The Origin and Development of Embryoids in Oil Palm (*Elaeis guineensis* Jacq) Embryo Culture

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ABSTRACT Mature zygotic embryos of *Elaeis guineensis* Jacq var *tenera* were excised and cultured on Eeuwens (1976, 1978) medium containing 2 mg/l 2,4-D. Callus was initiated from these embryos within 8 weeks. Embryoids were induced from the primary callus cultured on Murashige and Skoog (1962, MS) medium supplemented with 0.5 mg/l 2,4-D. For embryoid differentiation and plantlet regeneration, two successive media were employed. The first medium was MS-CAP devoid of 2,4-D but containing 0.05% activated charcoal . The second medium was MS-CAP containing 0.1 mg/l 2,4-D and 2.5 mg/l BA. The embryoids were harvested at various time, fixed, sectioned, stained and examined microscopically. The histological origin of embryoids was from single cells in the subepidermis along the surface of callus clumps. Embryoids proceeded in a standard development pattern to the globular-, heart- and finally to the cotyledon stage. Secondary embryoids occurred on the cotyledon of primary embryoids and originated from single, densely staining cells of the epidermis.

KEYWORDS: *Elaeis guineensis* Jacq, histology, oil palm, single cell origin, somatic embryogenesis.

INTRODUCTION

Oil palm is an arborescent monocotyledon and cannot be multiplied by conventional means of vegetative propagation. Somatic embryogenesis would be of great interest for this woody allogamous plant due to its high potential in high yield and plant breeding. Success in plant regeneration by means of somatic embryogenesis has already been reported on many species of palms.[1-5] Much research has been carried out from somatic callus cultures in oil palm.[6-16] The establishment of plant regeneration in oil palm by somatic embryogenesis is satisfactory. However, the origin and development on callogenesis and subsequent morphogenesis is inadequate. Few histological studies have dealt with palms.[2, 9, 13,17-19]

In this communication, we describe an histological study to determine the origin of the callus and the ontogenic stage in which somatic embryogenesis occurs in oil palm embryos cultured *in vitro*.

MATERIALS AND METHOD

Plant Material and Callus Initiation

Mature *Elaeis guineensis* Jacq var *tenera* embryos were excised and surface sterilized as described by Patcharapisutsin and Kanchanapoom.[16] Embryos were initially cultured on Eeuwens[20,21] medium supplemented with 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid). Callus initiated from embryos was observed for 8 weeks.

Induction of Embryoid and Plantlet Regeneration

For callus multiplication and embryoid production, callus was transferred to the culture medium containing a half strength of Murashige and Skoog[22] macroelements and iron, with (mg/l) NaH_2PO_4. H_2O 170; thiamine-HCl 0.4; pyredoxine-HCl 0.5; 2,4-D 0.5 (designated MS-P medium). For embryoid differentiation, all embryoids were transferred to the same medium without 2,4-D but supplemented with (mg/l) casein hydrolysate 100; adenine sulfate 40; activated charcoal 500 (designated MS-CAP medium). For plantlet development, differentiating embryoids were transferred to MS-CAP medium supplemented with 0.1 mg/l 2,4-D and 2.5 mg/l BA (6-benzyladenine).

All media were solidified with 0.15% Gelrite (Merck & Co, Kelco Division, NJ USA). The concentration of sucrose was 3% and the pH of all media was adjusted to 5.6 with 0.1 N NaOH or HCl before Gelrite was added. The media were autoclaved at 121°C for 15 min.

Environmental Conditions

Embryos and callus were incubated at 27°C under 20 $mmolm^{-2}s^{-1}$ photosynthetic photon flux density and 15-h photoperiod provided by Gro-lux lamps. All cultures were subcultured every 8 weeks.

One embryo was planted in 115 ml screw-topped jars. All experiments were carried out three replications with 40 cultures per treatment.

Histological Observations

For histological studies, zygotic embryos, callus and embryoids at various stages of development were fixed in FAA II solution of 90 ml 70% ethyl alcohol, 5 ml glacial acetic acid and 5 ml formalin solution. These tissues were dehydrated through an ethanol-tertiary butanol series for 48 h and embedded in Paraplast. Specimens were sectioned at 10 to 14 µm and stained with safranin and fast green.[23] All sections were mounted with Permount and were viewed under bright-field illumination with an Olympus microscope. Histological analysis was carried out on representative samples of the embryoid explants.

