PLANT REGENERATION THROUGH SOMATIC EMBRYOGENESIS FROM CULTURED ZYGOTIC EMBRYOS OF COCONUT

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A complete process of plant regeneration through callus induction and somatic embryogenesis from cultured zygotic embryos of two cultivars of coconut was established. Reproducible and high frequencies of callus induction with two distinct types of embryogenic callus were obtained. Development of somatic embryos through stages resembling those of zygotic embryos following a consistent pattern yielded complete plants with typical foliar features of the coconut palm. Histological studies provided a firm evidence of somatic embryogenesis in coconut which is reported for the first time. Implications of the reported findings to clonal propagation of coconut are discussed.

Key words: *coconut, embryogenic callus, somatic embryogenesis, plant regeneration, clonal propagation.*

INTRODUCTION

Despite the many problems besetting the world's coconut industry, in general, and the Philippines', in particular, the coconut still remains an important crop in many tropical countries. Its new uses for foods and oleochemicals have opened up a new prospect which is all dependent upon adequate nut production.

Compared to other oil-producing crops such as soybean and oil palm, coconut production does not appear to be increasing. The crop productivity has, in fact, continued to decline due to lack of suitable high-yielding cultivars, poor plantation management, and decrease in areas planted to coconut. Genetic improvement of coconut yield is a time-consuming task because of its open-pollination nature and mode of propagation, which is solely by the use of seed nuts. With open-pollination, the seed nuts are so variable that the annual production per palm in some plantations may range from 10 to over 100, with an average of not more than 30-40 nuts. Yet, some elite individual palms have been recorded to produce up to 400 nuts per year (Iyer 1981).

The successful development of the *in vitro* multiplication procedure for oil palm which increased its yield by 20%-30% (Corley et al. 1979) has greatly encouraged coconut researchers to explore the potential of clonal propagation to improve the productivity of the "tree-of-life." Indeed, Meunier et al. (1986) suggested that cloning of the best 13% of trees in a plantation of a coconut hybrid (e.g., PB 121) would provide a theoretical copra yield increase of 24% from a cultivar which already has a higher than average yield.

Tissue culture of coconut has been attempted by various laboratories around the world. In the early days of coconut tissue culture, there was an implicit assumption that the coconut would respond in a manner similar to that of the oil palm (Blake 1990). However, it was soon realized that it is more difficult to obtain plantlets in coconut and, so far, only limited success has been reported. Different sources of explant such as meristematic root and shoot tissues (Iyer 1981; Pannertier and Buffard-Morel 1982; Gupta et al. 1984; Raju et al. 1984; Verdeil et al. 1989); inflorescence tissues (de Guzman and del Rosario 1979; Blake and Eeuwens 1980; Branton and Blake 1983a; Sigimura and Salvana 1989; Verdeil et al. 1989); zygotic embryos (de Guzman et al. 1978; Bhalla-Sarin et al. 1986; Thanh-Tuyen and Dionzon 1986; del Rosario et al. 1989); endosperm tissues (Prakash-Kumar et al. 1985); and an ithers (Thanh-Tuyen and de Guzman 983; Monfort 1985) were used for duction of callus and/or development of organized structures.

Plant regeneration was reported from callus initiated from inflorescence tissues (Branton and Blake 1983b) and cultured zygotic embryos (Bhalla-Sarin et al. 1986; del Rosario et al. 1989), or directly from leaf tissue cultures (Raju et al. 1984). However, the complete developmental process from culture initiation to plant regeneration was not described and the reproducible frequencies of callus induction and formation of organized structures were not reported. Application of the published procedure for actual propagation of coconut is not also known. The reproducibility of the morphogenic phenomena and the frequency of plant regeneration, thus, appear to determine the eventual success in clonal propagation of coconut.

The paper reports the achievement in establishing the mode of plant regeneration through callus induction and somatic embryogenesis from cultured zygotic embryos of coconut and the reproducible frequencies of the observed developments. The implications of the developed procedure to clonal propagation of coconut are also discussed.

