THE USE OF BIOTECHNOLOGY FOR CONSERVATION AND DISSEMINATION OF COCONUT GENETIC RESOURCES: AN ASSESSMENT OF THE IRD/CIRAD CONTRIBUTION

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ABSTRACT

An assessment of twenty one years IRD/Cirad involvement in coconut (*Cocos nucifera* L.) biotechnology is presented. Research towards somatic embryogenesis and its mastering for clonal propagation of élite trees, *in vitro* conservation for short-, medium- and long-term through cryopreservation and its management and use for germplasm exchange is described. The high recalcitrance of coconut to *in vitro* culture has influenced the different directions of research. The different bottlenecks that appeared at various stages have stimulated the development of an international collaborative research programme between several Institutes, where IRD/Cirad has played an active role. Recent preoccupations relating to safe exchange of coconut germplasm, because of outbreaks of devasting diseases such as lethal yellowing, have reinforced the COGENT collaboration between coconut growing countries and Institutes such as IRD/Cirad.

Keywords: Cell cycle; *Cocos nucifera*; Cryopreservation; Embryo rescue; Somatic embryogenesis; Zygotic embryo.

INTRODUCTION

The coconut palm has, in most tropical regions of the world, a great importance as a food and cash crop. It is grown on more than 11 million hectares in 89 countries, where it is cultivated mainly for copra (dried endosperm) production, from which oil is extracted with a world production of 53 million tons (FAO 2003). It provides incomes for millions of smallholders on small (0.2 – 4.0 ha) plantations, and 95% of the world production is consumed locally (70% in Asia; 30% in Pacific region). In sub-tropical areas, it represents also a primary source of food, drink and shelter for millions of inhabitants for which reason it is generally called "the Tree of Life".

Since the 1980s the coconut industry has shown a decline of productivity and an increasing competition with other oil producing crops, where it now occupies the 7th place amongst oleaginous plants. Several factors are involved: (a) plantation ageing (two thirds of the individuals are over 60 years old) and low replanting rates, (b) poor cultural practices and material of poor genetic quality, (c) spread of several pests and diseases such as viroid disease (Cadang-Cadang in Asia) or phytoplasma disease (Lethal Yellowing in Caribbean and Africa), (d) natural calamities (typhoons, drought), and (e) competition with other lauric oils which are cheaper than coconut oil.

There has also been genetic erosion, for which Lethal yellowing has played a major role with millions of hectares completely destroyed by the spread of this disease in Central America, the Caribbean, and more recently in West and East Africa (Jones *et al.,* 1999). Due to its economic importance in sub-tropical areas, increasing farmers' demands, and the increasing genetic erosion, it is now essential to maintain and improve coconut cultivation by using the possibilities offered by biotechnology (Hocher *et al.,* 1999). This paper will review the facilities offered by the application of *in vitro* methods, through somatic embryogenesis, for clonal propagation of élite palms selected in the field, or through embryos rescue and cryopreservation for germplasm conservation and its safe international exchange, in the IRD/Cirad collaborative research programme (Verdeil *et al.*, 1995, 1998a, 1998b).

RESEARCH ON CLONAL COCONUT PRODUCTION THROUGH SOMATIC EMBRYOGENESIS

Twenty one years ago work first began on coconut *in vitro* culture, where IRD (exOrstom)-Cirad oil palm experience initially played a major role. The somatic embryogenesis program began in close collaboration with Côte d'Ivoire continues as an active partner without which this program would not have been sustained.

Because of the highly variability of the coconut varieties in cultivation (crosses between heterozygous individuals frequently allogamous) vegetative propagation of chosen individuals should improve plantation productivity significantly. The necessity to maintain the mother palm in

the field eliminates the possibility of collecting and culturing its only shoot meristem. Different *in vitro* techniques were used for coconut propagation, but somatic embryogenesis was definitely the most promising technique which allows coconut cloning which could lead to the production of homogeneous planting material.

