Research Article

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Histological studies of cellular differentiation during somatic embryogenesis of coconut plumule-derived calli

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Abstract

Since coconut is one of the most recalcitrant species to generate *in vitro*, it is necessary to study in detail about the cellular changes that occur during somatic embryogenesis to enhance our knowledge about this phenomenon. In the present study, coconut plumular tissues, the shoot meristem including leaf primordia, were used as explants for *in vitro* regeneration studies. Histological studies were carried out in different stages of plumule culture. No noticeable growth was observed in 15 days old cultures. After 30 days, meristematic cells could be identified. Abundance of meristematic cells, foremost to the development of callus structures, was observed after 45 days. After 75 days, globular friable calli were formed and histological studies revealed the presence of meristematic centers which eventually formed somatic embryos. The histological study of matured somatic embryos formed after 120 days of callus initiation showed a clear meristematic zone of parenchyma cells, surrounded by vascular bundles. Histological studies, carried out for certain abnormalities like compact calli, abnormal somatic embryoids with rudimentary shoots and multiplied roots, revealed the presence of intact cotyledonary leaves which seemed to inhibit the apical meristem development of somatic embryoids. The presence of vascular bundles in the early stages of callus formation might lead to the direct formation of meristemoids. These results could aid future studies leading to enhanced control of the somatic embryogenic process and greater efficiency of somatic embryo and plantlet formation in coconut.

Keywords: Coconut, leaf primordia, meristematic zone, meristemoids, somatic embryogenesis

Introduction

Coconut (*Cocos nucifera* L.) is a difficult tree crop to manipulate *in vitro*. Eeuwens (1978) standardized a media for successful callus induction from various explant sources like shoot, leaf and inflorescence. This led a few laboratories around the world to initiate histological research on coconut. Research on coconut tissue culture has remained challenging and difficult with only some of the problems being solved. With wide variations observed in coconut populations for productivity (Arunachalam and Rajesh, 2008), propagation of elite mother palms through somatic tissue culture will go a long way in solving the problem of production of adequate number of quality planting materials. In addition, this technique would also help in the rapid propagation of elite hybrids.

Studies on somatic embryogenesis in coconut have been reported for the past four decades. Several experiments have been conducted to test the ability of different tissues to undergo callogenesis. Embryogenic potential of leaf explants was limited to a particular size of young leaf (10-20 cm) and embryogenic capacity of short duration, which made percentage of embryogenesis low (<10%) (Karunaratne *et al*., 1991). Many researchers (Eeuwens, 1978; Branton and Blake, 1983; Buffard Morel *et al.,* 1992; Verdeil *et al.,* 1994) have reported immature inflorescence as a promising explant for callogenesis, but the success depended on the maturity of the inflorescence. Since there was no appropriate technique for assessment of maturity of inflorescence, the callusing rate was found quite low (around 30%) (Vidhanaarachchi and Weerakoon, 1997). Unfertilized ovaries of

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proper developmental stage were also reported to be an appropriate explant for callogenesis, but the mean percentage of callogenesis was found to be 41 per cent (Perera *et al*., 2007). The potential of plumule (the shoot apex excised from zygotic embryo) has also been tested for *in vitro* plant regeneration and the response of these tissues has shown to be better than that of vegetative tissues, in terms of callus formation and embryogenic capacity (Hornung, 1995). Chan *et al*. (1998) developed a protocol for coconut regeneration using plumules, extracted from mature zygotic embryos, as explants. Studies carried out in whole immature or sliced mature zygotic embryos gave a very good starting explant for somatic embryogenesis (Adkins *et al*., 1998). A protocol for plantlet regeneration from plumular tissues of West Coast Tall cultivar of coconut was standardized by Rajesh *et al*. (2005) and they observed an increased frequency of callus induction and a reduction in browning of the plumular explant when a cytokinin, thidiazuron (TDZ), was added along with the 2,4-dichlorophenoxyacetic acid (2,4-D). The exogenous supply of polyamines was also shown to enhance induction of somatic embryogenesis and plantlet regeneration from plumular explants of dwarf cultivars of coconut (Rajesh *et al*., 2014).