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MATERIALS AND METHODS

Plant Materials

The zygotic embryo explants were collected from tagged samples of 9-mo-old nuts of a tall cultivar, Baybay Tall and a dwarf cultivar, Lingkuranay. The dehusked nuts were broken horizontally across the median surface and the embryo explants, each enclosed in a piece of endosperm cylinder, were scooped out with a core borer. The materials were surface sterilized with 50% v/v commercial bleach (Chlorox) or an equivalent of 2.5% sodium hypochlorite solution added with 0.05% v/v "Tween 80" for 20 min with two changes followed by washing four to five times with sterile distilled water. The embryos were isolated aseptically and inoculated onto the culture medium for callus initiation.

Culture Media

The callus initiation medium was adopted from Branton and Blake (1983a), with modifications. In particular, the medium contained the macro-elements formulated by Murashige and Skoog (1962), the micro-elements of Eeuwens (1976), the vitamins of Nitsch and Nitsch (1965), 100 mg/L myo-inositol, 300 mg/L casein hydrolysate, 5% sucrose, 0.25% activated carbon, 20-40 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D), 1-5 mg/L N^6-benzyl adenine purine (BAP), 1-5 mg/L N^6-isopentenyl amino purine (2iP) and 0.6% agar. The medium was adjusted to pH 5.8 with 0.5 N NaOH before sterilization by autoclaving at 121°C and 1.05 kg/cm^2 for 20 min. One embryo each was inoculated on to 8 mL medium in a screw-capped vial (23x85 mm).

The cultures were transferred regularly at 4-wk intervals to fresh media with the auxin concentration being reduced gradually to stimulate somatic embryogenesis.

All chemicals used were of analytical grade, unless otherwise stated.

Culture Conditions

The cultures were maintained in the dark incubation condition at 28°-30°C for 2-3 mo for callus initiation and proliferation. When the initiation of somatic embryogenesis was observed, the cultures were exposed to light of 2,000 lux provided by two 40-watt fluorescent tubes at 28°-28°C following a lighting scheme of 16:8 hr of light and dark regime.

Histological Studies

The callus cultures and somatic embryos initiated from cultured zygotic embryos of coconut were examined histologically at different stages of development following the procedure for light microscopy described by Johansen (1940). The specimens were fixed in Craft III solution, dehydrated in a series of ethanol/tertiary butyl alcohol solutions, and embedded in paraplast-plus. Sections of 8-10 µm thick were cut with an American Optical rotary microtome, stained in safranin and counterstained with fast green.

RESULTS AND DISCUSSION

Callus Initiation and Proliferation

The formation of callus was initiated after 1 mo from inoculation, on the periphery of the cotyledonary sheath around the tissue zone enclosing the shoot and root apices of the cultured zygotic embryo (Fig. 1a). A cross section of the zygotic embryo explant cut across the neck-like intersection (Fig. 1b) shows the shoot apex with leaf primordia, both are enclosed by a semi-tubular structure, and the opposite root pole. The embryonic axis is surrounded by a mass of homogenous cells which are small, spherical, and non-vacuolated. When the explant was inoculated onto a callus initiation medium, "meristemoid" areas were initiated among the sub-epidermal cells on the periphery of the cotyledonary sheath (Fig. 1c) leading to the formation of the protuberances. The frequency of callus formation was recorded at 60%-70% after 2 mo in culture.

Three types of callus were observed on cultured zygotic embryos of coconut. These were classified as (1) nodular, (2) knobby, and (3) granular callus. The nodular callus was characterized as a mass of distinct, spherical bodies, which were compact and translucent to opaque white in color (Fig. 2a). The nodular callus most likely became embryogenic, that is, capable of developing into somatic embryos and regenerating plantlets in subsequent passages in culture. The knobby type callus consisted of white and compact outgrowths, which were of irregular shape and size (Fig. 2b). These bodies were partly coalesced at the base. The irregular proturberances of the knobby type callus could either develop into distinct nodular bodies which then became embryogenic, or expand and coalesce extensively to render the whole mass of callus spongy. The latter eventually lost its embryogenic potential. The granular callus appeared as a mass of granules (Fig. 2c) which often became soft and water-soaked. This type of callus then turned brown and degenerated. The nodular and knobby type calluses were often initiated concurrently on different embryo explants inoculated onto the same medium formulation, at a frequency of 40% and 60%, respectively. Since all the explants were collected from tagged sample nuts of the same age, but from different palms, the initiation of callus of either of these two types appeared to be dictated by some internal factors inherent to the mother palms. The non-embryogenic granular callus was occasionally observed on the embryo explants which were inoculated in culture vessels with limited gas exchange capacity. Verdeil et al. (1989) reported three types of callus which were induced on leaf and inflorescence explants of coconut, with different nomenclature from the aforementioned one.

