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## COCONUT CLONES THROUGH SOMATIC EMBRYOGENESIS

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### ABSTRACT

An important stage has been reached in the mastery of coconut *in vitro* vegetative propagation: reproducible formation of ramets from five clones belonging to different genotypes. Two sources of tissue taken from selected adult individuals were used: leaf explant and inflorescence explant. The procedure developed comprises the following steps: callogenesis induction on primary explants, embryogenesis induction on the calli obtained, embryo maturation and ramet production.

The final stage had not been mastered until now. The embryos obtained were incomplete and showed no stem meristem, which was the major obstacle in developing a coconut cloning procedure.

An analytical approach provided a better understanding of the causes behind embryogenesis obstruction. These new data led to the consistent production of complete embryos, described for the first time on a histological basis.

Work is now being directed towards the search for a culture medium that favours intense embryo proliferation for mass ramet production.

### INTRODUCTION

Productivity in coconut plantations could be significantly improved if uniform planting materials are obtained by cloning high-yielding individuals. Vegetative propagation of tolerant or resistant individuals could also be an effective way of controlling some diseases, such as lethal yellowing or *cadang-cadang*, which threaten coconut groves in South America and South-East Asia, respectively.

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Unfortunately, the coconut palm, which is usually allogamous, is only multiplied by seed at the moment. The only hope of asexual propagation, lies in *in vitro* culture techniques and, more particularly, in somatic embryogenesis.

Whilst the initial results of somatic embryogenesis research were promising (rapid growth of embryo-type formations in calluses), only three teams have so far reported complete regeneration of plants from somatic tissues (Raju *et al.*, 1984; Branton and Blake, 1984; Buffard-Morel *et al.*, 1988). Apparently, the ramets obtained came from a limited number of cultures. As far as we know, coconut cloning has still to be completely mastered.

An important stage was reached at the beginning of 1991 by the ORSTOM/IRHO-CIRAD team, who obtained reproducible ramets from five different clones. The experimental procedure followed was as follows:

- 1) Callogenesis from leaf or inflorescence fragments, leading to the formation of non-organized tissues (calluses).

- 2) Callus subculturing and embryogenesis induction.

- 3) Embryo maturation and conversion leading to ramet production.

These encouraging results (reproducible formation of complete embryos) were obtained through an analytical approach designed to provide a better understanding of the causes behind halts or deviation in somatic embryogenesis, which are often seen with coconut.

In this paper, we shall be describing this approach and giving the main results of:

- i) Histological studies (description of embryogenic calluses and for the first time on coconut, complete somatic embryos).

- ii) Nutritional studies (analysis of medium composition and determination of specific nutritional requirements in the initial phases of embryogenesis on calluses).

## MATERIALS AND METHODS

The planting material was supplied to us by the Marc Delorme Station in the Ivory Coast. It was taken from adult individuals (20 to 25 years) primarily the Malayan Yellow Dwarf variety and the Malayan Yellow Dwarf × West African Tall hybrid (PB 121 created by IRHO); a few trials were conducted with another hybrid (PB 111) and other dwarf varieties.

Samples can be taken without wounding the apex; growth resumption is therefore, possible and has actually been observed. The leaf explants used were fragments (1 cm long) of young non-chlorophyllous leaves surrounded by the petiole bases of older leaves.

The inflorescence explants used were fragments of rachilla (1 to 1.5 mm thick) from young inflorescences with an internal spathe length of between 15 to 40 cm. For each individual tested, 1,200 to 2,000 leaf explants, and as many inflorescence explants, were cultured.

The standard medium consisted of: Eeuwens mineral solution, Morel and Wetmore vitamins, sucrose (40 g/l) and agar-agar at 7.5 g/l, to which

2,4 dichlorophenoxyacetic acid (2,4-D) was added (30 to 80 mg/l), along with activated charcoal (2 or 3 g/l), in order to limit tissue browning.

The range of 2,4-D and activated charcoal quantities mentioned takes into account reactivity differences between individuals of the same genetic origin or different origins. The test tubes containing the cultured explants were placed in the dark at  $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

## RESULTS

### a) Callogenesis from Leaf and Inflorescence Explants

This stage has now been well mastered on the two explant sources worked on by the ORSTOM-IRHO team.

The first calluses appeared after four months' culturing (Figs. 27.3-1 and 27.3-2), and optimum callogenesis occurred around the ninth month (on an average, 35 to 40 per cent of leaf or inflorescence explants gave calluses). The callus production varied significantly according to the physiological age of the explant and the auxin concentration in the culture medium.