Plumule culture, therefore, holds a considerable potential as a model system for developing a clonal propagation method for coconut. During the development of a protocol for plantlet regeneration, both morphological and histological aspects need to be evaluated to understand the *in vitro* response, for instance, the basis and growth of the calli and somatic embryos and the characterization of the morphogenetic route.

Haccius and Philip (1979) were the first to use histological techniques to describe the development of coconut zygotic embryo. The histological changes of coconut explants and derived calli in *in vitro* culture were studied by Branton and Blake (1983), Verdeil *et al.* (1994), Hornung (1995) and Chan *et al.* (1998). Verdeil *et al.* (2001) reported the ultra-structural changes that occur during the formation of embryogenic cells in immature inflorescence of coconut. There are also reports dealing with events that occur during first day of coconut plumule culture (Hornung, 1995) and the formation of embryogenic callus (Chan *et al.*, 1998). Saenz *et al.* (2006) reported morphological and histological changes during the different developmental stages of somatic embryo formation, which gave evidences for the multicellular origin of the somatic embryos. Fernando *et al.* (2003) also reported pluri-cellular origin of somatic embryos through histological analysis of plant regeneration from coconut plumular explants. These studies have, however, provided only a partial knowledge about the somatic embryogenesis process in coconut. The present study was conceived to provide a complete documentation of both morphological and histological events during somatic embryogenesis, which we hope might provide better insights for modification of culture conditions for development of a successful regeneration protocol from coconut plumule explants.

Materials and methods

Explants and their preparation

Matured nuts (11-12 months old) harvested from West Coast Tall cultivar were used for excision of plumular region. Embryos with endosperm were excised from the cut-opened nuts using a cork borer and placed in distilled water. The endosperm plug, enclosing the embryos, were sterilized with 0.01 per cent $HgCl_2$ for 5 minutes and rinsed thrice with sterile distilled water to remove the traces of $HgCl_2$. Embryos were excised from endosperm plugs, surface sterilized with 20 per cent NaClO for 20 minutes and subsequently rinsed with sterile distilled water for 5-6 times. Plumule was directly extracted from the surface sterilized matured embryos in aseptic conditions and inoculated into the media containing different concentrations of callogenic chemicals.

In vitro culture media and condition

Y_3 medium (Eeuwens, 1978) supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} activated charcoal and 5.8 g L^{-1} agar was used as the basal medium. The growth regulator 2,4-D (74.64 µM) along with TDZ (4.5 µM) were used for the callus initiation. After adjusting the pH to 5.8, the media were autoclaved at 121 ^{0}C for 20 min and poured into Petriplates (100 x 15 mm) under asceptic conditions. Sub-culturing was done every 30 days to a media

containing decreasing concentrations of 2,4-D (45.23 > 22.67 µM) along with TDZ (4.5 µM) and spermine (100 µM). After 90 days, the embryogenic calli were transferred to Y_3 basal medium containing 2,4-D (0.04 µM), BAP (22.19 µM), L-glutamine (68.42 µM) and GA_3 (2.8 µM). After 120 days, the somatic embryos formed were transferred to a hormone-free maturation media (Y_3 basal media with 2.5 g L^{-1} charcoal).