Results

Culture Initiation

Eight weeks after culture initiation in Eeuwens[20,21]medium containing 2 mg/l 2,4-D, the embryos gave rise to compact, yellow creamy callus and the percentage of embryo forming callus was 29.36. The callus grew very slowly hence attempts were made to induce maximum callus production. The eight-week-old callus were transferred to MS-P medium supplemented with 0.5 mg/l 2,4-D and the multiplication rate was 7 fold the original size in 8 weeks. Prolonged culture in this medium from the eighth week onwards (upto 10-16 weeks) resulted in embryoid formation (8.33%). The embryoids were formed on the primary callus and could be distinguished by the presence of white, opaque, and compact nodules (Fig 1a). After being cultured in the MS-P medium for 16 weeks, the embryoids showed no further development. Differentiating embryoids were evidenced when they were transferred to MS-CAP medium devoid of 2,4-D with 0.05% activated charcoal for 5 weeks (Fig 1b). The number of embryoids slightly increased and most of them enlarged in size. They were polarized and shoot-like growth development emerged from these embryoids (Fig 1c). Transfer of these embryoids to MS-CAP medium supplemented with 0.1 mg/l 2,4-D and 2.5 mg/l BA allowed the differentiation of shoot-like growth structure and subsequent complete plantlet (Fig 1d).

Embryo Anatomy

The oil palm embryo from mature seed was white, ovate and averaged 3 mm in length. The embryo at this stage consists of a root pole, an epicotyl shoot apex and a single cotyledon (Fig 2a). The root pole is blunt and flattened. The shoot apex is surrounded by the cotyledon. An internal cavity or a small slit which separates the shoot tip zone from the cotyledon is evident in the base of cotyledon (Fig 2a,b). The shoot tip consists of a shoot apical meristem and two or three leaf primordia. The cotyledon is composed of three cell types namely parenchyma, procambial cells and protodermal cells. Procambial cells are narrow and elongate along the axis of the embryo. Procambial strands are visible as individual bundles in transverse section (Fig 2c).

Origin of the Callus

After 4 weeks of culture, an histological study showed that callus occurred from the subepidermis resulting in a layer of 3-4 cells with a markedly meristematic appearance (Fig 3a). These cells were small, had dense cytoplasm and contained well-stained nucleus. The callus composed of meristematic cells remained inside the explant; however, continuity of division led to intense proliferation which caused a rupture of the epidermis and hence emergence at the surface of the embryo (Figs 3b,c).

At the eighth week of culture, visual observation revealed several spherical nodular callus were adjacent. Histological examination showed numerous centers of meristematic activity. The cells in such centers were smaller than cells in the outer part of the callus, and they stained more intensely and contained distinctly prominent nuclei (Fig 3d). The callus cells outside of the meristematic centers were large and more highly vacuolated. When allowed to develop in MS-P medium for 12 weeks or longer, the meristematic centers appeared to differentiate into embryoids and some cells differentiated in which tracheids were present.

Early meristematic initials were located in the subepidermis regions of the callus (Fig 4a). It is presumed that single cells in this area were the precursors of the embroyids. These cells arose singly and easily distinguished by their darkly stained nuclei. The plane of first division was not uniformed oriented but could be periclinal or anticlinal to yield the two- and four- celled stages (Fig 4b). The next division creating the multicelled proembryo containing approximately eight to ten cells (Fig 4c). Subsequent divisions formed the globular- and heart-shaped embryos which often characterized by a prominent epidermal layer giving way to a bipolar embryoid (Figs 4d,e). Different stages of embryoid