The callus proliferated rapidly on the peripheral tissues of the culture explants (Fig. 3a) and subdivision of the callus was necessary after about 2 mo from inoculation in order to stimulate further proliferation (Fig. 3b). The process of callus initiation and proliferation on the cultured zygotic embryos as reported hereto was considerably faster than that recorded by Branton and Blake (1983a). The latter observed that initiation of callus on explants from both seedling and mature tissues is slow, taking about 16 wk before subculture is possible. Moreover, the callus obtained by the English scientists appeared fri-

able, cream colored, and small nodules in shape. They seemed to differ morphologically from the distinct compact and white nodular structures induced on the cultured embryos as mentioned above. Bhalla-Sarin et al. (1986) reported the formation of a white, compact nodular callus from cultured immature embryos of West Coast Tall coconut on Gamborg's B5 medium added with IAA-conjugates. The morphogenic potential of these callus cultures was reported to be destined for root formation.

Somatic Embryogenesis

The process of somatic embryogenesis commenced with the development of distinct globular bodies in the mass of callus (Fig. 4a) which was observed at about 3-4 mo after inoculation. Exposure of the callus to light and gradual reduction of auxin concentration in the medium were then essential to sustain the development of somatic embryos and prevent the conversion of the embryogenic callus to spongy tissues. The globular bodies subsequently differentiated into bipolar structures. In the succeeding stages of development, distinct heart-shaped embryos (Fig. 4b) showing a rounded end and a tapering end, and embryos at cotyledonary initiation stage (Fig. 4c) were observed.

The developmental stages of the zygotic embryos of monocot species have not been well-established as compared to that of the dicots. Blake (1990) was, thus, skeptical about the report on development of heart-shaped somatic embryos on the nodular callus initiated on cultured embryos of coconut (Thanh-Tuyen and Dionzon 1986). However, histological studies on the somatic embryogenesis in coconut (Thanh-Tuyen and Apurillo 1989) revealed the morpho-anatomical characteristics of the somatic embryos at the heart-shaped and advanced stage of development.

As shown in Figure 4d, the longitudinal section of a heart-shaped embryo induced on the callus of a cultured explant exhibits a bipolar structure with two cotyledon initials and a vascular strand running from the shoot to the root pole. In the advanced phase of embryogenesis, the embryos at cotyledonary initiation stage showed a suppressed development of one cotyledon. Assymetrical growth of one cotyledon was also noted by Thanh-Tuyen and de Guzman (1983) in androgenic embryos developed in cultured anthers of coconut. Apparently, the observed feature is an embryonal characteristic of coconut, being a monocot species. Figure 4e shows the longitudinal section of a somatic embryo having the remaining cotyledon developed to the extent that it seemingly occupied the terminal position with the shoot apical meristem located at the side of the cotyledon. The somatic embryos lacked the vascular connection with the explant tissue. The observations provided a firm evidence of somatic embryogenesis which is reported for the first time in coconut.

Somatic embryogenesis is well-documented for cells from tissue culture of carrot which produced embryos by a developmental sequence through globular, heart-shaped, torpedo, and cotyledonary stages, all strikingly like those occuring after fertilization of egg cells (Steward et al. 1954). For coconut, the pollen embryogenesis (Thanh-Tuyen 1990) and somatic embryogenesis reported hereto, in cultured anthers and zygotic embryos, respectively, exhibited the developmental sequence resembling that of the zygotic embryos. Although the sequence of coconut zygotic embryo development has not been documented, histological studies on the somatic embryos developed on callus cultures established a firm evidence of an orderly sequence of embryogenesis.