Histological studies

The tissue samples were fixed in a standard fixative Carnoy's 'B' solution (chloroform- 30 mL; absolute alcohol- 60 mL; glacial acetic acid- 10 mL) for 24 hrs. The materials were dehydrated serially using different concentration of alcohol alone (70%< 80%< 90%< 100%) and different concentrations of alcohol along with butanol (3:1, 1:1, 1:3). For infiltration and embedding, a mixture of paraffin wax and bee wax of melting point 58-60 ^{0}C was used. Paraffin blocks were prepared by fixed tissues and histological sections were taken using the microtome (Leica RM 2145). Slides were de-paraffinised using xylene. Subsequent dehydration was done with butanol and alcohol. The slides were transferred to 100 per cent alcohol and subjected to histochemical staining either directly or after hydration depending on the stain used for tissues. Periodic acid Schiff's reagent and Toluidine Blue were used for staining the sections. After subjecting the sections for histochemical staining, they were dehydrated subsequently using butanol. Dehydrated sections were mounted on DPX and observed under Wild Heerburg stereo and Leits Diaplan binocular microscope. Photos were taken and analyzed using Leica Application Suite.

Results

Initial callus formation

Coconut plumule is white in color with a soft texture (Fig. 1a) and contains a shoot meristem and two pairs of plumular leaf primordia formed of densely stained small actively dividing cells with meristematic appearance (Fig. 1b). The explants inoculated in the callus induction media did not show any apparent growth for about two weeks after inoculation, only morphological bulging could be noticed. After 15 days, the explants started growing steadily and formed initial calli, which were white in color with a smooth appearance (Fig. 1c). The histological observations of tissue fixed after two weeks showed noticeable growth of the plumular leaf primordia which were seen to form a ring around the inner leaves and the shoot meristem (Fig. 1d); this could be attributed to the formation of new meristematic cells by the division of provascular cells present in the plumular leaf primordia.

After 30 days, the size of the calli increased and the colour was pure white or beige (Fig. 1e). The transverse section showed initiation of cell division from actively dividing meristematic cells leading to calli initiation (Fig. 1f). The initial calli formed were finely chopped and inoculated into medium containing decreased concentration of 2,4-D (45.23 µM). After 45 days, the initial calli showed proliferation of meristematic cells leading to the development of white translucent structures (Fig. 1g). The histological observation showed that the basis of origin of the translucent structures were the actively dividing meristematic cells present in the provascular cells of plumular leaves and small cells presented in the peripheral layer with meristematic appearance. These peripheral meristematic zones ensured growth of the calli (Fig. 1h). These cells had a visible nucleus of irregular shape and densely stained cytoplasm (Fig. 1i) and were rich in polysaccharides too (Fig. 1j), indicating high metabolic activity of cells.

Embryogenic callus formation

After 60 days, the translucent structures of calli were well-defined, showing ear-like shape (Fig. 1k). The histological sections showed that the calli were formed by densely stained meristematic centers (Fig. 1l), which in turn formed meristematic nodules (Fig. 1m). The translucent calli were transferred to a media containing 2,4-D (22.67 µM) and TDZ (4.5 µM). After 75 days, slight morphological changes took place and the nodular structures became globular (Fig. 1n). Transverse sections showed that all the globular structures were formed of meristematic cells (Fig. 1o). After 90 days, globular embryogenic structures became larger and they covered most of the surface of the calli and were numerous (Fig. 2a). Histological sections showed that some of the globular embryogenic structures seemed to fuse with each other and form elongated structure. Proembryos were formed from this type of globular structures eventually (Fig. 2b).