Success of micropropagation in coconut was very dependant on the type of material used. Different types of explants were tried for coconut clonal propagation through somatic embryogenesis by IRD/Cirad: Stem and leaf (Pannetier and Buffard-Morel, 1982) and inflorescences (Verdeil, 1993; Verdeil *et al.,* 1989, 1993). Because of high recalcitrance of coconut to *in vitro* culture, progress in somatic embryogenesis still remains very slow and since the first incidence of somatic embryogenesis (Pannetier and Buffard-Morel, 1982), only a few micro plants have been obtained six years later (Buffard-Morel *et al.,* 1988). Coconut seems to be one of the most recalcitrant palms in respect of response to *in vitro* culture, and in consequence is difficult to propagate through somatic embryogenesis (Hocher *et al.,* 1999).

Although early work suggested that coconut cloning through somatic embryogenesis was feasible, subsequent efforts have shown great difficulties which could only be resolved through international collaboration. In 1994, some of the teams (Wye College, United Kingdom; Hanover University, Germany; ORSTOM/Cirad-CP, France; IDEFOR/DPO, Côte d'Ivoire; PCA, Philippines; CICY, Mexico) working on coconut embryogenesis joined together under an EU funded project (Hocher *et al.,* 1998, 1999). This collaboration contributed greatly to the important advances in different phases of the *in vitro* protocols (callogenesis, initiation of embryogenesis, maturation), and plantlets are now regularly obtained in most laboratories (Verdeil *et al.,* 1999).

Since the EU cooperation, identification of key features of coconut *in vitro* culture has been done for each step of the regeneration protocol. The use of immature explants (leaf, inflorescences) was recommended (Hocher *et al.,* 1998; Hornung and Verdeil, 1999). More recently, the plumule excised from mature zygotic embryo, was suggested as a better explants for somatic embryogenesis (Chan *et al.,* 1998; Saenz *et al.,* 1999).

To overcome the recalcitrance of coconut, different approaches are currently developed at IRD/Cirad: Growth regulator analyses in culture media and in tissue (Verdeil *et al.,* 1999), use of histology (Verdeil *et al.,* 2001), search for early protein markers (Sandoval, 2002), and study of the nutrition status of the seed during germination (Nowak, 1999; Malaurie *et al.,* 2002). This has led to a better formulation of the culture media components (activated charcoal, growth regulators, amino acid, sugars, etc) (Verdeil *et al.,* 1999). The IRD/Cirad *in vitro* protocol still achieved only a low rate of clonal plantlet regeneration (10%). However, several plantlets have been weaned, grown in greenhouse, and some established under field conditions in CICY (Mexico) (Verdeil *et al.,* 1999).

The Somatic Embryogenesis Steps

For the somatic embryogenesis process, different steps have to be accomplished such as callogenesis, embryogenesis induction, embryogenesis initiation and embryo maturation. For these steps nutrient requirements and historogical studies could also provide some important insights.

Callogenesis

Work on callogenesis was done mostly on the use of growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D), where it was shown that it was essential for activation and division of undifferentiated cells in the explants used to produce calli, when combined with a range (2 and 2.5 $g.l^{-1}$) of activated charcoal (Verdeil and Buffard-Morel, 1995). Work using another auxin, 2, 4, 5 trichlorophenoxyacetic acid (2, 4, 5-T), reported the formation of nodular calli on inflorescence explants (Buffard-Morel *et al.,* 1988; Verdeil and Buffard-Morel, 1995). Different concentrations (44, 55 and 66 $mg.l^{-1}$) of 2,4-D were tried allowing 20 to 40% explants to produce calli after six months culture (Verdeil *et al.,* 1994; Verdeil *et al .,* 1998; Hocher *et al.,* 1998).

