Initiation of somatic embryogenesis in coconut (*Cocos nucifera* L.)

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1. Introduction

The vegetative multiplication of high-performance individuals is a promising possibility for producing homogeneous planting material and improving the productivity and homogeneity of coconut plantations. Cloning would also result in more rapid exploitation of breeding results with rapid distribution of clones in breeding programs. Mastery of coconut regeneration by somatic embryogenesis would open the way to genetic transformation by introduction of genes bestowing resistance to certain diseases, thereby giving resistant plants.

In vitro culture remains the only approach to vegetative propagation of coconut and somatic embryogenesis appears to be the most promising technique.

The first results obtained by the principal teams working on the subject were promising (1980-1984). However, the scarcity of articles published on the subject between 1985 and 1988 illustrates the difficulties encountered by all teams in obtaining whole somatic embryos with shoot development. Between 1984 and 1988, only four teams achieved whole plant regeneration from somatic tissues [1, 2, 3, 10], but the ramets were apparently obtained from a limited number of cultures. The ramets obtained proved that coconut regeneration by somatic embryogenesis is possible, but simply has not been mastered. It is clear that coconut remains an extremely recalcitrant plant to *in vitro* culture.

The difficulties in coconut regeneration are numerous: intense browning of tissues linked to their high sensitivity to synthetic auxins (*e.g.* 2,4-D), considerable

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heterogeneity in tissue reaction, low capacity for embryogenesis and caulogenesis and an extremely slow response *in vitro*.

Activated charcoal is often used in plant tissue culture for its promotion of somatic embryogenesis and for its potent adsorption of growth inhibitors secreted by the tissues, such as phenolic compounds. Given the high sensitivity of coconut tissues to synthetic auxins, the use of activated charcoal is essential for the maintenance of the cultures. Coconut calli have low embryogenic potential, and embryogenesis is often incompletely expressed, leading to fused or incomplete embryos without shoots. Coconut is also characterised by the slowness with which the different events occur and by the highly determined tissue component. These difficulties complicate the search for mediators of induction and expression of somatic embryogenesis.

By adopting an analytical approach (histological study, investigation of culture medium composition), the joint ORSTOM/CIRAD team at Montpellier acquired a clearer understanding of the reasons that embryogenesis was blocked or deviated. In this way, the Montpellier team obtained reproducible whole embryos. In total, approximately forty plantlets have been obtained from several clones belonging to different genotypes [11]. The different steps for development of somatic embryogenesis are described below.

2. Callus induction

The explants were received from the Ivory Coast (Marc Delorme Station, IDEFOR). This material was taken from adult individuals selected for their agronomic value but were predominantly from 'Malayan Yellow Dwarf' and the 'Malayan Yellow Dwarf' x 'West African Tall' ('PB-121') and 'Cameroon Red Dwarf' x 'West African Tall' ('PB111') hybrids created by CIRAD-CP. Two types of explants were used in the laboratory: young non-chlorophyllous leaves and immature inflorescences.

The basal medium consisted of Eeuwens inorganic nutrients [6] and the vitamins of Morel and Wetmore [9], supplemented with 30 gl^{-1} sucrose, 2,4-D (40, 50, 60, 70 mgl^{-1}) and 2 gl^{-1} of neutralised activated charcoal (Sigma). The medium was gelled with 7.5 gl^{-1} agar (Sigma). The pH was adjusted to 4.5 before autoclaving for 20 minutes at 120°C.

Calli appeared after four months of culture, with an optimum of 6-8 months. Histological studies identified sites of callus growth on both explant sources and provided a better understanding of the mechanisms involved in its growth. Neoformed tissues have two possible origins; a deep origin for calli obtained from leaf explants where the calli formed on undifferentiated perivascular sites, and a superficial origin, observed for calli forming on floral areas. Whatever the tissue origin, the growth and multiplication of calli were ensured by a peripheral meristematic zone frequently exhibiting cambium-like organisation. Callus multiplication could be maintained for long periods without any obvious histological change.

3. Initiation of somatic embryogenesis

In order to circumvent the difficulties encountered in coconut regeneration, homogeneous calli strains were obtained by the multiplication of the primary calli. In the presence of optimum 2,4-D concentration, callus strains were selected on the basis of their embryogenic competence. This material was suitable for improving the understanding of the induction of embryogenesis and for studying different factors that may promote embryogenesis.

