ADVANCES IN OIL PALM AND COCONUT MICROPROPAGATION

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SUMMARY: *Oil Palm (*Elaeis guineensis *Jacq.) and Coconut palm (*Cocos nucifera *L.), two major oleaginous crops cultivated in the intertropical area, are only seed propagated.* In vitro *culture techniques allow vegetative propagation of these species through somatic embryogenesis on calluses obtained from immature leaves or inflorescences.*

With oil palm, 15-year development of this propagation process has yielded experimental plantations in Africa, Latin America and South East Asia. Three different patterns were defined regarding the evolution of calluses, based on different anatomical and physiological characteristics. They led to three different ways of regeneration. Embryogenesis on nodular calluses is routinely used in pilot-scale laboratories. The plants produced show a good true-to-type rate, but this propagation process is costly, essentially due to its low productivity. Regeneration through somatic embryogenesis on friable, fast-growing callus produces always produces sterile palms bearing abnormal inflorescences. This phenomenon is due to a malfunctioning of cytokinin metabolism induced by the level of growth regulators in the culture media. A third type of callus, termed granular callus, can be used to produce embryogenic suspensions. This system shows a very high productivity, can be automated, and leads to the production of single somatic embryos with good synchronization and with at low production cost. An experimental plantation of palms produced through embryogenic suspensions was set up in Côte d'Ivoire and the first available findings do not indicate any abnormality.

Experiments performed on coconut by the ORSTOM/CIRAD team in Montpellier have led to regeneration from mother plants of different genotypes. Analytical approaches to culture media, nutrient uptakes and systematic histological examination, have recently allowed mastery of a regeneration process, with production of complete somatic embryos, which developed into plantlets. Work is now being directed towards the search for culture conditions that favour intense proliferation for mass plantlet production.

CIRAD : Centre de Coopération International en Recherche Agronomique pour le Développement.

ORSTOM : Institut Français de Recherche Scientifique pour le Développement en Coopération.

IRHO : Institut de Recherche pour les Huiles et Oléagineux.

INTRODUCTION

Oil palm and coconut palm are the two major oleaginous crops cultivated in the intertropical area. Their breeding by conventional methods is limited by several factors. Firstly, they are perennial crops, and about ten years are required in order to assess the value of a progeny. Secondly, they are allogamous and large heterogeneity is observed in hybrid planting material. Lastly, oil palm and coconut are only seed propagated and there is no classical vegetative propagation technique avalaible. For these reasons, *in vitro* propagation has been investigated for more than twenty years with the aim of propagating the best hybrid palms on a large scale. The *in vitro* culture of apices was studied with juvenile oil palms (Staritsky, 1970) but did not produce any valuable results. An alternative is the reversion of flower bud sampled on adult plants. In an important study of coconut, Blake and Eeuwens (1981) concluded that the most promising approach was somatic embryogenesis, which today is used by all teams involved in the vegetative propagation of these palms (see Blake, 1990, and Wooï, 1990, for reviews).

The aim of the ORSTOM/CIRAD team at the ORSTOM Research Centre in Montpellier is to set up processes for oil palm and coconut true-to-type regeneration, for large-scale propagation on the best material produced by breeding programme. The first studies of oil palm were carried out at the beginning of the 70's (Rabéchault and Martin, 1972) and several pilot units were developed during the 80's, in Côte d'Ivoire, Malaysia and Indonesia. Several methods of regeneration by somatic embryogenesis were studied ; the advantages and drawbacks of each are presented in this paper. Our experiments on coconut started in the 80's. There was an implicit assumption that coconut would respond in similar manner to oil palm (Blake, 1990). In fact, the behaviour of coconut tissue differs greatly from that of oil palm and repetitive regeneration was only achieved in 1991 (Verdeil *et al.*, 1992).

MATERIAL AND METHODS

In vitro culture procedure:

I - Oil palm:

Plant material was nursery or adult *tenera* palms chosen for their high agricultural value, generally *Dura* x *Pisifera* or *Dura* x *Tenera* hybrids. The basal media used at all stages described in this paper contained Murashige and Skoog macroelements modified by Rabéchault et Martin (1976), Nitsch's microelements (1969), Morel and Wetmore vitamins (1951), sodium ascorbate (100mg l^{-1}), casein hydrolysate (500mgl^{-1}) and agar (8 gl^{-1}).