Morphologically, a mature zygotic embryo of coconut has the shoot and root ends facing the stony endocarp and the modified cotyledon or haustorium end facing the central cavity. During germination, the haustorium expands considerably and eventually fills up the cavity of the seednut (Child 1974). The spongy haustorium tissue is not a common structure in germinating seeds of other monocots. Its existence is apparently associated with a nutritive role during germination of the coconut embryo as it absorbs the nutrients from the solid endosperm for translocation to the developing seedling. *In vitro* germinated seedlings of coconut, which were adequately supplied with nutrients from the culture medium, did not produce the spongy haustorium (de Guzman and del Rosario 1964). The coconut somatic embryos as shown in this paper were also devoid of the spongy tissue, in contrast to the report of Branton and Blake (1983a). The spongy tissues which the latter ascribed as representing haustorial tissue may probably be discrete bodies which expanded considerably on medium lacking 2,4-D rather than an analogy to a component of a germinating zygotic embryo. Under the reported experimental conditions, development of spongy tissues in a mass of callus for prolonged culture on a medium lacking 2,4-D, was indicative of loss of embryogenic potential. Otherwise, the embryogenic callus was destined for an orderly process of somatic embryogenesis which was induced more favorably than organogenesis, on the cultured zygotic embryos. More interestingly, root development was largely suppressed until shoot development had established.

In a total number of eight experiments in which somatic embryogenesis was successfully induced on the zygotic explants of both cultivars, for example, Baybay Tall and Lingkuranay, it was computed that about 30-50 somatic embryos were produced per cultured explant. The figure marked a promising rate of multiplication through this mode of plant regeneration.

Direct formation of embryogenic structures on the cultured zygotic embryos (Fig. 5a) was occasionally observed. Similar phenomenon was reported on inflorescence explants (Verdeil et al. 1989). Figure 5b shows two distinct somatic embryos regenerated on the periphery of the zygotic explant with their distal ends fused together.

Plant Regeneration

Mature somatic embryos derived from the cultured explants possessed a pearlike shape (Fig. 6a) with a single cotyledon occupying the terminal position that resembles the scutellum of grass

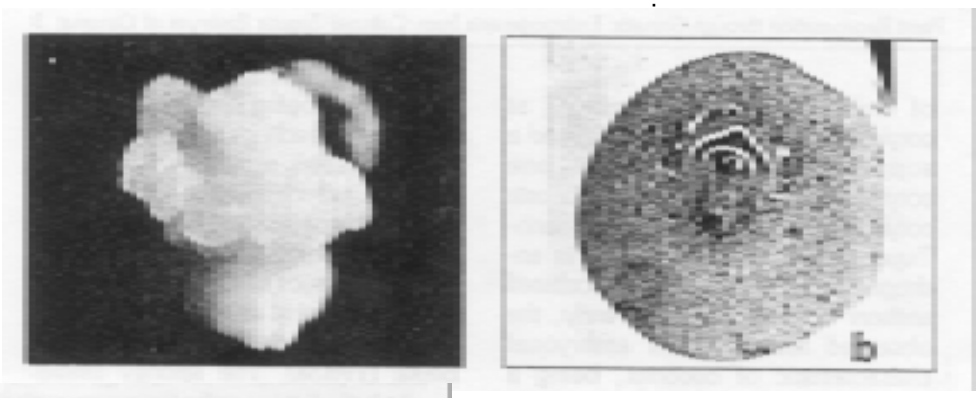


FIGURE 1
Initiation of embryogenic callus from a cultured zygotic embryo of coconut (a) Early stage of callus initiation b) Cross-section of a zygotic embryo at the neck-like intersection (c) Cross-section of a cultured zygotic embryo with a mass of meristemoid areas.