Histological studies showed that there were two possible origins for the newly formed tissues:

i) An internal origin 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 fourth month of culturing, on the perivascular cells. This origin is identical to that described for oil palm.

ii) An external origin observed for calluses forming on floral sites. These calluses appeared later (after the fifth month of culturing). They were derived from pre-existing floral meristems and were similar to the calloids described by Branton and Blake (1984).

Whatever their tissue origin, callus growth and multiplication are ensured by the establishment of a peripheral meristematic zone organized all the way through as a cambial type meristematic zone.

### b) Embryogenesis Induction

Calluses were isolated from the sixth month of culturing onwards. They were placed on media containing activated charcoal and enriched with 2,4-D (compared to the callogenesis media).

Under these culturing conditions, the callus growth and multiplication ensured by the peripheral meristematic zone can continue for months, with no notable change. It can lead to secondary callus growth. These calluses were used to make up homogeneous tissue strains on which a large number of embryogenesis induction trials were conducted.

#### 1) Histological Description of Embryogenic Calluses

Two types of embryogenesis processes were observed:

a) One from organogenetic type of epidermal complex meristematic structures which can lead to embryogenesis of multicellular origin. This process leads to embryo type structures that are usually incomplete (haustorium with or without root pole, embryos with a leaf-like appearance).

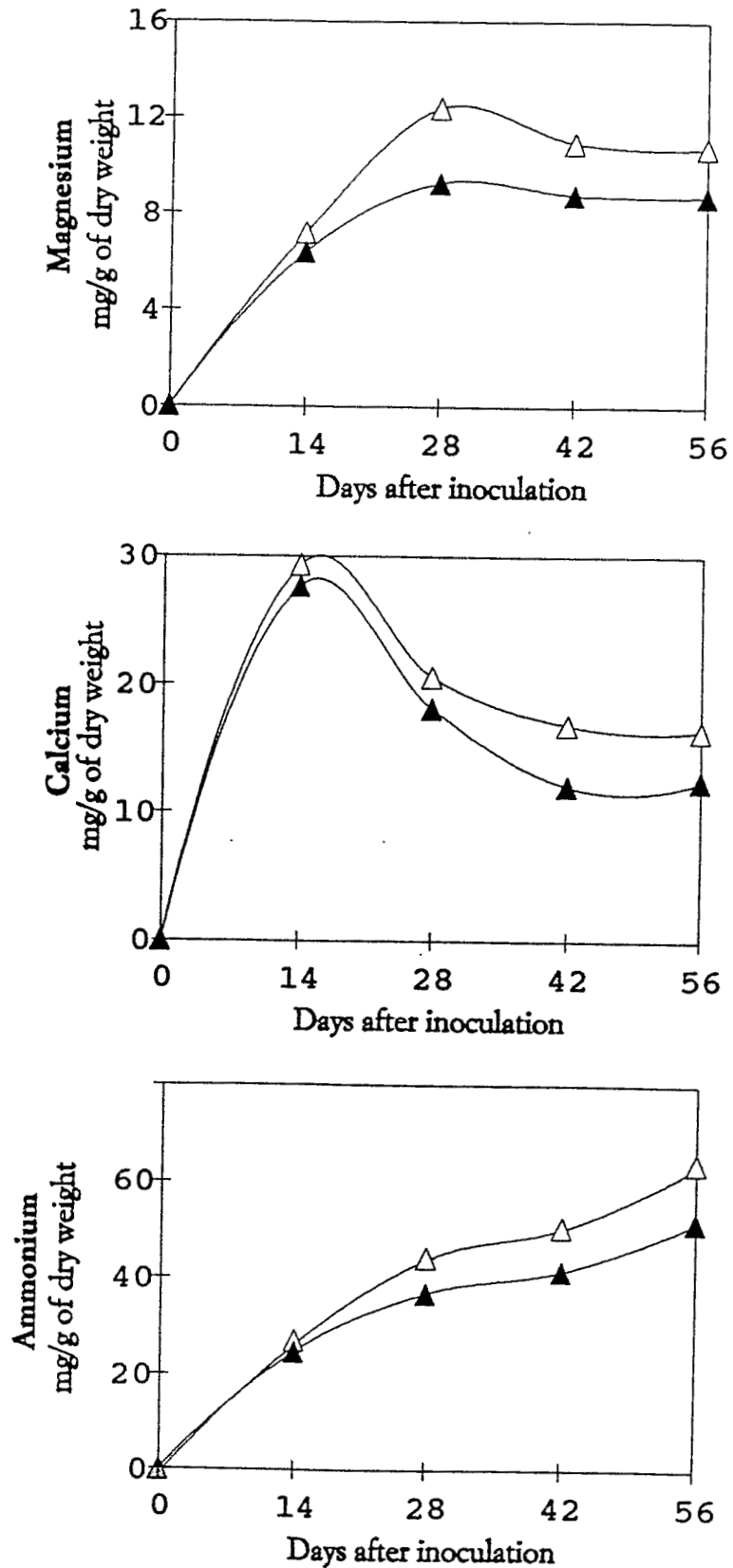


Fig. 27.1: Coconut somatic embryogenesis. Evolution of magnesium, calcium and ammonium during callus multiplication (▲) and embryogenesis initiation (Δ).

