRD).

Survival of non-frozen embryos remained very high (>70 %) after pretreatment. By contrast, no survival was noted after cryopreservation without pretreatment. For varieties with relatively larger embryos (RT, IT and PB 121), survival after cryopreservation increased in line with increasing pretreatment durations, whereas it reached an optimum after 17 h for the variety CRD, which has the smallest embryos. Under optimal pretreatment conditions, survival ranged between 76-100 % with non-frozen embryos and between 73-93 % with cryopreserved ones. Most embryos considered alive after one month germinated and the same proportion of non-cryopreserved and cryopreserved embryos developed into whole plantlets. The main differences between control and cryopreserved embryos were the non-development of the haustorium and a delay of 1 to 2 months in the development of cryopreserved ones.

These results were validated by N'Nan (1997) with embryos of two ecotypes, West African Tall and Malayan Yellow Dwarf, and recently confirmed (N'Nan *et al*. 2003) on a total of 10 ecotypes, including 5 Talls and 5 Dwarfs, originating from Africa, Latin America-Caribbean, South Asia, Southeast Asia and the South Pacific, with 44-100% of cryopreserved embryos giving rise to whole *in vitro* plantlets.

Plumules

Plumules represent a potentially interesting material for cryopreservation because they are of small size (< $1mm^3$), they are mostly composed of meristematic cells and it is possible to regenerate whole plantlets from *in vitro* cultured plumules (Malaurie *et al*. 2002b). Cryopreservation experiments were performed using the encapsulation-dehydration and encapsulation-vitrification techniques.

With the encapsulation-dehydration technique, excised plumules were encapsulated in alginate beads, pregrown for 2-3 days in medium containing 0.5 to 1.0 M sucrose, desiccated to 0.5-0.2 % moisture content and cryopreserved. Depending on the experiments, survival after cryopreservation could reach up to 67%, but only a limited number of frozen plumules could give rise to whole *in vitro* plantlets (Malaurie and Borges 2001; N'Nan *et al*. 2002). Preliminary experiments performed with the encapsulation-vitrification technique (Sakai *et al*. 2000) showed that up to 20% of cryopreserved plumules could survive after freezing

(Malaurie *et al*. 2002a).

Conclusion and prospects

These preliminary results demonstrate the great potential of cryopreservation for the long-term conservation of coconut genetic resources. Additional research has to be performed to further refine and standardize the protocols developed for embryos and plumules, to test the improved protocols with additional genotypes before their large-scale application in the genebank context can be envisaged. Long-term storage of coconut pollen under cryopreservation would represent an important additional technique for allowing conservation of genes. Research is needed to further develop and refine an appropriate technique.

In view of the very positive results described above, it is clear that, in a not too distant future, cryopreservation will play a greater role in the overall approach in the conservation of coconut genetic resources.

References

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

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

Assy-Bah, B, T Durand-Gasselin, F Engelmann and C Pannetier. 1989. Culture *in vitro* d'embryons zygotiques de cocotier (*Cocos nucifera* L.). Methode, révisée et simplifiée, d'obtention de plants de cocotiers transférables au champ. Oléagineux 44:515-523

Batugal, PA and F Engelmann (eds.). 1998. Coconut embryo *in vitro* culture. Proceedings of the First Workshop on Embryo Culture, Banao, Guinobatan, Albay, Philippines, 27-31 October 1997. IPGRI-APO, Serdang, Selangor, Malaysia.

Chin, HF and EH Roberts (eds.). 1980. Recalcitrant crop seeds. Tropical Press Sdn. Bhd., Kuala Lumpur, Malaysia.

Chin, HF, B Krishnapillay and YL Hor. 1989. A note on the cryopreservation of embryos of coconut (*Cocos nucifera* L. var. *Mawa*). Pertanika 12:183-186.

Engelmann, F. 1992. Cryopreservation of embryos. Pp. 281-290. *In:* Y Dattée, C Dumas and A Gallais (eds). Reproductive biology and plant breeding. Springer Verlag, Berlin.

Engelmann, F. 1997. *In vitro* conservation methods. Pp. 119-162. *In:* BV Ford-Lloyd, JH Newburry and JA Callow (eds). Biotechnology and plant genetic Resources: Conservation and use. CAB International, Wallingford, UK.

Engelmann, F. 1999. Cryopreservation of coconut germplasm. Pp. 289-236. *In:* C Oropeza, JL Verdeil, GR Ashburner, R Cardeña and JM Santamaria (eds). Current Advances in Coconut Biotechnology. Kluwer Academic Publishers, Dordrecht.