To quantify plant growth regulators in culture media containing charcoal, techniques have been used that allow a better understanding of the role of charcoal. The strong adsorption of 2, 4-D and cytokinins by charcoal was confirmed. The adsorption capacity is affected by charcoal particle size and can depend on the charcoal brand used. The choice of a new medium composition is based on the auxin-cytokinin ratio that can be modified by the introduction of charcoal. The optimum 2, 4-D level for the formation of embryogenic calli also depends on the genotype (Verdeil, 1993; Verdeil *et al.,* 1999).

Embryogenesis induction

For somatic embryogenesis induction in coconut, auxin concentrations remain the determining factor, but presence of activated charcoal is also considered to be an essential element for embryogenesis induction (Pannetier and Buffard-Morel 1986; Verdeil *et al.*, 1996). As work shows that embryogenesis from calli isolated from explants is non-synchronous (needing 2 to 24 months after callus isolation from the explant to be observed) and it is induced on less than 10% of calli, the study of the phenomenon remains complicated and explains the paucity of data concerning embryogenesis induction in coconut.

Embryo maturation and germination

Somatic embryo maturation is achieved after 2,4-D treatment and introduction of cytokinin in the medium, such as thidiazuron (TDZ) or 2iP, which were found to be the most efficient cytokinins (Verdeil *et al.*, 1996). Differentiation of the shoot meristem in the somatic embryo is a cytokinin dependent phenomenon (Verdeil *et al.,* 1994). Work on endogenous cytokinin in coconut tissue

cultured *in vitro* has shown an important level in isopentenyl forms of cytokinin during early somatic embryo development (Hocher *et al.,* 1998). Under these culture conditions, 28% of embryonic structures developed into whole plants (Verdeil *et al.,* 1998a).

Nutrient requirement

Different studies of nutrient requirement have been developed on IRD/Cirad. A kinetic comparison of callus nutrient requirements in media for the multiplication step and embryogenic step was developed, where protein accumulation in embryogenic cells was observed (Dussert *et al.,* 1995 a, b; Magnaval *et al.,* 1997). Analysis of the free amino acid in calli under somatic embryogenesis induction showed that the proline, valine and serine contents of the calli decrease as 2, 4-D concentration is increased. Magnaval *et al.* (1995) have shown that increase in proline, valine and leucine contents were related to accumulation of protein in the cells.

Histological studies in coconut

Many studies have been done using histological tools in coconut by the IRD/Cirad coconut team. Histological studies were very useful in investigations of calli origin as well as somatic embryo origin. Histological studies allowed to determine callus origin sites when calli were obtained from leaf explants (Buffard-Morel *et al.,* 1992) or when cross-sections of inflorescence fragments were used (Verdeil *et al.,* 1992); as well as to provide a better understanding of the mechanisms involved. Two pathways have been described for the evolution of somatic embryogenesis.

The first one is considered a multicellular origin. It happens generally when media contain low 2,4-D concentrations (40 to 60 $mg.L^{-1}$) and 2 $g.l^{-1}$ charcoal (Buffard-Morel *et al.,* 1992; Verdeil *et al.,* 1992, 1994; Hocher *et al.,* 1998) or even when ABA is added to the media (Fernando *et al.,* 2003). The presence of meristematic and epidermized structures is an indication of the embryogenic capacity of calli, where very active cell divisions in the cambium-like zone leading to the formation of meristematic nodules is the strongest characteristic.

The second path way was observed when high 2,4-D concentration (80 to 120 $mg.L^{-1}$) and 2 to 3 $g.L^{-1}$ charcoal is used. This led to individualization of embryogenic cells and formation of typical pro-embryos (Verdeil *et al.,* 1994; Hocher *et al.,* 1998). In further work, Verdeil *et al.* (2001) have shown by a detailed histological study, the more precise events which occur the cells such as invaginations of the nuclear membrane and proliferation of dictyosomes (emission of golgi vesicles), that are linked to embryogenic status.