b) The other of unicellular origin, with individualization of typical embryogenic cells, similar to those described for oil palm (Schwendimann *et al.*, 1990). These cells have a large, very active nucleus, dense cytoplasm rich in soluble proteins and numerous small vacuoles. They are isolated from the rest of the callus by a thick wall.

The second process led to the formation of typical pro-embryos with all the characteristics of the first stages of zygotic embryos as described by Haccius (1978).

## 2) Determining the Specific Nutritional Requirements for Embryogenesis

A kinetics study conducted on two strains of homogeneous calluses revealed the specific mineral nutrient requirements during induction and during the initial phases of somatic embryogenesis.

The cations  $\text{NH}_4^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  and the anions  $\text{NO}_3^-$ ,  $\text{Cl}^-$ ,  $\text{H}_2\text{PO}_4^-$  and  $\text{SO}_4^-$  were titrated in the culture media using HPLC (DIONEX ionic chromatograph). For the same tissue strain, a comparison of callus nutrient requirements on multiplication media and on the embryogenesis medium revealed greater ammonium, calcium and magnesium absorption per g of dry matter for embryogenesis (Fig. 27.1).

These specific nutritional requirements are accompanied by an increase in protein synthesis (Fig. 27.2) reflected in an accumulation of proteins in the embryogenic cells (see results of the histological approach). This study has largely contributed to better knowledge of somatic embryogenesis factors in coconut. It therefore, provides the opportunity of controlling it better by acting upon medium composition and culture transfer frequency.

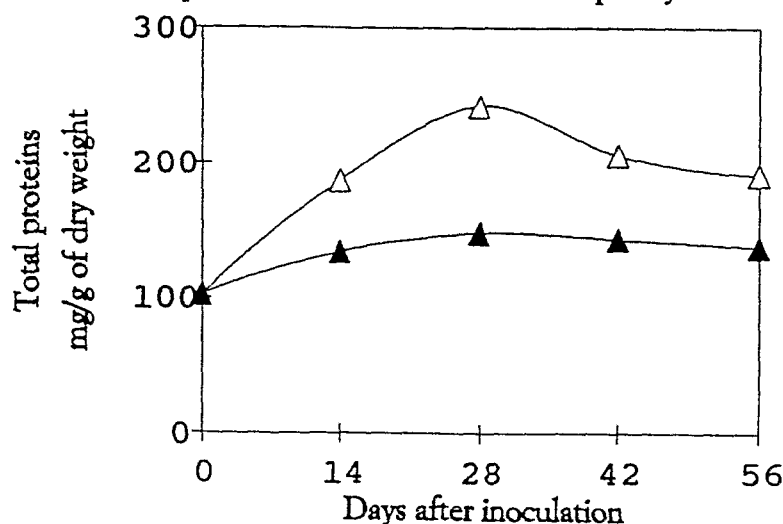


Fig. 27.2: Coconut somatic embryogenesis. Evaluation of total protein content in callus during multiplication (▲) and embryogenesis initiation (△).

## 3) Embryo Maturation/Conversion and Shoot Development

Somatic embryo maturation was obtained by gradually reducing the 2,4-D concentration compared to the auxin level enabling embryogenesis induction (Fig. 27.3-3). After elongation of the haustorium, a cotyledonary notch appears at the base of the embryo; this immediately precedes root-pole differentiation.