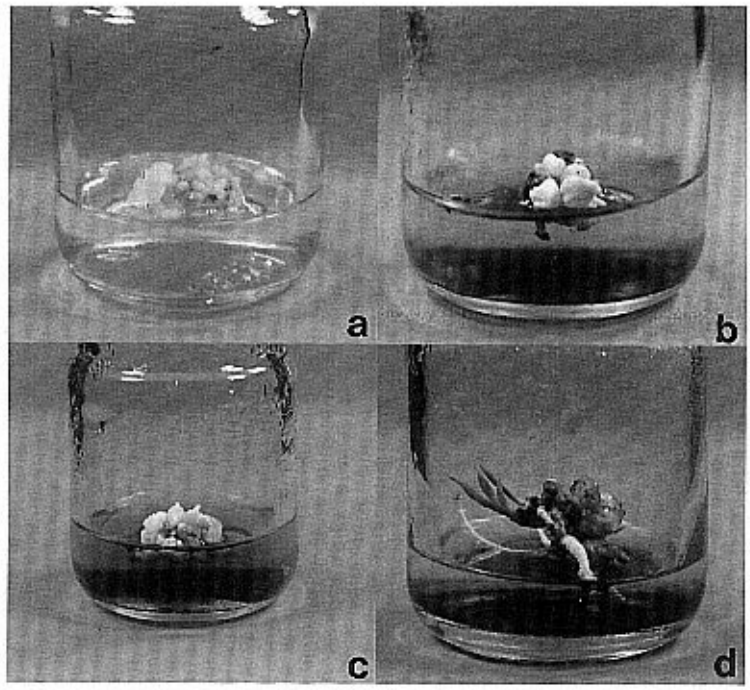

Fig 1. Somatic embryogenesis in oil palm embryo-derived callus. (a) Morphological appearance of embryogenic friable callus cultured on MS-P medium supplemented with 0.5 mg/l 2,4-D. x165. (b) Opaque white and chlorophyllian embryoid formed on MS-CAP lacking 2,4-D with 0.05% activated charcoal. x165. (c) Shoot-like growth emerge from embryoids in (b) after a prolonged culture on the same medium. x125. (d) Complete plantlet cultured on MS-CAP containing 0.1 mg/l 2,4-D and 2.5 mg/l BA. x165.

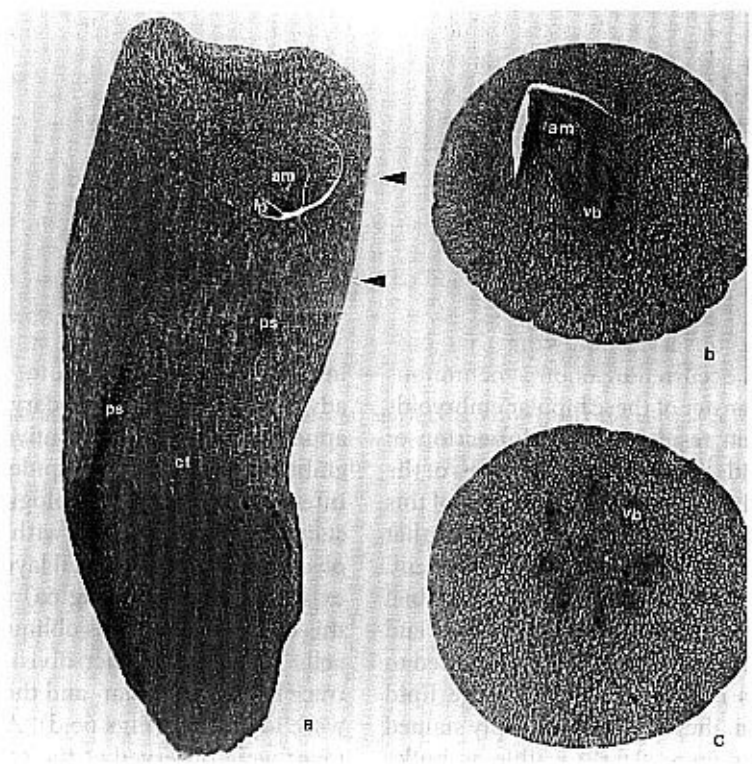

Fig 2. Histological aspects of zygotic embryos. (a) Longitudinal section through the epicotyl showing apical meristem and leaf primordia. Arrowheads indicated approximate level of (b) and (c). x295. (b-c) Transverse section through epicotyl and base of cotyledon, respectively. x86. (am = apical meristem; r = radicle; ct = cotyledon; ps = procambial strand; lp = leaf primordia; vb = vascular bundle).