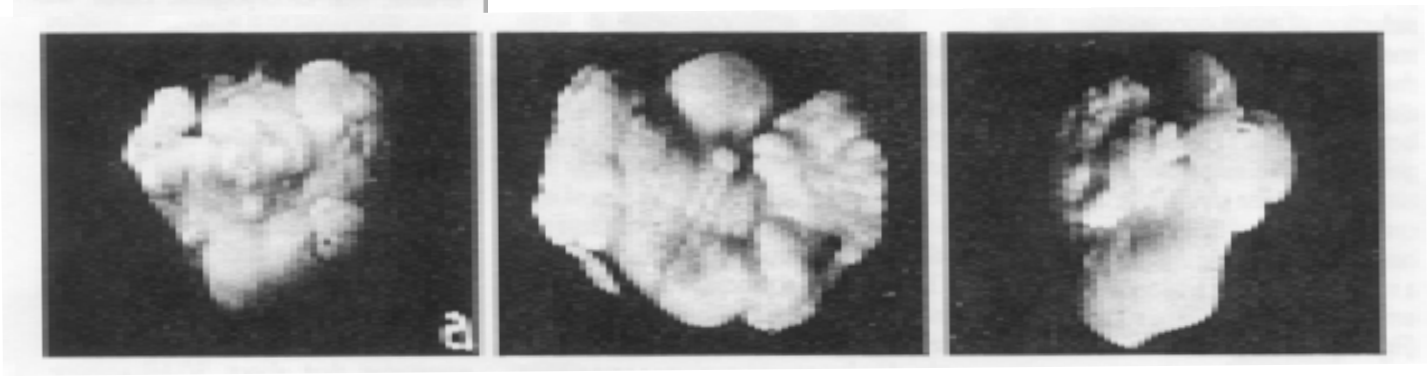


FIGURE 2
Three types of callus formed on cultured zygotic embryos of coconut (a) Nodular (b) Knobby (c) Granular.

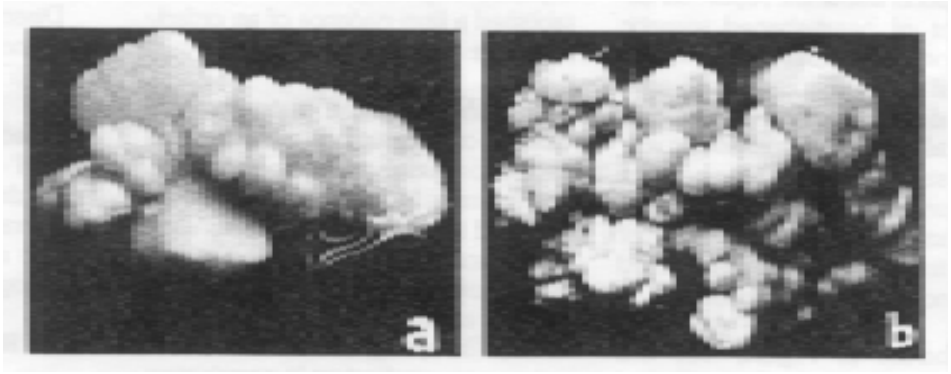
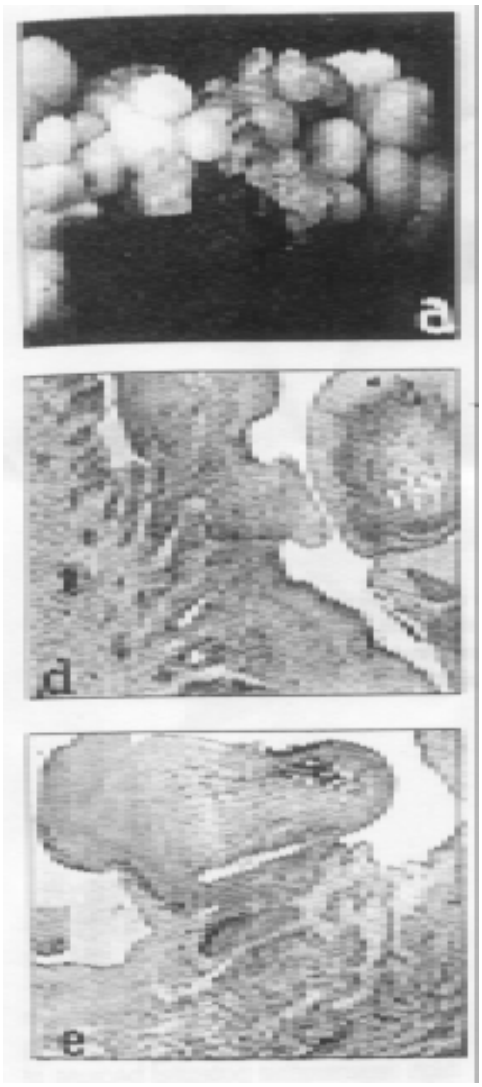