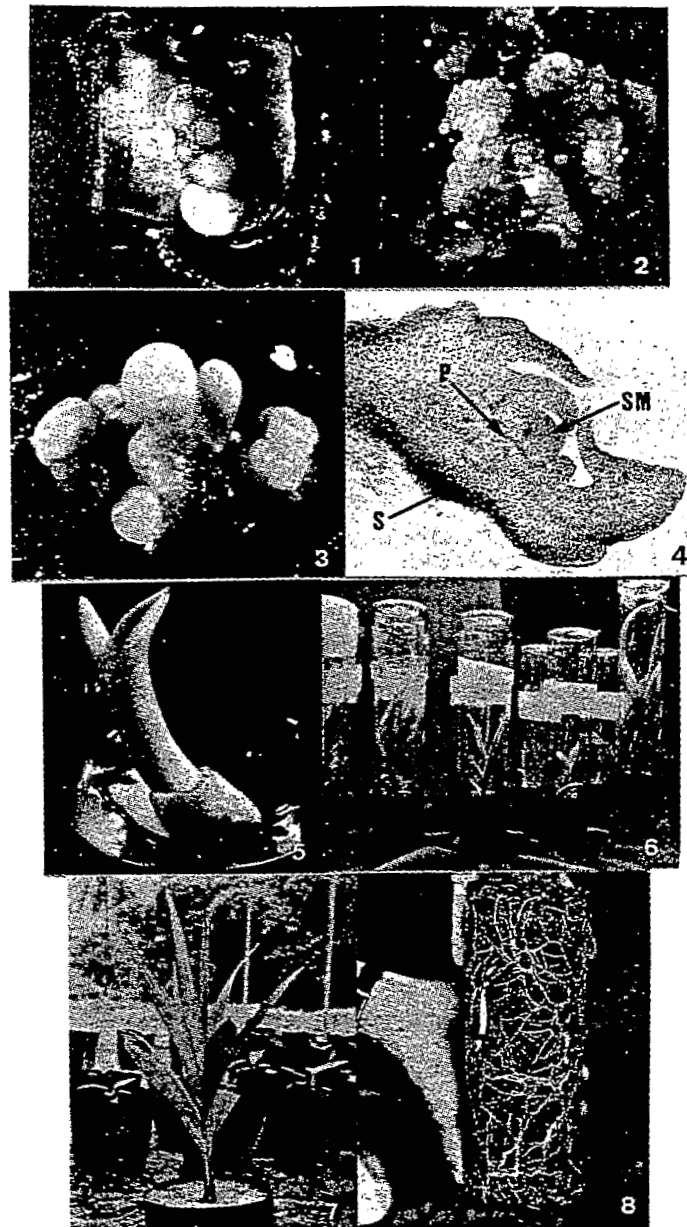


Fig. 27.3: Somatic embryogenesis from leaf and inflorescence tissue of coconut.

Fig. 27.3-1: Callogenesis from leaf explants: nodular calluses after around four months' culturing.

Fig. 27.3-2: Callogenesis from inflorescence explants: globular white calluses arising from floral areas.

Fig. 27.3-3: Clump of somatic embryos at the beginning of the 'maturation' phase.

Fig. 27.3-4: Histological cross-section (just below the notch) of a somatic embryo.

SM = domed shoot meristem, P = provascular strands, S = starch grains.

Fig. 27.3-5: Isolated somatic embryo, emission of first two leaf sheaths.

Fig. 27.3-6: Overall view of some ramets arising from somatic embryos.

Fig. 27.3-7: Ramet after weaning, in pre-nursery (Côte d'Ivoire).

Fig. 27.3-8: Root system of the same weaned ramet.

For the first time in coconut, histological cross-sections, made at the cotyledonary notch, revealed somatic embryos with perfectly structured stem meristem, consisting of a protruding meristematic dome (Fig. 27.3-4) surrounded by leaf primordia.

The haustorial tissue located opposite the shoot meristem contains starch reserves. The positioning of starch grains, which is a good indication of embryo polarity, is identical to that found in zygotic embryos.

Shoot development (Figs. 27.3-5 and 27.3-6) from isolated embryos takes place in the light, on a hormone-free medium with activated charcoal.

Once the first leaf sheaths have emerged, the embryo root can develop spontaneously (without hormone treatment) which indicates the existence of a functional bipolar axis within the embryonal structures.

However, in the majority of cases, rooting requires treatment with an auxin namely, naphthalene acetic acid (NAA) (Figs. 27.3-7 and 27.3-8).

### CONCLUSION

The results recently obtained by the ORSTOM/IRHO-CIRAD team have led to a procedure for reproducible development of complete somatic embryos.

Twenty or so ramets from five different clones of Malayan Yellow Dwarf and PB 121 have been obtained in this way. They were obtained from leaf or inflorescence explants taken from adult trees. Other cultures are currently making good headway.

However, the number of ramets regenerated per clone remains low, due to absence of an embryo multiplication phase, as research has so far concentrated on achieving complete embryogenesis.

The main purpose of our future work will be to determine the conditions required for achieving adventive embryogenesis. Successful multiplication of the somatic embryos obtained should make it possible to satisfy ramet mass production requirements.

We hope these promising results will pave the way for the application of recent plant biotechnology techniques to coconut genetic improvement in the near future.

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