Physiological studies of microplants developed from zygotic embryos

Development of plantlets during *in vitro* phase and acclimatization is still a major concern because of various problems associated with it. To understand this problem, physiological studies of the development of plantlets using zygotic embryos as a model, were performed by IRD/Cirad (Rival *et al.,* 1999). Different photosynthetic parameters were studied: 1) chlorophyll fluorescence for the

efficiency of photosystem II; 2) activity of two carboxylases (PEPC, RubisCO) as an indicator of autotrophic $C0_2$ fixation; 3) net photosynthesis rate; 4) chloroplast ultra structure (Triques *et al.,* 1997 a, b). From these works, it was shown that the photosynthetic metabolism of *in vitro* cultured coconut establishes early. Meanwhile, further work showed a lower rate of net photosynthesis in *in vitro* grown plantlets as compared with acclimatized coconut plantlets (Rival *et al.,* 1999; Triques *et al.,* 1998).

INVESTIGATIONS ON SLOW GROWTH OBSERVED IN *IN VITRO* COCONUT TISSUE CULTURE

As slow growth of plantlets still remains the major problem, a molecular approach through cell cycle studies and through differential display was attempted. Shoot plantlets from rapidly-growing and slow-growing coconut apices were used for comparative analysis and should provide molecular markers.

Control of the Cell Cycle

The IRD/Cirad team has first started the cell cycle studies on coconut five years ago. Flow cytometry has revealed that most of the meristematic cells (>90%) of different tissues (calli showing a rapid or a slow growth, caulinary apex, immature leaves) were in G0/G1 phase of the cell cycle. The work on the characterisation of the cell cycle reinforced the hypothesis of a blocking of the coconut cells in the G_0/G_1 and G_1/S phases. A time course study revealed that this blocking takes place very early following the introduction of the material *in vitro* (Sandoval *et al.,* 2003).

Identification of Molecular Markers *of* Shoot Meristem Activity

A study was conducted on the characterisation of the cell cycle and the search for molecular markers of meristematic potential by gene candidate and ddRT-PCR approaches. Based on this knowledge, the IRD/Cirad team has started the search for genes implicated in coconut cell cycle regulation (Fernando, 1999; Sandoval, 2002).

Two full length clones, CnCycD1 and CnRb1, showing strong homology with D type cyclin and genes encoding retinoblastoma protein respectively, were isolated though a gene candidate approach. A semi-quantitative Rt-PCR analysis of the CnRb1 expression was done in shoot meristems with active and retarded growth and calli with fast and slow growth. The accumulation

of CnRb1 transcripts was compared with that of CnH4;1 (coconut gene for H4). Results suggest a relationship between meristematic activity and profusion of CnRb1transcripts. *In situ* hybridization of CnRb1 expression in shoot meristems with normal and inhibited growth confirmed this relationship, since CnRb1 mRNAs are localised mainly in the meristematic cells and tissues. The study by differential display of the expression of shoot apex with inhibited or normal development made it possible to identify and to validate by Northern blotting, eight potential markers. The sequence homologies obtained and their possible function are discussed (Sandoval, 2002). Understanding the ell cycle regulation in coconut tissues cultured *in vitro* should allow a better mastering of the cloning protocol and thus increase the multiplication rate of coconut (Sandoval *et al.*, 2003).

Embryo Germination and Plantlet Recovery

The remaining difficulties are the heterogeneity of embryo behavior and the slow in the development of plantlets (compared to seedlings). An assessment of photosynthesis during the development *in vitro* of zygotic embryos suggests that the slow development could be due to a nutritional problem (Triques *et al.*, 1997b). A study of the role of the haustorium during germination has been started by IRD/Cirad. Using HPLC, the levels of sugar, amino acid and lipids have been measured in the haustorium and endosperm during the germination phase of coconut seeds. Results are under analysis and should give important information for improving the composition of *in vitro* culture media.