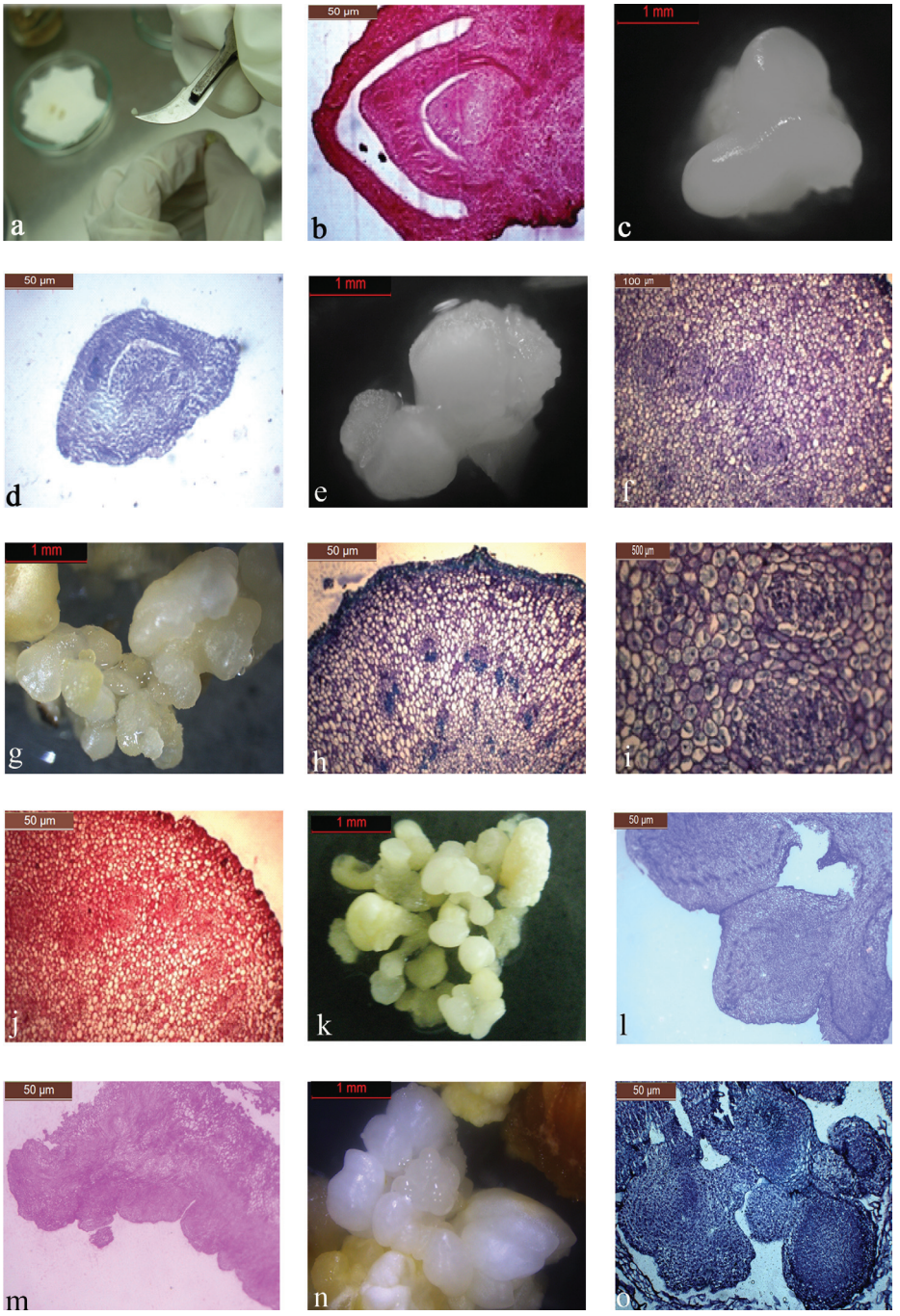

Fig. 1. Histological analysis of different stages of callogenesis (a) Fresh plumule extracted from embryo; (b) Plumule showing shoot meristem and two plumular leaves; (c) Morphological bulging observed after 15 days of inoculation; (d) Plumular leaf forming a ring around the shoot meristem; (e) Callus initiation observed after 30 days; (f) Actively dividing meristematic cells; (g) Cell proliferation observed after 45 days; (h) Meristematic centers in the periphery (i) Meristematic cells with densly stained cytoplasm and visible nucleus; (j) Cells rich in polysaccharides showing high metabolic activity; (k) Callus formation after 60 days; (l) Callus with densly stained meristematic centers; (m) Transverse section showing meristematic nodules; (n) Globular calli formed after 75 days; (o) Transverse section showing the globular calli formation

