Coconut micropropagation

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Introduction
The coconut palm (Cocos nucifera L.) is a very important crop providing cash and subsistence to millions of smallholders in 86 countries where about 12 million ha are planted with this palm (Santos 1999). However, most coconut groves require replanting because of loss due either to palm senescence or to diseases such as lethal yellowing in America (Arellano and Oropeza 1995), the lethal diseases in Africa (Eden-Green 1995) and cadang-cadang in Asia, in particular the Philippines (Hanold and Randles 1991). Research on genotype selection for disease resistance or other traits of interest, such as high yield, are being carried out worldwide with positive results (Santos 1999; Zizumbo et al. 1999). However, propagation of selected genotypes, or even more conveniently, individuals within these genotypes to satisfy the rapidly growing demands will be very hard to fulfill through natural coconut propagation that occurs only sexually, producing very few seeds per palm within its long life cycle. Therefore, alternative approaches for rapid propagation of improved planting materials must be considered. In this respect, in vitro cloning via somatic embryogenesis seems to provide a convenient alternative for the future due to its potential for massive propagation. Unfortunately, coconut is a species that responds very poorly to in vitro culture, being one of the most recalcitrant species to regenerate in vitro (George 1996). This paper summarizes the efforts that have been carried out to develop protocols for the micropropagation of coconut through somatic embryogenesis, presenting the first work carried out during the 20th century and the research advances obtained during the past five years. The paper focuses particularly on research leading to sustained developments such as those related to the use of inflorescence and embryo explants.

Research from the 1970’s to the 1990’s
Initial developments started with the work of Eeuwens (1976) on improving callus formation and growth by optimizing the mineral composition of the culture media following factorial design experiments.
CHAPTER 5: Germplasm use

Testing of the different types of explants followed: young roots of mature palms (Justin 1978), stem and leaf (Pannetier and Buffard-Morel 1982; Gupta et al. 1984; Raju et al. 1984), embryos (Karunaratne and Periyapperuma 1989) and inflorescences. The first promising results involving somatic embryogenesis were obtained at Wye College (UK) with the first clonal plants produced in 1983 (Branton and Blake 1983) and similar findings were obtained with young leaf explants (Buffard-Morel et al. 1992). Low percentage of callus formation and the development of abnormal plants were common occurrences (Branton and Blake 1984; Dublin et al. 1991). However, these studies demonstrated that coconut regeneration by somatic embryogenesis was possible.

The use of inflorescence explants

Coconut tissue culturists were initially interested in the use of immature coconut inflorescences as explants because they contain meristematic tissue, which was encouraged to form callus tissue with the addition of an auxin to the culture medium. Immature inflorescences from mature palms could be excised non-destructively from the palms (Rillo 1989). Initial callus formation started at about three months after culture initiation and was observed until about nine months (Hornung and Verdeil 1999). The most commonly used auxin was 2,4-D at varying concentrations, depending on the amount and type of activated charcoal employed. Cytokinins were usually not added to the medium for callus initiation. For instance in Montpellier, the Eeuwens Y3 mineral solution (Eeuwens 1976) was used with the addition of Morel and Wetmore’s vitamins (1951), 2.5 g l⁻¹ activated charcoal, sucrose at 40 g l⁻¹ and agar at 7.5 g l⁻¹, 2,4-D between 13 and 36 mM, due to the variable sensitivity of different palms and the developmental stage of inflorescences to the auxin. Murashige and Skoog medium (1962) with the addition of sucrose, activated charcoal and auxin was also employed for callus production. Callus grown on media with gradually reduced auxin levels (Blake 1990), or by an increase followed by a reduction (Verdeil et al. 1992), produced nodular somatic structures that subsequently developed into proembryos and finally into embryos (Verdeil et al. 1994). However, the production of good somatic embryos still presented a problem due to the development of fused embryos, fused leafy structures with or without roots and haustorial type tissues (Sugimura and Salvaña 1989; Blake 1990; Verdeil et al. 1992).