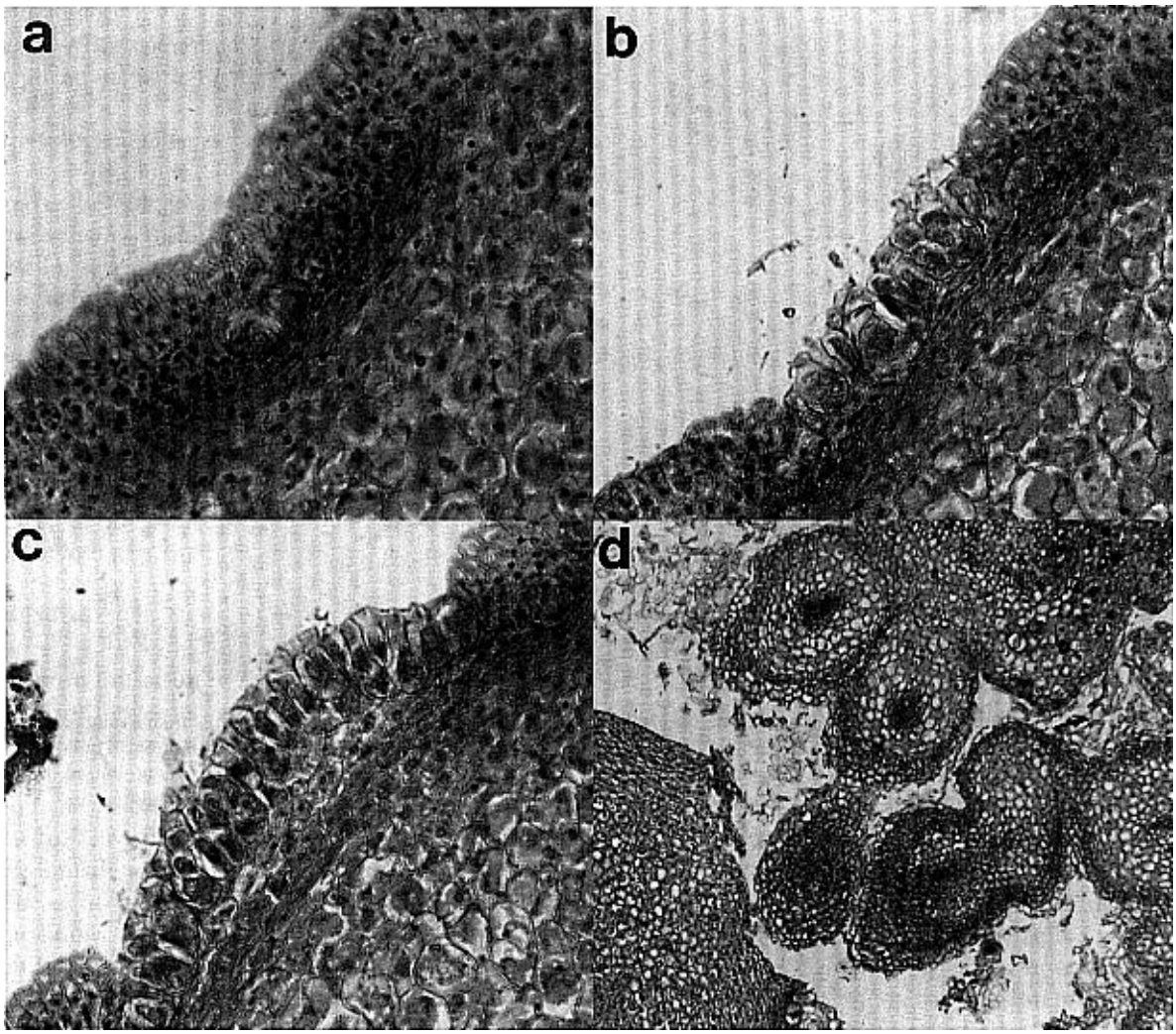

Fig 3. (a) Transverse section of zygotic embryo showing induction of small meristematic cells. x172. (b) Increase mitotic activity to form meristematic zones at the embryo periphery. x172. (c) Rupture of the epidermal layer to allow a group of meristematic cells emergence. x172. (d) Fragmentation of nodular callus from embryos composed of centers of meristematic activity. x86.

development were observed showing that pro-embryogenesis had occurred in a non-synchronous fashion (Fig 4f). Transfer of these bipolar embryoids to MS-CAP medium resulted in the formation of cylindrical embryoids which resembled stages of the excised zygotic embryos (Fig. 5a). Sectioning of this embryoid revealed its rudimentary cotyledons that became vascularized. At this stage of development, they acquired a protodermal cells and procambial strands, then shoot and root meristem. At the end of the culture period, very active cell division led to differentiated cells containing high storage lipid content. In addition, these cells were deeply stained and starch reserves were clearly visible as bulky grains within the cytoplasm (Fig 5b).