Callogenesis was performed on immature leaflets sampled from the spike, on basal culture media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and

trichloropropionic acid (TCPP) (0.1 to 1mg l^{-1}), and glucose (20gl^{-1}). Explants were incubated for 12 to 20 weeks at 27 °C in the dark.

Plant regeneration from nodular calluses: Calluses were isolated from explants and transfered on basal media supplemented with glucose (20g l^{-1}) and activated charcoal (3 g l^{-1}). 2,4-D, adenine sulfate, benzylaminopurin (BAP) were added at various concentrations. Embryogenic structures derived from nodular calluses were transferred on to hormone-free basal media containing sucrose (30 g l^{-1}). These conditions ensured mass propagation of embryos by proliferation through adventitious embryogenesis with shoot development. Shoots were manually separated when they reached a height of 5-7cm and were rooted on basal media containing naphthalenacetic acid (NAA) (1 mg l^{-1})

Plant regeneration from Fast Growing Calluses (FGC): Nodular calluses cultured on basal media with high auxin content gave rise to a second type of callus termed FGC. FGC were grown on basal media supplemented with 2,4-D and NAA. Embryogenesis was obtained by addition of BAP and/or antiauxin (Hanower and Hanower, 1984). Plantlets were produced from somatic embryos as described above.

Plant regeneration from embryogenic suspensions: A third type of callus of small meristematic nodules was used for embyogenic suspensions. Callus (approximately 500 mg) was placed in a 100 ml erlenmeyer flask containing 20 ml of liquid basal medium without agar, supplemented with 2,4-D (100 mg l^{-1}), glucose (20 g l^{-1}), adenine sulfate, BAP, and activated charcoal. Flasks were placed on a gyratory shaker at 90 rpm. After sieving, cell aggregates were transfered to liquid basal medium supplemented with sucrose (30 g l^{-1}) for 1 month for proembryo maturation, then plated on the same medium solidified with agar (8 g l^{-1}) until germination.

II- Coconut:

Plant material consisted of adult palms (20-25 years) belonging to the Malayan Yellow Dwarf variety and the Malayan Yellow Dwarf x West African Tall hybrids.

Basal media used at all stages described below comprised Eeuwens macroelements and microelements (1976), Morel and Wetmore vitamins, sucrose (40g.l^{-1}), activated charcoal (2-3g l^{-1}) and agar (7,5g l^{-1}).

Callogenesis was performed with two different types of explant, immature, leaf and inflorescences. Leaf explants were fragments of immature leaves, sampled from the spike without wounding the apex. Inflorescence explants were fragments of rachillae from young inflorescences with an internal spathe length of between 15 and 40 cm. They were cultured on basal media supplemented with 2,4-D (30 to 80 mg l^{-1}) in test tubes placed in the dark at 27°C. Embryogenesis was induced by increasing the level of 2,4-D. Somatic embryo maturation was obtained by gradually reducing the 2,4-D concentration. Shoot development took place in light, on hormone-free medium with activated charcoal. Rooting required an auxin treatment with NAA.

RESULTS AND DISCUSSION

I - Oil palm:

Plant regeneration from nodular calluses:

Appearance and growth of the nodular calluses:

In young leaves, the callus originates close to the veins (Ahée *et al.*, 1981) (Figure 1), and callogenesis has been studied histologically in great detail by Schwendiman *et al.* (1988). A transverse section in the primary explant revealed that callus emerges *via* multiplication of perivascular cells, resulting in a layer (four or five cells thick) of very meristematic tissue close to the xylem. This layer evolves into a cambium-like zone which differentiates rapidly into a sclerenchyma towards the inside of the nodule, and into quiescent cells towards the outside (Figure 2). Pseudocambium activity ensures the growth of the callus by invaginations of the pseudocambium, which lead to the formation of individualized nodules.