The main difficulties encountered in coconut regeneration by somatic embryogenesis from inflorescence explants were: intense browning of tissue linked to the oxidation of polyphenols, considerable heterogeneity in tissue response, a strong tendency to produce roots only, low
embryogenic potential, poorly developed embryo formation, poor shoot formation and slow growth rate in vitro. The first one could be overcome to a great extent by the use of activated charcoal. The others were more difficult to overcome. However, in 1993, several groups (from the University of Hanover, Wye College, L’Institut de Recherche pour le Développement-Centre de Coopération Internationale en Recherche Agronomique pour le Développement (IRD-CIRAD), Philippine Coconut Authority (PCA) and Centro de Investigación Científica de Yucatán (CICY)) involved in coconut regeneration research gathered together for the first time to start a joint effort to overcome the major difficulties encountered in coconut regeneration. This was made possible through a project funded by the European Commission within the Science and Technology for the Development 3rd Period Programme (STD-3) (ERBTS3*CT940298) that started in January 1993. This project increased the fundamental knowledge on the different aspects of somatic embryogenesis in coconut: morpho-histological development (and infrastructural changes (Verdeil et al. 2001); hormone studies (Verdeil et al. 1994; Hocher 2003); nutritional studies (Dussert et al. 1995a,b; Magnaval et al. 1995; 1997); protein phosphorylation during somatic embryogenesis (Islas-Flores et al. 2000); and plantlet photosynthesis characterization (Triques et al. 1997a,b; Rival et al. 1999). These studies increased the understanding of the regeneration process and helped to overcome some of the difficulties encountered in coconut regeneration and improvement of protocols using either inflorescence or plumule explants. Several experimental protocols using inflorescences or plumules are now available. For a more detailed account on coconut regeneration from inflorescence explants, see Hornung and Verdeil (1999). Studies using plumule are described below.

The use of embryo explants
As mentioned above, whole coconut embryos had been used as explants to induce embryogenic calli and somatic embryo formation without success. As an alternative, researchers from Wye College and CICY tested embryo isolated parts: plumules, root poles and cotyledonary tissues. Plumules were the only explants that readily formed embryogenic callus and embryos that developed shoots (Hornung 1995; Oropeza and Chan 1995). Further studies within the EC-STD3 project (reported in Chan et al. 1998) were carried out to improve somatic embryogenesis and shoot formation from plumule explants, and ultimately, ex vitro plantlet establishment. Plumules from the embryos of nuts harvested 12-14 months after pollination were used and cultured on Y3 medium (Eeuwens 1976) containing activated charcoal and gelledite. Different parameters were
tested to optimize callus and somatic embryo formation: concentration of phytohormones, subculture frequency and light conditions. Callus formation required auxin (2,4-D) at an optimum concentration of 0.1 mM 2,4-D. These calli developed meristematic centers and when kept at the same auxin concentration (0.1 mM), the calli developed embryogenic structures. A greater proportion of plumule explants developed into calli bearing embryogenic structures when cultures were kept undisturbed and no subculturing was practised. Histological observations showed that formation of somatic embryos had already started in calli bearing embryogenic structures, but development of embryos occurred when the auxin concentration was reduced by a hundred-fold and cytokinin was added (50 µM 6-BAP), performing better under illumination (12 h photoperiod) than in the dark. Keeping cultures in these conditions and subculturing every three months, allowed embryos to germinate and the resulting shoots eventually developed into plantlets that, after acclimatization, grew successfully under ex vitro conditions. Following this protocol, different batches of cultures were tested and the performance was found to be reproducible.

As reported by Chan et al. (1998), with plumule explants shorter times were required to obtain somatic embryos (7-9 months) than those previously reported with inflorescence explants (14-20 months, Verdeil et al. 1994), and the yields were higher (nearly two-fold for calli and over ten-fold for calli bearing somatic embryos) than those reported with inflorescences (Verdeil et al. 1994). Acclimatization has been successful and plantlets did well in open environmental conditions since they continued producing new leaves, sexual organs and fruits. This protocol by Chan et al. (1998) reported that 60% of the explants formed initial calli using a local brand charcoal. In Montpellier, by using a different brand (Sigma acid washed charcoal), an improvement in callus induction of nearly 100% was obtained (J.L. Verdeil and V. Hocher, Montpellier, unpublished). Similar results were obtained afterwards at CICY. Therefore, careful selection of charcoal is very important. Clonal plantlets are now produced in most of the participating laboratories within the STD-3 project.

The use of embryo slices as explants and different treatments were evaluated at Queensland University. Polyethyleneglycol and ABA were tested for somatic embryo maturation and had very little or no effect when these chemicals were added separately, but when added simultaneously, they inhibited the growth of non-embryogenic calli and improved the maturation of somatic embryos (Samosir et al. 1999). Conditions that increased ethylene concentrations in the above coconut cultures were found to affect callus growth and somatic embryogenesis.
(Adkins et al. 1999). Accordingly, embryogenic calli were incubated with a number of additives that could reduce ethylene production (aminoethoxyvinylglycine), protect from ethylene (silver thiosulphate) or help combat ethylene-induced stress (polyamines). Coconut somatic embryogenesis was promoted (100%) by the addition of the polyamines putrescine and spermidine to the medium (Adkins et al. 1999). Unfortunately, when tested with plumule explants, no promotion of somatic embryo formation was obtained (CICY, unpublished data).