IN VITRO CONSERVATION OF GENETIC RESOURCES AND SAFE INTERNATIONAL EXCHANGE OF COCONUT GERMPLASM

Conservation of Coconut Genetic Resources

Amongst four regional field collections, germplasm of coconut genetic resources located in the following four countries: (Côte d'Ivoire, India, Indonesia and Papua New-Guinea), Côte d'Ivoire remains the most important in term of genotypic diversity (Verdeil *et al.*, 1996). With coconut, important problems occurred in transporting and storing germplasm because of the size of the coconut seed, the biggest in the plant kingdom, with no dormancy (Assy-Bah *et al.*, 1987). This type of recalcitrant seed, which is not tolerant to dehydration and to low temperatures leads to problems linked to short-term, medium-term and long-term conservation (Engelmann and Dussert 2000).

Short-term conservation

Short-term conservation is important for storage of embryos during collecting missions of endosperm cores, where combinations of disinfection and culture process for zygotic embryos in tropical conditions were studied (Assy-Bah *et al.,* 1989; N'Nan *et al.,* 2002b). A short-term preservation technique was developed for keeping embryos in KCL for up to 14 days *in vitro* (Assy-Bah *et al.,* 1989).

This technique was also adapted for collecting zygotic embryos under prospecting conditions and used routinely. The different conditions needed for sampling, disinfecting, directly in field in tropical conditions and for further transport up to laboratory have been described (Assy-Bah *et al.,* 1987; N'Nan *et al.,* 2002b). Although this conservation method allows resolving immediate problems, it does not assure conservation for longer durations.

Medium-term conservation

Medium-term conservation of coconut germplasm was described in 1993 (Assy Bah and Engelmann, 1993) where germination of coconut zygotic embryos held *in vitro* from 6 to 12 months was observed. The role of two factors (sucrose and activated charcoal) on slow growth was observed (Assy-Bah, 1992; Verdeil *et al.,* 1998b). Plant material needs a suppression of their metabolism to ensure genetic stability when conservation is done for a long duration (Dereuddre, 1985). The use of liquid nitrogen for long term conservation through cryopreservation allows such a metabolic stoppage and so avoids the loss of important genetic material.

Long-term conservation: Cryopreservation in liquid nitrogen

At present, cryopreservation is the only way, which permits a complete arrest of cell divisions and metabolic processes without causing cell damage (Dereuddre, 1985; Engelmann, 1999) and assures long-term conservation. Compared to other *in vitro* conservation methods, cryopreservation requires less space and less maintenance to preserve material during entire conservation period (Engelmann and Dussert, 2000).

Cryopreservation of Coconut Zygotic Embryos

From the first work on coconut embryo cryopreservation (Bajaj, 1984; Chin *et al.,* 1989), only one plantlet has been obtained after 15 months culture. Further work developed between 1989 and 1992 by Assy-Bah and Engelmann on cryopreservation used a pretreatment in the presence of glucose and desiccation under laminar air flow. Investigations of immature and mature embryos

showed that immature embryos were not suitable for this cryopreservation (Assy Bah and Engelmann, 1992a).

Cryopreservation of mature embryos was feasible using a four hours desiccation pretreatment in a laminar air flow followed by 11 to 20h culture in the presence of 600g.L^{-1} glucose and 15% glycerol (Assy-Bah and Engelmann, 1992b). Four coconut varieties (hybrid PB121, Indian Tall, Cameroon Red Dwarf, Rennell Island Tall) gave 10 to 93% germination after cryopreservation, depending on the ecotype. Later, these results were validated with two other ecotypes: West African Tall (WAT) and Malayan Yellow Dwarf (MYD) (N'Nan, 1997).