Engelmann, F. 2000. Importance of cryopreservation for the conservation of plant genetic resources. Pp. 8-20. *In:* F Engelmann and H Takagi (eds). Cryopreservation of tropical plant Germplasm: Current research progress and applications. JIRCAS, Tsukuba and IPGRI, Rome.

Engelmann, F and B Assy-Bah. 1992. Maintenance of coconut genetic resources: *In vitro* techniques for medium and long-term conservation. Pp. 63-69. *In:* Coconut genetic resources. Papers of an IBPGR Workshop, Cipanas, Indonesia, 8-11 October, 1991. International Crop Network Series No.8. IBPGR, Rome.

Engelmann, F and H Takagi (eds). 2000. Cryopreservation of tropical plant germplasm: Current research progress and applications. JIRCAS, Tsukuba and IPGRI, Rome.

Engelmann, F, P Batugal and JT Oliver (eds). 2002. Coconut embryo *in vitro* culture: Part II. IPGRI-APO, Serdang, Selangor, Malaysia.

Hornung, R, R Domas and PT Lynch. 2001. Cryopreservation of plumular explants of coconut (*Cocos nucifera* L.) to support programmes for mass clonal propagation through somatic embryogenesis. CryoLetters 22:211-220.

Malaurie, B and M Borges. 2001. Cryopreservation of coconut (*Cocos nucifera* L.) plumules by encapsulation/dehydration. Pp. 59. *In:* Abstracts "Bioveg 2001". International Workshop on Plant Biotechnology – Plant Breeding and Biotechnology. Centro de Bioplantas, Ciego de Avila, Cuba, April 16-20, 2001.

Malaurie, B, M. Borges and O. N'Nan. 2002a. Research of an optimal cryopreservation process using encapsulation-osmoprotection-dehydration and encapsulation-osmoprotection-vitrification techniques on caulinary meristems of coconut (*Cocos nucifera* L.). Abstracts IV Jornada Científica IIA"Jorge Dimitrov", Bayamo, Cuba, 19-21 Sept. 2002.

Malaurie, B, O N'Nan, V Hocher, P Ilbert, F Grosdemange, JL Konan, N Zakra and JL Verdeil. 2002b. State of research on culture and cryopreservation of zygotic coconut embryos at IRD/CIRAD (France). Pp. 146-156. *In:* F Engelmann, PA Batugal and JT Oliver (eds). Coconut embryo *in vitro* culture: Part II. IPGRI-APO, Serdang, Selangor, Malaysia.

Maxted, N, BV Ford-Lloyd and JG Hawkes. 1997. Complementary conservation strategies. Pp. 15-39. *In:* N Maxted, BV Ford-Lloyd and JG

Hawkes (eds). Plant genetic resources conservation. Chapman and Hall, London.

N'Nan, O. 1997. Recherche d'une méthode de déshydratation simple, favorable à la survie et à la régénération des embryons zygotiques matures cryoconservés de cocotier (*Cocos nucifera* L.). Diplôme d'Etudes Approfondies, Université Abidjan-Cocody.

N'nan, O, JL Verdeil, V Hocher, JL Konan, N Zakra and B Malaurie. 2002. Mise au point d'une méthode de cryoconservation d'apex caulinaires de cocotier (*Cocos nucifera* L.). Pp. 152-153. *In:* I El Hadrami (ed). Biotechnologies Végétales: Valorisations pour une Agriculture Durable, VIII[èmes] Journées Scientifiques du réseau "Biotechnologies, Amélioration des Plantes et Sécurité Alimentaire" de l'Agence Universitaire de la Francophonie, 7-9 Octobre 2002. Marrakech, AUF, Paris.

N'Nan, O, M Borges, JL Verdeil and B Malaurie. 2003. Is cryopreservation of mature zygotic embryos of coconut (*Cocos nucifera* L.) the easiest and safest way to preserve coconut germplasm? Paper presented during the 4[th] International Workshop on Plant Biotechnology and Sustainable Development "BioVeg 2003", 14-19 April 2003, Centro de Bioplantas, Universidad de Ciego de Avila, Ciego de Avila, Cuba.

Ramanatha Rao, V and P Batugal (eds). 1998. Proceedings of the COGENT Regional Coconut Genebank Planning Workshop, Pekanbaru, Riau, Indonesia, 26-29 February 1996. IPGRI-APO, Serdang, Selangor, Malaysia.

Sakai, A, T Matsumoto, D Hirai and T Niino. 2000. Newly developed encapsulation-dehydration protocol for plant cryopreservation. CryoLetters 21:53-62.

Withers, LA and JMM Engels. 1990. The test tube genebank: A safe alternative to field conservation. IBPGR Newsletter for Asia and the Pacific 3:1-2.