**Recent advances**

**The use of plumule explants**

During the past five years, research on the plumule system has continued in order to further improve its performance and different approaches have been tested. The compound 22(S), 23(S)-homobrassinolide was found to increase embryogenic calli and somatic embryo formation (1.5 and 2 fold respectively, compared with the controls) when applied as a pretreatment to plumule explants (Azpeitia et al. 2003). Cytokinins have been found to decrease embryogenic callus formation in plumule explants and therefore, the anticytokinin 8-azaadenine was tested. It increased somatic embryo formation 1.5 fold in relation to the control treatment (Azpeitia, 2003). There are other two approaches that resulted in even larger increases of yields: secondary embryogenesis and multiplication of embryogenic calli (CICY, unpublished results). Secondary embryogenesis is based on the use of somatic embryos as explants to produce more embryos. This process can be repeated several times. Therefore, it can be useful to increase the total somatic embryo yield obtained per original explant. Embryogenic calli multiplication allows increasing the yield of this type of calli several fold. Unpublished results (CICY) presently show that by combining these two approaches, thousands of embryogenic calli and tens of thousands of somatic embryos can be obtained from one plumule and the amounts depends on the number of multiplying cycles carried out. Furthermore, if these two approaches were combined with the use of 22(S),23(S)-homobrassinolide (Azpeitia et al. 2003), yields could be potentially increased even more. This three-approach strategy is being tested in collaboration with COGENT.

Regarding germination and post-germinative development of somatic embryos, studies had been limited by the low yields obtained. Therefore, these studies were approached using the coconut zygotic embryo as a model system. At CICY, this system showed that aerobic respiration was required for embryos to germinate (Pech y Ake et al. 2004). Percentage of
germination increased from 66% in liquid medium where embryos were submerged to 93% on solid medium where embryos could be placed with their micropylar end facing upwards exposed to the ambient atmosphere of the vial (Pech y Ake et al. 2004). This also resulted in increased conversion from 46% to 89% for liquid and solid media, respectively. In addition, the use of gibberellic acid (GA₃) further promoted both germination and conversion into plantlets (Pech y Ake et al. 2002). The use of ventilated vessels (with filter paper windows) when compared with sealed vessels, improved the leaf water loss control of plantlets formed from zygotic embryos cultured in these vessels (Talavera et al. 2001). Ex vitro survival of plantlets was found to be over 90% if proper development was allowed, plantlets should have a minimum of three bifid leaves and three main roots when transferred from in vitro conditions to ex vitro acclimatization conditions (Pech y Ake 2004). Some of the information obtained using zygotic embryos has been used to help define the optimal germination and post-germination development conditions for somatic embryos. This way, when plantlets derived from somatic embryos were allowed to develop three bifid leaves and three main roots, ex vitro survival was over 90% (CICY, unpublished results). Micropropagated palms established in permanent field conditions over four years ago have done well under and some are already bearing fruits. Similar (unpublished) observations have been noted at the Coconut Research Institute in Sri Lanka.

The use of other explants (leaf and inflorescence)
The information obtained on coconut regeneration using plumular explants can be useful to for research on the use of other explants. Research on micropropagation based on inflorescence or leaf explants has not been abandoned and it is one of the main objectives of a project supported by the Australian Centre for International Agricultural Research (ACIAR) involving laboratories at the University of Queensland (Australia), the Research Institute for Coconut and other Palmae (Indonesia), the Philippine Coconut Authority (Philippines), the University of Philippines at Los Baños (Philippines), the Cocoa and Coconut Research Institute (Papua New Guinea) and the Oil Plant Institute (Vietnam). It would be interesting to test the combined use of secondary embryogenesis and embryogenic callus multiplication with inflorescence and leaf explants.

Genetic engineering
In addition, there are new areas of research intended to open new avenues for coconut micropropagation improvement and probably applications. These studies are based on molecular techniques and presented below.
A different approach to increase coconut micropropagation efficiency, not tried before, is to improve the embryogenic capacity of coconut tissues by inserting genes related to this capacity. Hence, the genes and the protocols for their insertion through transformation techniques are needed. Therefore, through a collaborative effort, researchers from Max Planck and Fraunhofer Institutes (Germany) and CICY are attempting to develop transformation protocols for coconut tissues. *Agrobacterium tumefaciens*-mediated transformation and particle gun (Biobalistic) DNA delivery methods have been applied to transform coconut cells using the reporter genes *uidA* that codify for b-glucuronidase (GUS), *gfp* for the green fluorescent protein (GFP) and *rfp* for the red fluorescent protein (RFP) under the control of constitutive promoters such as 35S CaMv and Ubiquitin from Maize. Transient transformation was successfully obtained with both transformation methods and the three reporter genes. However, the use of *uidA* was hampered by the finding of endogenous GUS activity in coconut calli. Stable transformation has been confirmed for *gfp* in *A. tumefaciens*-mediated transformed calli. In addition, the effect of hygromicin and bialaphos has been evaluated as selective agents for transformed cells. The former was shown to be useful, whereas the latter was not. These results have yet to be published.