Embryogenesis:

During the embryogenesis, the cambium-like zone is markedly modified. The meristematic cells of the nodular callus evolve into embryogenic-type cells, according to their histochemical characteristics: high nucleocytoplasmic ratio, large central nucleus with a single nucleolus, numerous lipid droplets, dense cytoplasm intensely stained by naphtol blue black, gelification of the median lamella which individualizes isolated cells and cell clusters. This differentiation of the cambium-like zone, which is not visible to the naked eye, seems to be the first phase of embryogenesis in nodular calluses. This phenomenon is followed by an intense multiplication of the embryogenic cells. The cell clusters grow, become epidermized and give rise to somatic embryos.

Proliferation:

The embryogenesis phenomenon observed in nodular calluses leads to a complex system termed polyembryonic culture. It consists in a group of proembryonic formations comprising meristematic cells and of somatic embryos at various developmental stages. On hormone-free medium, these polyembryonic cultures produce new adventive somatic embryos and simultaneously the most advanced embryos develop into shoots (Figure 3). Closely linked to the culture conditions and the genotype of the explant, the phenomenon of adventive embryogenesis is rare and leads to the production of a limited number of shoots. In the most favourable cases, however, polyembryonic cultures proliferate continuously and ensure mass propagation.

The shoots are separated manually and rooted. Plantlets are weaned under controlled conditions in order to avoid water stress, which is common at this stage for all plants

produced *in vitro*. The observations made by our team show that it is necessary to develop the root system during the *in vitro* phase. Indeed, rooting during hardening could never be obtained, even after an auxin treatment of the shoots. After a 4-week period of acclimatization, the plantlets are transfered to the prenursery (Figure 4) and cultivated like seedlings and then transfered to the field.

The efficiency of this process were recorded for 442 palms sampled in Côte d'Ivoire between 1982 and 1992. Embryogenesis was observed in 95 % of palms, but large-scale embryogenic proliferation was obtained in only 124 differents palms. Total plantlet production reached over 630,000 plants which were used in different planting programmes. In particular, clonal trials were set up with 204 clones over about 250 ha (Durand-Gasselin, pers.comm.).

The value of a given clone produced through somatic embryogenesis depends on the agricultural value of the donor plant, and on the value of the process in terms of genetic stability.

As concerns the yields of clonal material, data were recorded in Côte d'Ivoire for 180 hectares of comparative field trials involving 85 clones (Durand-Gasselin *et al.*, 1990). The first results indicated a higher homogeneity in the clones and a 14 % increase in Fresh Fruit/Bunch production in comparison with seedling material. More recent results obtained with young clonal palms (Le Guen *et al.*, 1991) indicated a 25 % maximum increase in oil production compared with the best seeds, for the four best clones.

Plant regeneration from Fast Growing Calluses (FGC)

When nodular calluses are cultivated on media of high auxin content, a new type of callus can appear, termed Fast Growing Callus (FGC) (Smith and Thomas, 1973; Ahée *et al.*, 1981; Hanower and Pannetier, 1982). FGCs show a very high growth rate, are whitish, soft and friable, and are made of dispersed clumps of meristematic cells, large cells and lacunae (Ahée *et al.*, 1981). They originate from a disorganization of the procambial zone, in which the meristematic cells show a disorganized proliferation. When FGCs are transfered to media with reduced auxin concentration and/or added with cytokinin, intense embryogenesis was observed, sometimes involving the entire FGC. This phenomenon can be accentuated by the addition of antiauxins (2-o chlorophenoxyisobutyric acid (10^{-6} M) or 7-azaindol (10^{-5}M) or phenolic compounds (10^{-3} M phloridzin or phloroglucinol) to the medium (Hanower and Hanower, 1984). The meristematic cells become embryogenic, multiply and give rise to embryogenic structures.

The production of plantlets through the FGC pathway was investigated by the ORSTOM/CIRAD research group at the end of the 70's (Ahée *et al.*, 1981). Mass production is ensured by the proliferation of FGCs on media with high auxin content, followed by the differentiation of meristematic clumps into somatic embryos after the addition of cytokinins to the medium. These embryos can develop directly into plantlets or give rise to polyembryonic cultures. The major drawback of this pathway

as regards true-to-type regeneration is the multiplication of undifferentiated tissues for extended periods on media with high auxin concentration Observations on this type of tissue revealed a high proportion of polyploid and aneuploid cells (Smith and Thomas, 1973), which is unfavourable to the clonal fidelity of the regenerated plants. For these reasons, the process through FGC has not been developped on a large scale (Duval *et al.*, 1988).