Somatic embryo formation and shoot initiation

After 120 days, the proembryos formed were transferred to hormone-free maturation media containing 2.5 g L^{-1} charcoal for the maturation of somatic embryos. Somatic embryos observed were translucent white with torpedo shape (Fig. 2c). The transverse section of torpedo shaped embryo showed a germinative pore (Fig. 2d), a well defined meristematic zone (Fig. 2e) formed of parenchyma cells (Fig. 2f) and vascular tissues (Fig. 2g) in matured somatic embryos. Four to five somatic embryos could be observed per callus. By the end of sixth month, the shoot meristem was completely differentiated and more than one plumular leaves were present (Fig. 2h). At this stage, the somatic embryo started to germinate and form the shoot pole. The transverse section of somatic embryo showed a shoot meristem and root meristem (Fig. 2i), which were beginning to define.

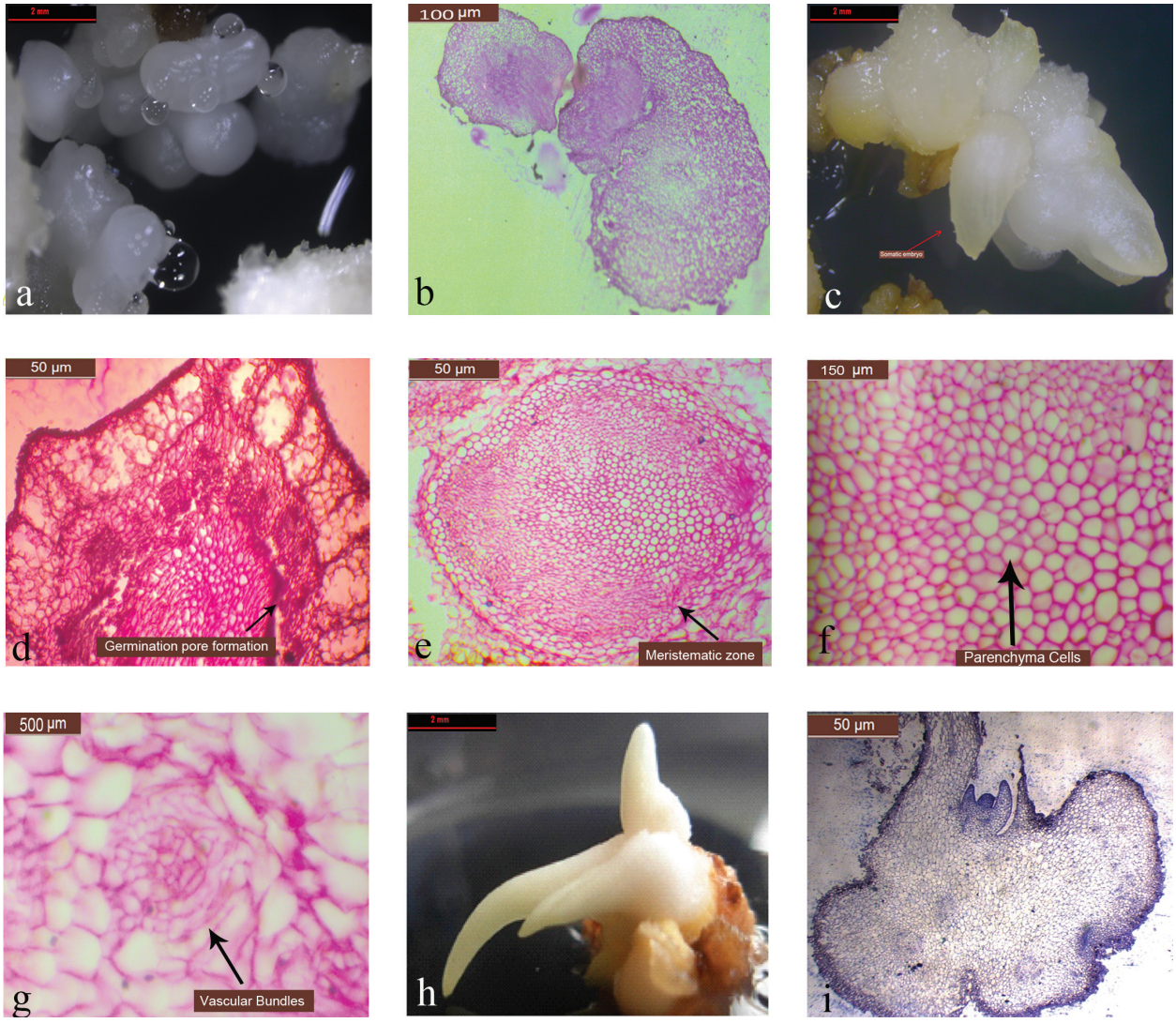

Fig. 2. Histological analysis of different stages of somatic embryogenesis
(a) Globular shaped proembryos; (b) Transverse section of proembryos; (c) Translucent white torpedo shaped proembryo; (d) Germination pore formation inside mature somatic embryo; (e) Well defined meristematic zone inside somatic embryo; (f) Inner layer of parenchyma cells; (g) Transverse section showing the formation of vascular bundles inside the differentiated somatic embryo; (h) Differentiated somatic embryo forming more than one plumular leaf; (i) Transverse section showing shoot meristem with plumular leaf and root meristem