In vitro manipulation of coconut tissues is limited by the scarce knowledge of their cellular behaviour. A major problem is the difficulty to maintain the meristematic potential of tissues and to further control their capacity for cell division. Therefore, the L’Institut de Recherche pour le Développement - Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement (IRD-CIRAD) started a study on the cell cycle status of in vitro coconut cells. Using flow cytometry, most of the cells were found to be in G0/G1 phase (around 90% in nodular calli and shoot meristems), with a low mitotic index (less than 0.5%) (Sandoval et al. 2003). These results are in agreement with those obtained by Jesty and Francis (1992) with microdensitometry. Adding aphidicolin (a synchronisator of cell cycle) to the media, around 80% of cells were blocked in G0/G1 and only 20% of meristematic cells were cycling cells (Sandoval et al. 2003). Using immature inflorescences and immature leaves, the study showed that flow cytometry methods could be used to rapidly assess the ability of tissues cultured in vitro to divide. It appears to be a useful tool for a more effective monitoring of the meristematic potential of tissues cultured in vitro, in relation to culture conditions.

The basic components of the cell cycle machinery appear to be conserved in all eukaryotes and particularly those controlling the re-entry of cells in the cell cycle (transition between G0 and G, transition between G1 and S). Screening a coconut shoot meristem cDNA library with
heterologous probes (from Maize, *Arabidopsis* and mouse) allowed to isolate cDNA (Cyclin D, cyclin dependant kinase, E2F and retinoblastoma) involved in the retinoblastoma pathway known to control the re-entry of cells into cell cycle and the early cell cycle phases. Among the genes isolated from coconut, those encoding D-type cyclins are of great interest because they are known as favourable candidates for linking the perception of the environment (culture conditions) with cell cycle activity in plants. The study of the accumulation of these cDNA in *in vitro* coconut tissue is now on the way through collaboration between IRD-CIRAD and CICY. It should help to understand the mechanisms controlling the switch from non-cycling cells to cycling cells.

**Perspectives and conclusion**

The account presented here of the research for the development of efficient coconut micropropagation protocols via somatic embryogenesis shows that solid progress has been made and that this has been possible because there has been collaboration among institutions all over the world, particularly in the last ten years. Rapid progress has been made using plumule explants, but there is still work to be done. For instance, there is a need for improving embryo development, mastering germination and post-germination development and continuing genetic stability/integrity testing. Interest in plumule micropropagation started because this could be a useful model system, thus developments obtained with this system should now be tested with other explants such as immature inflorescences and young leaves. From a practical point of view, plumule micropropagation cannot be used for elite plant propagation. However, it can be applied for superior population propagation such as genotypes that are disease resistant. Countries affected by the phytoplasma-associated diseases need urgently at least hundreds of thousands of resistant palms. In the near future, plumule micropropagation could be the way to obtain them. Another application for the plumule system is the multiplication of the Makapuno coconut currently produced by rescuing the embryo of the non-germinating Makapuno nut.

On the other hand, research work on coconut somatic embryogenesis should also incorporate the latest trends in developmental biology as they become available and in particular those concerning the control of embryogenesis and shoot meristem differentiation and functioning. As mentioned above, transformation protocol development is already under way based on plumule micropropagation. Regarding the search for genes, there are recent reports on interesting genes that have been isolated from *Arabidopsis* such as BAYBY BOOM (Boutilier *et al*. 2002) and LEC1 (Lotan *et al*. 1998; Stone *et al*. 2001) encoding transcription factors involved in
the conversion from vegetative to embryonic growth. The over-expression of these genes in Arabidopsis led to the formation of somatic embryos at the surface of the leaves with a high rate. Such genes are attractive and are promising tools for improving somatic embryogenesis and clonal propagation in coconut.

Finally, it is very important that for future research efforts, collaboration among institutions in different countries is intensified, not only to sustain current progress in coconut micropropagation research, but also to allow it to take place rapidly. To successfully achieve this, the continuing support of Asian Pacific Coconut Community (APCC), Bureau for the development of research on tropical perennial oil crops (BUROTROP) and the International Coconut Genetic Resources Network (COGENT), is absolutely necessary.

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