Plant regeneration from embryogenic suspensions

Embryogenic suspensions are established from a rarely observed type of callus, which corresponds to development of nodular calluses cultivated under standard conditions and which is termed granular calluses. These are made of small (< 1 mm) meristematic nodules located at the periphery of the parenchymatous callus to which they are only slightly connected. Histological examination revealed meristematic nodules of variable sizes, from two to several tens of cells. The most advanced developmental stage reached by these structures is a protoderm, even though culture conditions are identical to those used for the induction of somatic embryos on nodular calluses. When these nodules are transfered to a liquid medium containing 2,4-D and activated charcoal, they can give rise to suspensions of embryogenic cell clusters (Figure 5). These suspensions produce individualized somatic embryos after one subculture in liquid hormone-free medium and plating on solid medium (de Touchet *et al.*, 1991) (Figure 6). Like most of the embryogenic suspensions from various crops cultured in liquid media, productivity is much higher than in the standard procedure on gelified media (Redenbaugh, 1991). Observations made in our laboratory with oil palm embryogenic suspensions indicate a density of 10^5 cell aggregates per litre and a mass multiplication rate of 4 per month during the proliferation stage. Transfer to hormone-free media induced the differentiation of cell clusters into proembryos without multiplication. The density of inoculation is 5.10^4 aggregates per litre. One month later, plating of proembryos allowed the maturation and conversion of somatic embryos at a rate of 40%. Estimations made from our cultures indicate a productivity of 45,000 plantlets per month from one litre of embryogenic suspension in the proliferation stage. This method of regeneration has been tested on 17 different clones in Montpellier, and a first trial was set up in 1991 at La Mé Station in Côte d'Ivoire. Initial data indicate good genetic stability, but it is too early to draw any firm conclusions.

Genetic stability

The vegetative abnormalities mentioned (curled leaves, erected palms, thick leaflets) in oil palm propagated through somatic embryogenesis are rare and are also observed with seedlings. The culling percentage is therefore equivalent for clonal and sexual material.

The occurrence of a floral morphogenesis abnormality on clonal palms was first mentioned in 1986 by Corley *et al.* in Malaysia and then by Duval *et al.*, (1988) in clonal trials in Côte d'Ivoire. This so-called "mantled" abnormality (Corley *et al.*, 1986) induced by *in vitro* culture consists of feminization of the male organs on

female flowers. This leads to the formation of abnormal (mantled) fruits or to the partial (or complete) sterility of the palms, depending on the intensity of this phenomenom. In the most severe cases, besides the sterility of female flowers, male flowers are affected, stamens developing into pseudocarpels. This abnormality is reversible, and in many cases definitive recovery is observed after several months or years in the field (Durand-Gasselin *et al.*, 1990). The avalaible information indicates that the abnormality is probably a consequence of the micropropagation technique. In Côte d'Ivoire, all the palms produced through the FGC pathway exhibited a severe abnormality (Duval *et al.*, 1988). By contrast, the abnormality was observed on a limited number of plants originating from somatic embryos obtained on nodular calluses. Indeed, an average of only 5 % of severely mantled palms was observed in 1992 among 21,000 plants from 92 clones. However, very high variability was observed among clones, the most affected clone displaying 67 % of abnormal palms (Durand-Gasselin, unpubl. res.).

As this abnormality is expressed in the reproductive organs and during sexual differentiation, it suggests that the hormone metabolism may be involved. Numerous studies have underlined the importance of growth regulators in the sexualization processes in plants (Chailakhyan, 1979; Durand and Durand, 1984), particularly in the case of oil palm (Corley, 1976). Analyses of endogenous growth regulators in immature inflorescences sampled on normal and abnormal palms revealed cytokinin deficiency in the abnormal inflorescences (Besse *et al.*, 1991). Hormone analysis confirmed observations made in Côte d'Ivoire stressing the importance of the type of callus as regards the regeneration of true-to-type plants were confirmed by hormone analyses. Indeed, they showed that the metabolism of cytokinins from the zeatin group was disturbed in FGCs (Besse *et al.*, 1992), probably due to too high levels of 2,4-D in the embryogenesis media (Besse, 1992). These recent results specify the determinism of the mantled abnormality and raise the possibility of controling this phenomenon in the near future (early markers).