During development continuing normally, a large number of embryoids became the site of adventive change to secondary embryogenesis. This appeared to be direct adventive change to secondary embryogenesis from the epidermal cell without the intervening callus. Histologically, single densely stained, nonvacuolated with thick wall cell was observed in the epidermal layer of the cotyledon of existing embryoids (Fig 6a). The first division of this competent cell was oblique and resulted in two cells (Fig 6b). Further divisions were difficult to trace but the globular- and the heart-shaped stages were identified (Figs 6c,d). All stages of development were observed at the same cotyledon site of the embryoid.

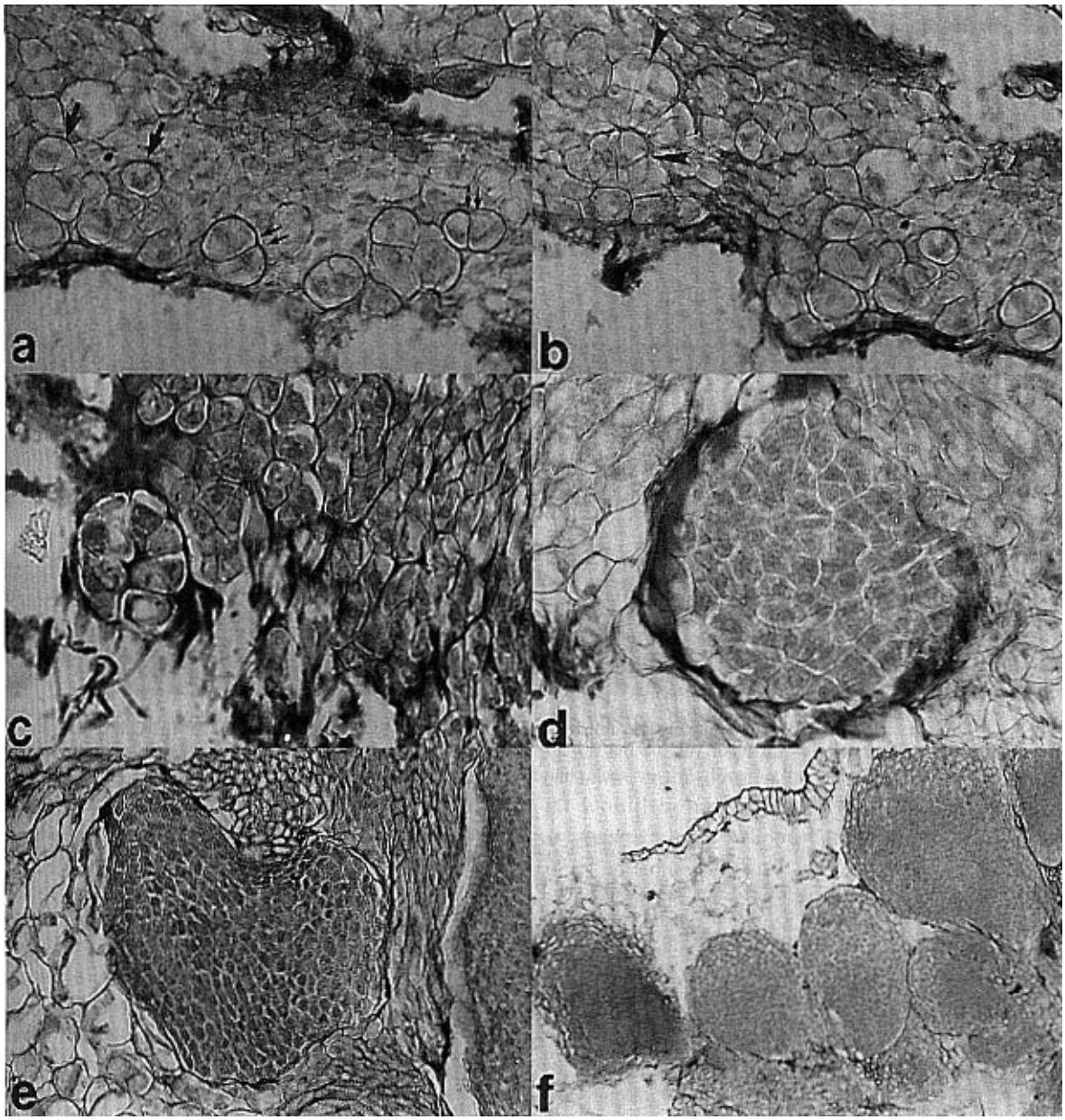