The need of a reliable process for conservation of coconut in the zygotic embryo form led to validation of previous results (Assy-Bah and Engelmann 1992a, b). This was done with a technical process (Assy-Bah and Engelmann, 1992b) and a modified protocol (N'Nan, 1997), on ten ecotypes, five "Dwarf" [BGD, Brazilian Green Dwarf; CRD, Cameroon Red Dwarf; MYD, Malayan Yellow Dwarf; NLA, Niu Leuka Dwarf] and five "Tall" [PNT, Panama Tall; SGD, Sri Lanka Green Dwarf; SLT, Sri Lanka Tall; TAGT, Tagnanan Tall; VTT/VNT, Vanuatu Tall; WAT, Wes African Tall] covering the main areas under coconut cultivation [Africa; Latin America & Caribbean; South Asia; South-East Asia; South Pacific] (N'Nan *et al.,* 2003a).

The results confirmed the feasibility of using the protocol with all the ecotypes with success rates varying from 20 to 70% (N'Nan, 2004). The ranking order of ecotypes for rates over 40% was as follows: TAGT< PNT < SGD < BGD < CRD < MYD. A treatment allowing an optimal action on the development of all the ecotypes, except for the SGD, was shown (N'Nan *et al.,* 2003; N'Nan, 2004). These results indicated that conservation, safeguarding and exchange of ecotypes is feasible between areas free of well-known and dangerous coconut pathogens.

Cryopreservation of Plumules

In spite of the successful results obtained on cryopreservation of mature complete embryos, it appeared that cells of the haustorial part of the embryo were damaged while meristematic cells from the shoot meristem were still surviving (Assy-Bah, 1992). This caulinary meristem, with one or two leaf primordia, called the plumule, appeared to be an interesting material for cryopreservation. Its small size (around 1 millimeter), its structure (cells in meristematic state) makes it use conceivable for obtaining a safe material, disease-free, as previously observed with this type of explant (Morel and Martin, 1952).

Investigations in plumule cryopreservation led to the use of several techniques, all using encapsulation of plumules in alginate beads. These techniques differed from each other by their pretreatment step (liquid or solid medium) or the dehydration step (osmotic dehydration or physical dehydration) such as (a) encapsulation/osmoprotection/vitrification (Sakai *et al.,* 2000), (b) encapsulation/osmoprotection/dehydration (Sakai *et al.,* 2000), and (c) encapsulation/ dehydration (Dereuddre *et al.,* 1990).

Techniques using osmoprotection

In this case several morphological factors were observed after one month of culture, such as browning, swelling, survival and growth recovery. In the case of technique [I], with dehydration durations ranging from 2h to 10h growth recovery (after one month of culture) was observed only in 3h dehydration treatment. Plumular tissues remain sensitive to dehydration and freezing stress leading to tissue browning (79%) (Malaurie *et al.*, 2002a). Later observations (5-6 months) have shown 13% survival between 3 and 5h freezing. In the case of growth recovery it was only observed at 3h dehydration with 10% however dehydration with freezing (+AL) or dehydration alone (-AL).

In the case of technique [II] using PVS2 for 20, 60 and 100min., 100% browning was observed with (+AL), meanwhile this phenomena linked to dehydration duration increase from 14 to 65% with (-AL). Swelling and growth recovery were observed too in case of (-AL) and 1 month culture, with 96% and 11% respectively (Malaurie *et al.*, 2002a). At 5 months culture, growth recovery was up to 20% after 20min. dehydration (Malaurie *et al.*, 2003; N'Nan, 2004).

Techniques using encapsulation /dehydration

Preliminary work has shown that it was possible to regenerate plantlets from plumules using this technique after immersion in liquid nitrogen (N'Nan 1999; Malaurie and Borges 2001; Malaurie *et al.*, 2002b). Survival after cryopreservation was observed with rate up to 67% in some experiments (Malaurie, 2001). This rate has been revised downwards because some explants later developed abnormalities. These explants did not develop into complete plantlets *in vitro* (N'Nan *et al* ., 2002c).