II - Coconut:

Different methods of regeneration through somatic embryogenesis were investigated in coconut, using two different types of explant: immature leaf fragments and immature inflorescences.

Appearance and growth of callus:

On the two types of explant, the first callus appeared after 4 months of culture. Optimal callogenesis occurred around the ninth month with an average frequency of 35 to 40 %. Histological studies showed that there were two possible origins for the neoformed tissues. An internal origin was observed for calluses derived from leaf explants and for those formed on the rachis cross-section of inflorescence fragments. In both cases, they appeared in the 4^{th} month of culture, on the perivascular cells. This origin is identical to that described for oil palm and the callus showed nodular-type growth.

Another type of callus of external origin issued from the floral sites of inflorescence later (after the fifth month of culturing). These calluses derived from pre-existing floral meristems and were similar to the calloids described by Branton and Blake (1984). Within the subcultures, these calluses became very homogenous and of granular texture. This was also observed with calluses of internal origin.

Embryogenesis:

A first type of differentiation could be obtained on every sort of callus with the appearance of an epidermized complex meristematic structure when the level of 2,4-D in the culture media was lowered (40-60 mg l^{-1}). This led to embryogenesis of multicellular origin which produced embryo-type structures that were usually incomplete (haustorium with or without root pole, embryos of leaf-like appearance).

With granular calluses, increasing the 2,4-D concentration in the culture media (60-80 mg l^{-1}) led to embryogenesis from unicellular origin, with individualization of typical embryogenic cells, similar to those described for oil palm. These cells had a large, very active nucleus, dense cytoplasm rich in soluble proteins and numerous small vacuoles. They were isolated from the rest of the callus by a thick wall.

The formation of typical pro-embryos with all the characteristics of the young zygotic embryos described by Haccius (1978) was observed later. They appeared within twenty months of callus isolation in about ten per cent of the calluses in the best cases.

Embryo maturation:

After elongation of the haustorium, a cotyledonary notch appeared at the base of the embryo and this immediately preceded root pole differentiation.

Histological cross-sections at the cotyledonary notch, revealed somatic embryos with perfectly structured stem meristem, consisting of a protruding meristematic dome surrounded by leaf primordia. The haustorial tissue located opposite the stem meristem contained starch reserves. The positioning of starch grains, which is a good indication of embryo polarity, was identical to that found in zygotic embryos. The embryogenic structures were subcultured every two months until the first leaf appearance. Shoot development from isolated embryos took place in the light, on a hormone-free medium with activated charcoal.

Once the first leaf sheaths were emitted, the embryo root developed spontaneously (without hormone treatment), thus indicating the existence of a functional bipolar axis within the embryo structures. However, in the majority of cases, rooting required a treatment with naphthalen acetic acid.

Regeneration of forty plantlets belonging to five genotypes was achieved in the Montpellier laboratory (Figure 8). Two palms were transfered under natural conditions, and no vegetative abnormality was detected. Research in progress is

aimed at the large-scale multiplication by adventitious embryogenesis on somatic embryos and the setting up of embryogenic suspensions from granular calluses.

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Legends

Figure 1: Emergence of calluses on leaf explant.

Figure 2: Cross-section of nodular callus. (magnification 500).
-CL: cambium-like zone
-SC: schlerenchyma
-QC: quiescent cells

Figure 3: Polyembryonic culture of oil palm.

Figure 4: Prenursery of clonal planting material.

Figure 5: Cross-section of embryogenic cell cluster from liquid suspension. (magnification 160).

Figure 6: Single somatic embryo germinated.

Figure 7: Cross-section of embryogenic cells of coconut. (magnification 500).

Figure 8: *In vitro* coconut palm plantlets.