Fig 4. Origin of embryoids in embryo-derived callus culture. (a) Single cells (arrow) and two-celled stage (double arrows). x137. (b) Four-celled stage (arrowhead). x137. (c) Multicelled proembryo containing eight to ten cells. x137 (d) Epidermized globular stage. x34. (e) Heart shape embryoid that remain in the callus. X34. (f) Several compact embryoids in different stages of formation. x14.

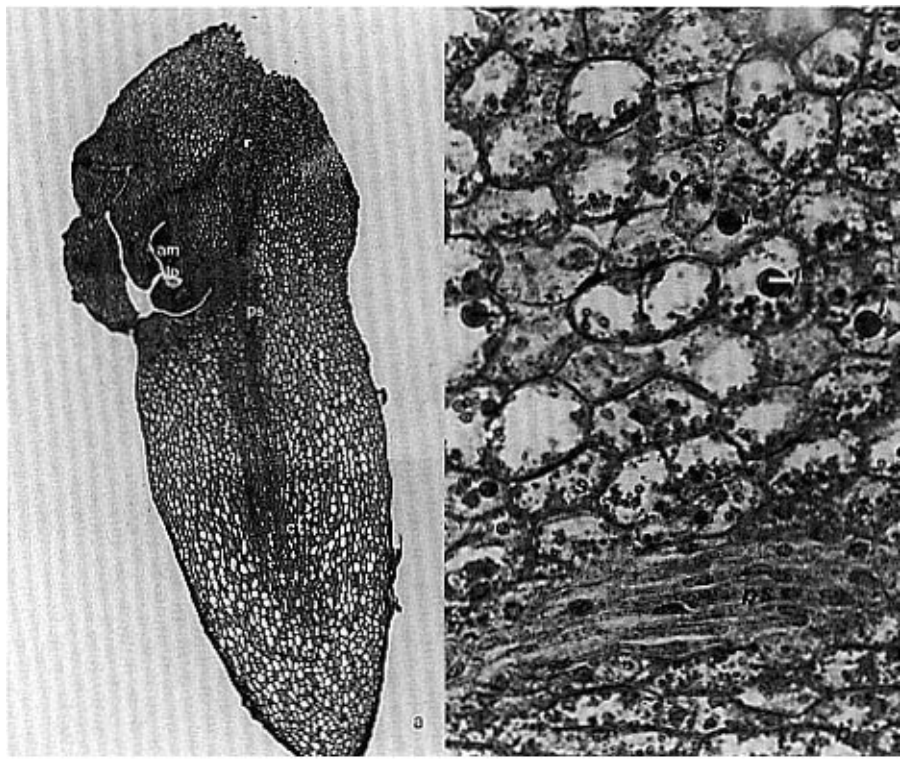

Fig 5. (a) Longitudinal section of bipolar embryoid showing structures resemble to zygotic embryo. x295. (b) Cell differentiation from procambial strands with starch reserve and storage lipid. x172 (am = apical meristem; r = radicle; ct = cotyledon; ps = procambial strand; lp = leaf primordia; vb = vascular bundle; s = starch reserve; l = storage lipid).