Using one ecotype (MYD), different investigations have been conducted to define the best conditions for success after dehydration and freezing in liquid nitrogen. The best growth recovery (20%) was obtained with combinations of treatments (sucrose concentration, pre-treatment duration, dehydration duration) with best values as follows [0.75/3/16 and 1/2/8] (N'Nan, 2004).

Protocol confirmation with several ecotypes

The protocol was applied to five other ecotypes, two Dwarfs (BGD, CRD) and 3 Talls (PNT, VNT, TAGT). Four of them have shown a growth recovery after cryopreservation ranging from 4 to 36% and are arranged in increasing order as follows: TAGT < PNT < MYD < CRD (N'Nan 2004). The confirmation of the suggested protocol has been validated for three of the ecotypes which have obtained growth recovery higher than 20%. These first results provided a positive beginning to a protocol validation. Further, work is planned with other ecotypes which could represent well the genetic diversity and the geographic range of coconut.

A histological study using optical and electronic microscopy on cryopreserved plumules and zygotic embryo's revealed treatment effects on the structural integrity of cells and tissues, yielding a good idea of cell behaviour under different stress conditions (N'Nan *et al.*, 2003b).

Safe International Exchange of Coconut Germplasm

To avoid spread of diseases such as Cadang-Cadang in the Philippines or Lethal Yellowing (LY) (in the Caribbean parts of Central America, and observed in Ghana recently) there is a need for safe movement of germplasm, for distribution of material *in vitro*, as required by the technical guidelines for safe movement of coconut germplasm (Frison *et al.*, 1993). Zygotic embryos were until now preferred for coconut germplasm exchange, and follows the coconut guidelines instructions for germplasm transfer (Diekmann, 1999; Ramanatha Rao and Batugal, 1998). IRD/Cirad developed this form of transfer, through collecting and disinfecting endosperm cores with zygotic embryos which could be cultured *in vitro* directly or after mailing (Assy-Bah *et al.*, 1987; N'Nan *et al.*, 2002b; Malaurie, 2003). For mailing between distant countries, an adapted storage in sterile potassium chloride solution was proposed allowing storage up to 14 days and later *in vitro* introduction in the aseptic conditions of a laboratory (Assy-Bah *et al.*, 1987; N'Nan *et al.*, 2002b).

Collaborative Work with Coconut Research Institute, Sri Lanka (CRISL)

Collaboration between with CRISL and IRD/Cirad started in 1999 with Mrs S. C. Fernando's fellowship with an IRD grant. Her work was followed in 2001 with an UNESCO grant. Since 2002, this collaboration continued with Mrs Prasanthi Perera who obtained an IRD grant for a training of 1 year spread over three periods (3 months in 2002, 4 months in 2003 and 5 months in 2004). The following items were developed:

- Histological studies on plumular somatic embryogenesis (Fernando *et al.*, 2003).
- Research on the expression of the retinoblastoma gene in coconut somatic embryogenic structures, using RT-PCR (Fernando, 1999).
- Histological studies of somatic structures from ovary culture, with histological description of flower ontogenesis, from primordial initiation to maturity (Perera, 2002).
- Study of the ploidy level of somatic structures from ovary through flow cytometry (Perera, 2003).
- Apprenticeship of western blot technique for the research of the protein retinoblastoma expression in somatic embryonic tissue (Perera, 2003).
- In 2004 work is planned, for Mrs. P. Perera to work on *in situ* hybridization research of the coconut retinoblastoma gene expression in somatic embryonic structures obtained from ovule + RTPCR. This work will be developed in collaboration with J-L Verdeil.
- In 2004 and 2005, a fellowship granted by BRG (Bureau des Ressources Génétiques, Paris, France) should allow this collaboration to continue through training on coconut cryopreservation with the visit of Mrs H.D. Dharshani Bandupriya, researcher from CRI.SL.