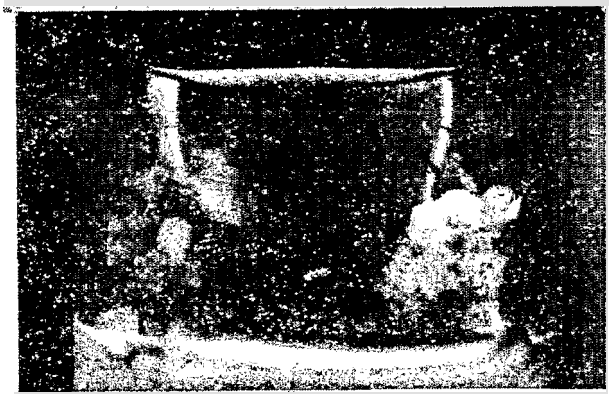


Figure 1

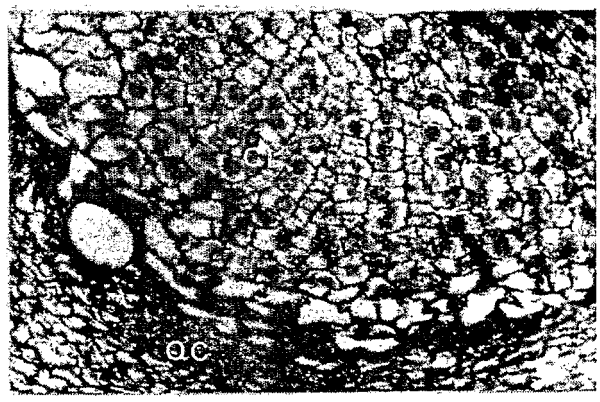
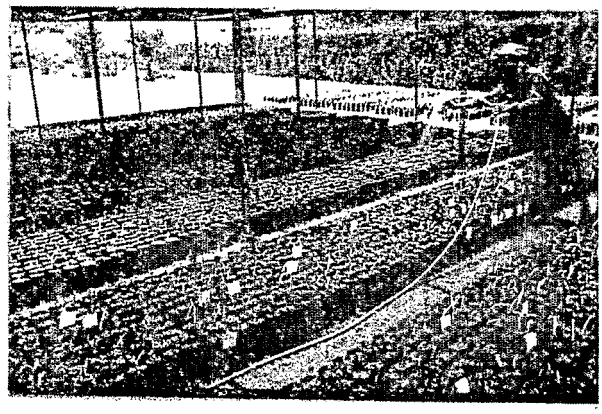


Figure 2

Figure 3



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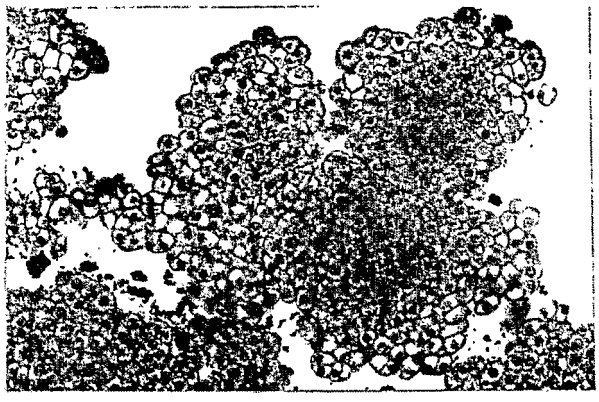

Figure 5

Figure 6

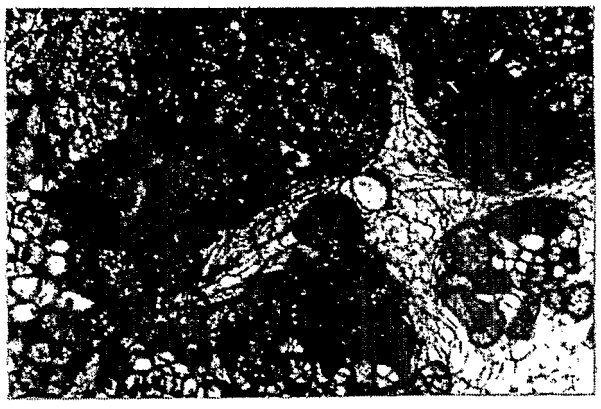

Figure 7

Figure 8

Adapted Propagation Techniques for Commercial Crops of The Tropics

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