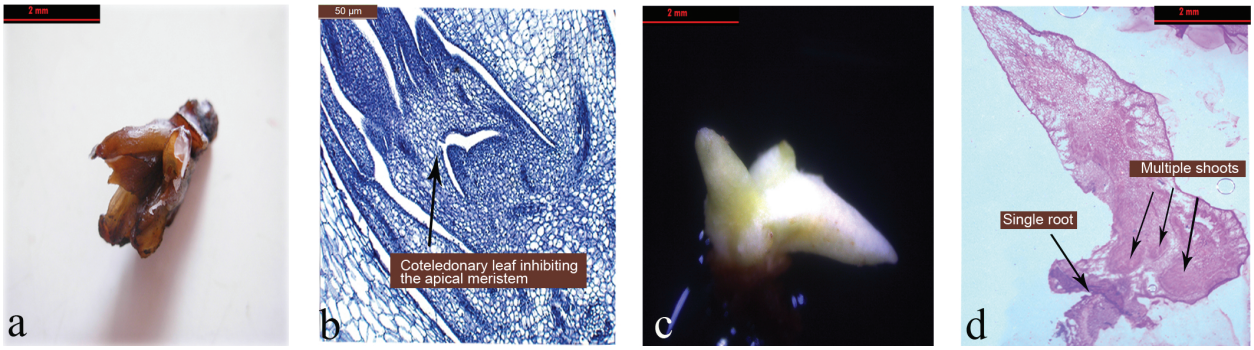

Fig. 3. Histological analysis of abnormalities observed (a) Aberrant somatic embryo; (b) Intact cotyledonary leaf inhibiting apical meristem development; (c) Somatic embryo with multiple shoots; (d) Transverse section of meristemoid with multiple shoots and single root

Some of the embryos showed irregular development with aberrant structures like rudimentary shoot with multiple roots (Fig. 3a). The histological studies revealed the presence of fused cotyledonary leaves which seemed to inhibit the apical meristem development of somatic embryoids (Fig. 3b). Another abnormality observed was the formation of somatic embryos with multiple shoots (meristemoids) (Fig. 3c). Transverse section showed a single root for all the shoots (Fig. 3d), because of which it was difficult to separate each shoot as it grew.

Discussion

During somatic embryogenesis, several histological and morphological changes take place. Since coconut is considered as a most recalcitrant species it is important to study in detail about the cellular changes that occur during somatic embryogenesis. The present histological study gives a detailed view of the internal cellular changes taking place during the formation of somatic embryoids from the coconut plumule-derived calli.