3.1 Histological study

A histological study of embryogenic calli revealed two embryogenic pathways [4]:

a) a multicellular pathway which led to the formation of meristematic and epidermised structures obtained with low 2,4-D concentrations (40 to 60 mgl^{-1}, and 2 gl^{-1} charcoal). The first stage of development of these structures was characterised by the fragmentation of the cambium-like zone and the formation of complex meristematic structures followed by their epidermisation. Too low a concentration of auxin sometimes resulted in the incomplete or deviated embryonic structures (*e.g.* a haustorium only; with or without a root pole; foliaceous embryos).

b) a unicellular pathway which led to the appearance and individualisation of embryogenic cells isolated by a thick wall, with dense cytoplasm, a high nucleo-cytoplasmic ratio, a single large nucleolus, and starch and protein reserves. This second pathway was obtained in the presence of high 2,4-D

concentrations (80-120 mgl^{-1} with 2-3 gl^{-1} charcoal) and led to the formation of typical pro-embryos characteristic of the initial stages of zygotic embryogenesis described by Haccius and Philip [8].

3.2 Mineral nutrient requirements

Specific nutritional requirements during initiation of embryogenesis were observed irrespective of the embryogenic process. This study revealed greater NH_4^+, Ca^{2+}, Mg^{2+} and sucrose absorption per gram of dry weight during embryogenesis. These specific nutritional requirements were accompanied by an increase in cellular protein reserves.

3.3 Hormonal study

Charcoal strongly adsorbs the growth inhibitors secreted by *in vitro* tissues, such as polyphenolic compounds. Fridborg and Eriksson [7] suggested that charcoal might also adsorb certain growth regulators, particularly synthetic auxins. The use of activated charcoal introduces a further source of variability in culturing, and under certain conditions of medium preparation, may be responsible for variable tissue performance in callogenesis and non-reproducible results.

A quantitative study of 2,4-D adsorption was carried out in the Montpellier laboratory using HPLC in a solid medium with 2,4-D detection under UV light. Under our culturing conditions, the results confirmed high 2,4-D adsorption by the activated charcoal. Thus, with 4.5×10^{-4} M 2,4-D and 3 gl^{-1} activated charcoal, 0.47% of the total auxin initially present in the medium remained available 21 days after the media were prepared.

A kinetic study showed that 2,4-D adsorption by the activated charcoal was a gradual process. The time taken for the 2,4-D level to stabilise depended on the initial 2,4-D concentration and the activated charcoal content. Using 3 gl^{-1} activated charcoal and 100 mgl^{-1} 2,4-D, the free 2,4-D/adsorbed 2,4-D equilibrium was reached 21 days after media preparation.

4. Somatic embryo maturation

According to most authors, the essential factor in embryogenic expression is the gradual drop in 2,4-D concentration in relation to the auxin level used for induction of embryogenesis. However, the initial concentration from which this gradual reduction occured was the essential factor; it ensured good initiation of embryogenesis likely to yield whole embryos. If the drop in auxin rate was too rapid, the maturation of the embryogenic structures usually led to incomplete or deviated forms.

The frequent deviation of embryonic morphogenesis could be linked to a hormonal imbalance [5]. Addition of an exogenous cytokinin (BAP) resulted in complete embryogenesis. A strictly defined sequence of events had to be followed to obtain complete embryogenesis: haustorium elongation, cauline pole differentiation and root pole differentiation. Without exogenous cytokinins, premature differentiation of the root pole led to a morphological dead-end, with irreversible inhibition of cauline pole differentiation.

5. Embryoid conversion

Conversion of embryos with shoot emission was carried out on an auxin-free medium. Establishment of the cauline meristem preceded root-pole differentiation. The root could develop spontaneously, indicating the presence of a functional bipolar axis, but in most cases addition of NAA was necessary to obtain roots. The haustorial tissue located opposite the stem meristem contained starch reserves. The location of starch grains, which is a clear indicator of embryo polarity, was identical to that found in a zygotic embryo.

6. Conclusions

Clones of several genotypes have been obtained in our laboratory. However, the number of ramets regenerated per clone remains low due to the lack of an intense multiplication phase. The main objective of future work will be to determine the conditions required for achieving adventive embryogenesis. In 1992, embryogenic suspensions were established with competent friable calli. This technique has the potential for the mass production of individual embryos in synchronous growth.