FIGURE 3
Callus proliferation on cultured zygotic embryos of coconut (a) Before subculture (b) After subculture.



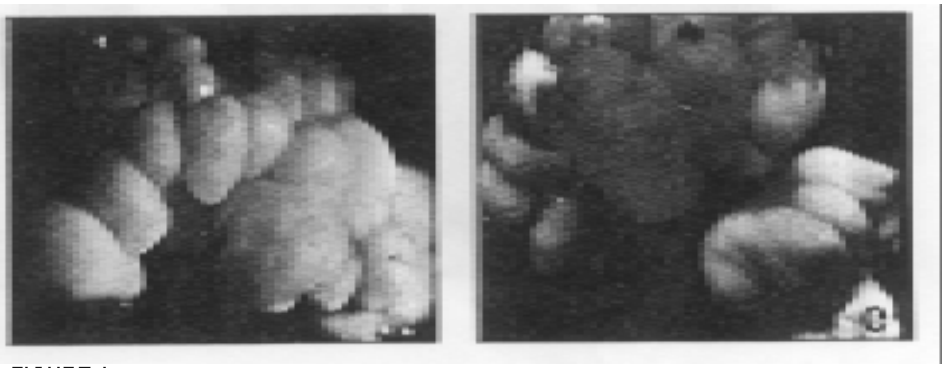


FIGURE 4
Somatic embryogenesis in coconut (a) Development of globular bodies in a mass of callus (b) A cluster of somatic embryos with distinct heart-shaped embryo (arrow) (c) Two somatic embryos at cotyledonary initiation stage showing suppressed cotyledons (arrow) (d) Longitudinal section of a heart-shaped embryo (e) Longitudinal section of a somatic embryo at advanced stage of development.

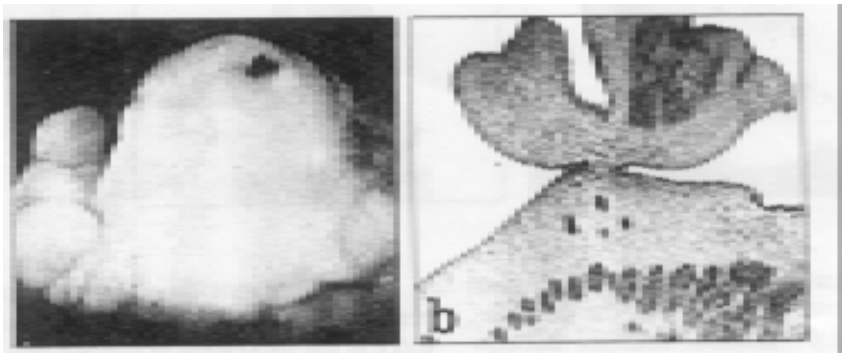


FIGURE 5
Direct somatic embryogenesis on a cultured zygotic embryo (a) A cluster of somatic embryos (b) Cross-section of the cultured explant showing two fused somatic embryos generated at the periphery of the zygotic embryo.