Histological analysis showed that the initial cell divisions occur in cells adjacent to vascular tissue, resulting primary calli. In palms, cells adjacent to the vascular tissue apparently have higher morphogenetic capacity. Studies with leaf explants of oil palm (Schwendiman *et al*., 1988), young and matured embryos excised from seeds and inflorescences of juccara palm (Guerra and Handro, 1998), the seed explants of date palm (Sane *et al*., 2006) and the zygotic embryos of peach palm (Steinmacher *et al.,* 2007) showed that the early events of cell division were always observed in cells adjacent to the vascular tissue, resulting in primary calli or meristematic nodules similar to those observed in the present study.

The origin of formation of callus could be traced out by observing the changes taking place in the plumular leaves, particularly the external ones. After 15 days of culture initiation, the plumular leaves grew and formed a ring around the shoot meristem. This was mainly due to the division of the provascular cells present in the plumular leaves which in turn formed meristematic cells of actively dividing capacity, resulting in initiation of calli after 30 days as reported in the present study, which were similar to earlier reports in leaf explants of oil palm (Schwendiman *et al*., 1988) and zygotic embryos of peach palm (Steinmacher *et al.,* 2007).

The meristematic cells, formed by continuous division of provascular cells, gave rise to peripheral meristematic masses. These peripheral meristematic zones ensured growth of the calli, which could be observed after 45 days of callus initiation in the present study. Similar results have been reported in palms, particularly in inflorescence explants of coconut (Buffard-Morel *et al*., 1992) and leaf explants of oil palm (Schwendiman *et al*., 1988). The occurrence of peripheral meristematic zones associated with callus growth has also been observed in calli originating from inflorescence explants of coconut (Verdeil *et al*., 1994; 2001) and leaves of coffee (Berthouly and Michaux-Ferriere, 1996).

After 60 to 75 days, the meristematic masses formed meristematic nodules which in turn gave rise to ear-like translucent structures. After 75 to 90 days, these meristematic nodules became

globular embryogenic structures. The development of globular embryogenic structures was characterized by the breaking up of meristematic masses, which were comparable with the results observed in leaf explants of oil palm (Schwendiman *et al*., 1990) and coffee (Berthouly and Michaux-Ferriere, 1996).

After 90 days, the active cell division in the discontinuous zone led to the formation of meristematic cells followed by the formation of epidermis. After four months, the globular embryogenic structures fused and formed structures with elongated appearance (torpedo shape). The detailed observation of globular embryogenic structures showed nuclei with irregular morphology, which were also observed in an earlier study in embryogenic cells of coconut calli derived from inflorescence explants (Verdeil *et al*., 2001).

After a period of four to five months, it was possible to observe germination pore, meristematic zone of parenchymal cells and vascular bundles in the transverse section of matured somatic embryos and further formation of shoot meristem and root meristem in the present study, which is comparable with the results obtained by Fernando *et al.* (2003) and Saenz *et al.* (2006). Formation of aberrant somatic embryos, reported earlier by Saenz *et al.* (2006), was also observed in the present study and this could be attributed to the absence of shoot meristem and the presence of fused cotyledonary leaves which seemed to inhibit the apical meristem development.

As previously reported (Fernando *et al.*, 2003; Saenz *et al*., 2006), the sequence of events observed in the present study supports the evidence for the multicellular origin of the somatic embryos. But these observations are contrary with earlier studies (Verdeil *et al*., 1994; Verdeil and Buffard-Morel, 1995), which highlighted the unicellular origin of embryo formation in coconut callus obtained from inflorescence explants.

The present study reports that the sequence of events involved in the complete formation of normal somatic embryos capable of germination was the same as that of zygotic embryo *ie.,* formation of protoderm, individualization of provascular strands and meristematic areas giving rise to the shoot meristem and further to the root meristem (Haccius and Philip, 1979). To conclude, this study provides a better knowledge about cellular changes occurring during somatic embryo formation from the plumular explant of coconut. The results obtained could aid future studies leading to enhanced control of the somatic embryogenic process and greater efficiency of somatic embryo and plantlet formation in coconut.

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