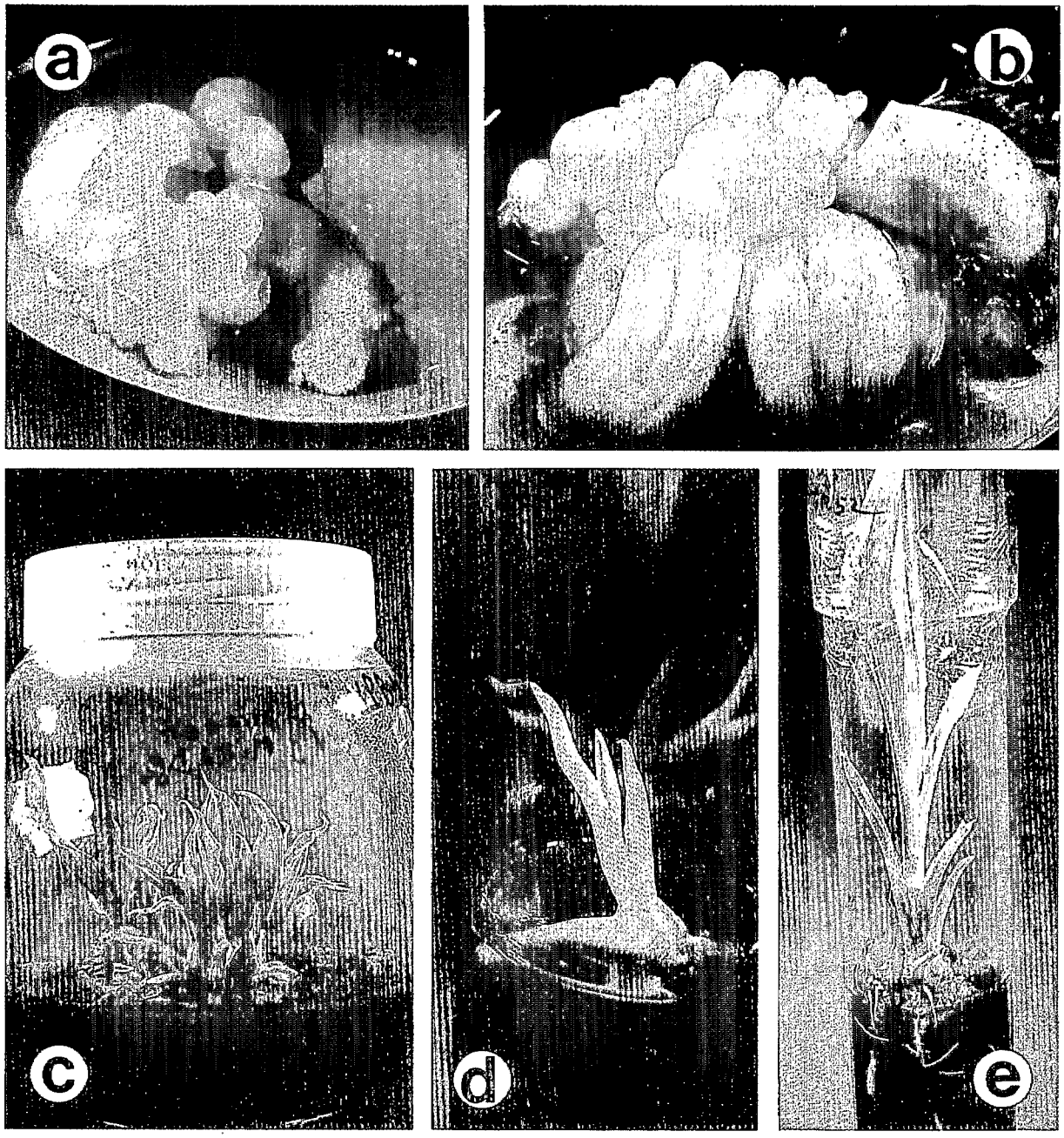

Fig. 1. a: Embryogenic structure (x6). b: Initiation of adventive embryogenesis from embryos with developed haustoria (x3). c: Shootlets from a clump of somatic embryos (x3). d: Somatic embryo conversion with first leaves and root emergence on an auxin-free medium (x3). e: Vitroplant from somatic embryo (x1).

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