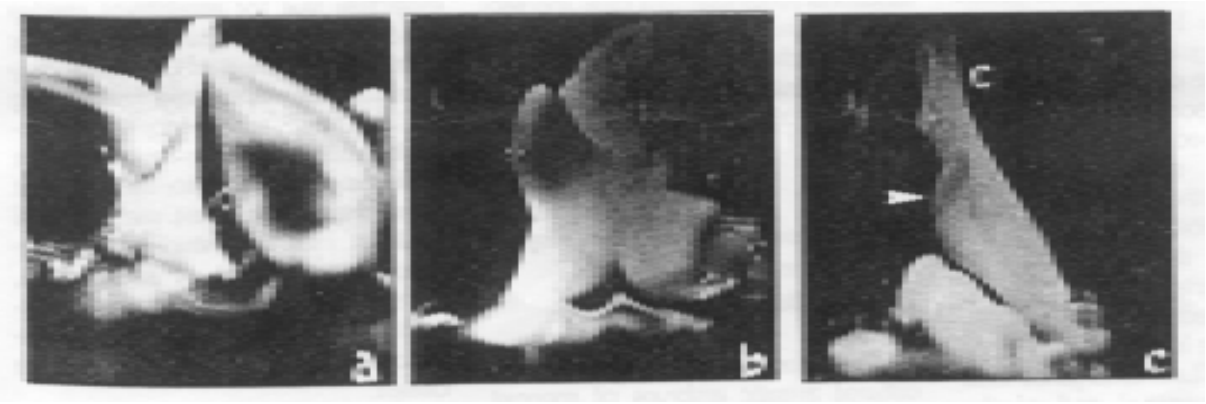


FIGURE 6
Plant regeneration process (a) Two mature somatic embryos (b) Splitting at the adaxial side of the cotyledon (c) Emergence of a tubular structure (arrow) from the side of the cotyledon (d and e) Germination pattern of coconut somatic embryos (f) Emergence of opened leaves with foliar features of coconut.