Other IRD/Cirad International Collaborations

IRD and Cirad play an important part in development research programs under international collaboration. They provide facilities for trainees to learn techniques developed in IRD/Cirad. During the last years, more than 10 persons were trained by IRD/Cirad. Funds were granted by IRD/Cirad, French Research Ministry, UNESCO, CONACYT (Mexico), and COGENT.

Different partners benefited from these fellowships. They were CICY & INIFAP, Mexico; CRI, Sri Lanka; PCA, The Philippines under EU STD funded project (Hocher *et al.,* 1998. Verdeil *et al.,* 1999); Wye College under EU STD funded project; CNRA & Cocody University, Côte d'Ivoire; IIA "Jorge Dimitrov" and Granma University, Cuba.

The IRD/Cirad Involvement in Support and Training

The joint Research Unit team, UMR BEPC, located at IRD Montpellier, has been chosen by COGENT (Coconut Genetic Research Network) and IPGRI (International Plant Genetic Resources Institute), to organize training on the coconut zygotic embryo cryopreservation process, from 13 to 17 October 2003. The stay of five researchers, chosen from among the different coconut network partners, has been funded by COGENT/IPGRI for four of them, the fifth and the sixth getting an IRD grant. These researchers belonged to Embrapa (Brazil), CICY (Mexico), MARI (Tanzania), PNGCCRI (Papua New-Guinea) and to CRISL, and to Côte d'Ivoire for a PhD student. This training allowed them to be initiated to the techniques developed by the UMR BEPC team. These techniques could be put into practice to equip cryopreservation units of coconut genetic resources in their respective countries. Thus they could ensure the more secure coconut germplasm safeguarding in their original area: South America for Embrapa, Central America and Caribbean for CICY, East Africa for MARI, Oceania and South Pacific for PNGCCRI, South Asia for CRI. West Africa was represented through O. N'Nan who is at present working with CNRA germplasm.

CONCLUSIONS

Achieving high yielding coconut through vegetative propagation still remains the main prospect for obtaining and distribution of élite trees. For that several lines of research have been investigated. Undeniably important advances have been made on different aspects of *in vitro* coconut culture, where IRD/Cirad has played an important role. However, several remaining bottlenecks need to be resolved.

Regarding zygotic embryo culture, recent concern relating to safe exchange of coconut germplasm, because of devastating diseases such as lethal yellowing, have reinforced the

COGENT collaboration with coconut growing countries and Institutes such as IRD and Cirad (N'Nan and Malaurie, 2003; N'Nan, 2004). Results obtained on zygotic embryo cryopreservation, allowing routine use on all ecotypes conservation and exchange of ecotypes should be feasible between areas free of known dangerous pathogens for coconut, such as lethal yellowing, because of its possible spreading through nature embryo transfer (Cordova *et al .*, 2003). In this context, International collaborative research could play, through COGENT facilities, an important role to overcome this serious threat for millions of people who depend closely on coconut for food and cash (Batugal, 1999).

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APPENDIX 1

The list of abbreviations used

CICY	Centro de Investigación Cientifica de Yucatán
CNRA	Centre National de Recherche Agronomique
Cirad	Centre de coopération internationale en recherche agronomique pour le développement
COGENT	International Coconut Genetic Resources Network
CONACYT	Consejo Nacional de Ciencia y Tecnología ;
CRISL	Cococnut research Institute Sri Lanka
IIA-JD	Instituto de Investigaciones Agropecuarias "Jorge Dimitrov";
INIFAP	Instituto Nacional de Investigaciones Forestales, Agrícolas Pecuarias;
IPGRI	International Plant Genetic Resources Institute;
IRD	Institut de Recherche pour le Développement ;
MARI	Mikocheni Agricultural Research Institute;
PCA	Philippines Coconut Authorities ;
PNGCCRI	Papua New-Guinea Cocoa Coconut Institute;
UMR	BEPC, Unité Mixte de Recherche sur la Biologie du développement des Espèces Pérennes Cultivées;
UNESCO	United Nations Educational, Scientific and Cultural Organization.

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