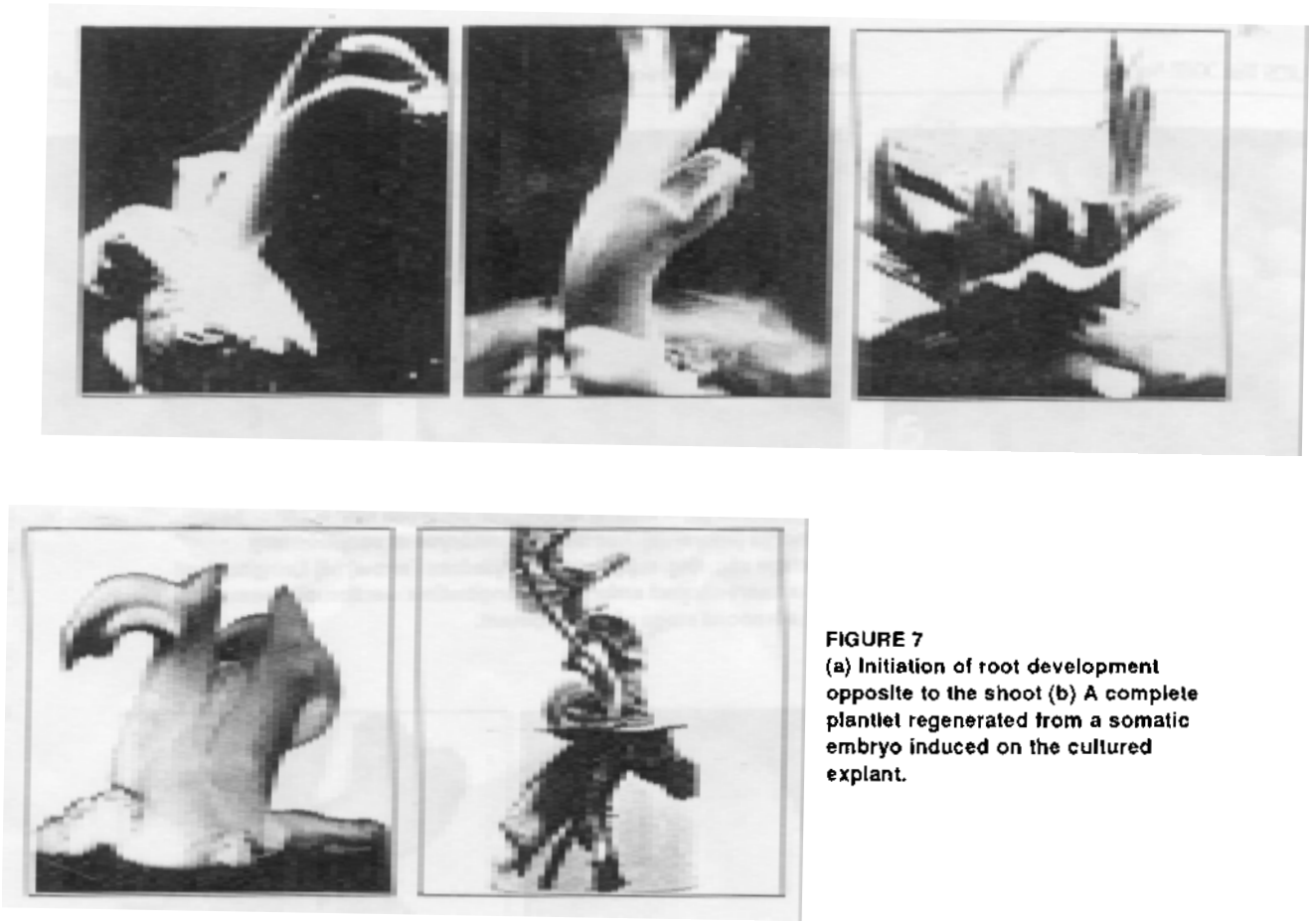


FIGURE 7
(a) Initiation of root development opposite to the shoot (b) A complete plantlet regenerated from a somatic embryo induced on the cultured explant.

embryos. The process of plant regeneration started with the germination of the somatic embryos through splitting at the adaxial side of the cotyledon (Fig. 6b) to expel the tubular structure (Fig. 6c). The latter appeared to be similar to the coleoptile of a grass embryo, but had never been indicated in coconut. However, in the cross section shown in Figure 1b. the semi-tubular structure is recognized to enclose the shoot apex of the coconut zygotic embryo. Clusters of somatic embryos were frequently observed to fuse at their distal ends. The somatic embryos regenerated from the embryogenic callus of coconut exhibited repeatedly the typical features during germination which were characterized by the splitting of centrally-located cotyledon and the emergence of the locally-located shoot (Fig. 6d and 6e). Leaf emergence took place at the terminal end of the coleoptile-like structure to yield the first rolled leaf which was followed by the opened leaves showing the typical foliar features of coconut (Fig. 6f).

Initiation of root development was observed at the opposite end to the shoot at the early stage of somatic embryo germination (Fig. 7a), but its growth was slightly suppressed until the first pair of true leaves were produced to yield a complete plantlet of coconut (Fig. 7b). The developed root system showed functional roots with root caps and secondary branched roots. A passage to a culture medium favoring profused root development was necessary to prepare the *in vitro* plants for their establishment in soil.

Implications of the Developed Procedure to Clonal Propagation of Coconut

The reported plant regeneration from cultured zygotic embryos of coconut through a well-defined process of somatic embryogenesis, points to the eventual success in clonal propagation of the "tree-of-life." The statement is substantiated by the reproducibility of the reported phenomena, the high frequency of culture response, and the possibly wide application of the developed procedure for other cultivars. In particular, immature embryos possessing high regenerative potential because of their juvenile nature are excellent materials for *in vitro* propagation. This is especially true for recalcitrant species like coconut and Gramineae. The newly germinated seedlings of Gramineae may have lost their totipotency (Hu and Wang 1983). The high frequency of embryogenic callus induction as reported above could be attributable to the fact that immature embryos were used as initial explants. However, since zygotic embryo is a product of fertilization, controlled/assistant pollination is essentially important to maintain the genetic makeup of the mother palm to be cloned.

In addition, the mode of plant regeneration through somatic embryogenesis from cultured zygotic embryos offers two advantages. One is that so