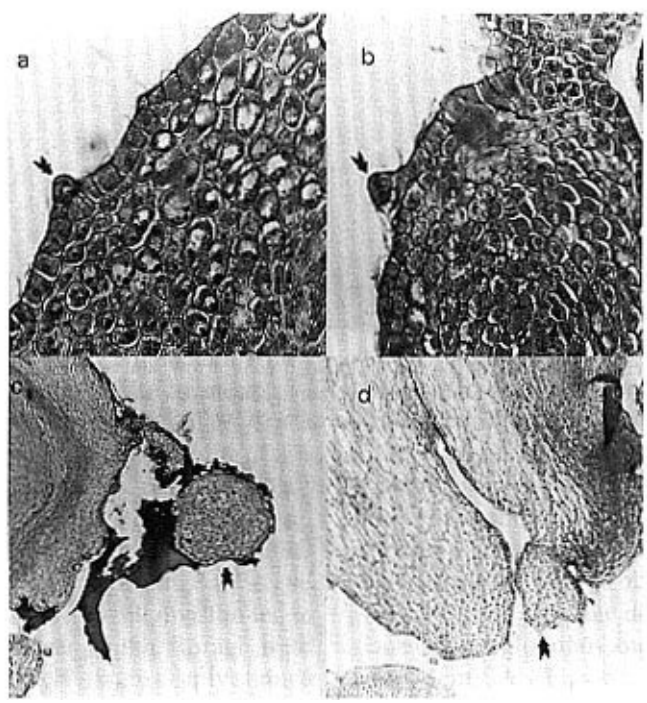

Fig 6. Adventive embryogenesis arising from the epidermal cell of the cotyledon of pre-excisting embryoids. (a) Single cell origin with densely stained cytoplasm and nucleus. x86. (b) Two-celled stage derived from oblique division of the single cell. x86. (c) Globular stage. x34. (d) Heart shape stage. x34.

Discussion

This research has shown conclusively that somatic embryogenesis occurs in oil palm embryo culture. Conditions for a reproducible initiation of embryogenic callus were studied. Media with different plant growth regulators were needed for induction and development of embryoids. It is well documented that the presence of auxin is critical for embryo initiation and the reduction of the auxin concentration, or its absence is important for both initiation and maturation.[16] In our experiments, a callus is first obtained in the medium rich in 2,4-D (2 mg/l). It is then transferred to another medium with the reduction of 2,4-D (0.5 mg/l) to achieve embryoids. Differentiation takes place when transferred these embryoids to the medium lacking 2,4-D in the first phase of culture followed by the addition of 0.1 mg/l 2,4-D and 2.5 mg/l BA in the second phase. Gradient of 2,4-D and addition of BA, a cytokinin, may have influenced somatic embryo development.

Histological studies of palm somatic embryogenesis have been carried out by several research groups. The present study demonstrates without ambiguity that somatic embryogenesis arises from single cells and corresponds to the development morphology of zygotic embryos of oil palm. Somatic embryos of this single cell origin are widely encountered *eg* date palm,[17] *Ranunculus*,[24] carrot,[25] guinea grass,[26] *Trifolium*,[27] borage,[28] cork oak,[29] *Vanda* orchid.[30] This finding deviates from the result reported by Schwendiman et al.[19] that the origin of an oil palm somatic embryo is multicellular. This is probably due to the different source of explant used since the plant material was fragments of non-chlorophyllian leaflet from inside the spear. Moreover, the physicochemical conditions of the culture differred from the present study. Therefore it would appear that culture conditions favor the uni- or multicellular mode of embryogenesis in callus with embryogenic potential.[31]

The somatic origin of the embryoids was obtained from single cells which were highly differentiated with numerous starch grains. Such cells contain starch grain, a source of energy, in the cytoplasm. Thomas *et al*[32] considered starch to be an indicator of the development of tissue towards somatic embryogenesis. Starch accumulation has been detected in the callus and bipolar embryoids of the oil palm indicating that starch accompanies the formation of somatic embryos. Other main feature of the somatic embryos is the presence of storage lipids indicating that physiological processes associated with embryogenesis occur.[28]

Secondary somatic embryogenesis has also been demonstrated. This process is another source of somatic embryos from pre-existing embryos. Recent studies on different species have shown the production of somatic embryos by adventitious budding as in cork oak,[29] *Trifolium*,[33] cocoa.[34,35] The appearance of adventive embryogenesis can be considered as a result of the escape of some particular cells from integrated group control which allows them to express their totipotency,[36] El Maataoui *et al*[29] stated that repetitive embryogenesis was often associated with a failure of the embryonic axis to develop as a normal seedling probably reflecting a loss of the bipolar control of embryo ontogeny. Kim and Janick[37] also reported secondary embryo occurred with high frequency on the cotyledons and the hypocotyl of celery, which inhibited growth of the apical meristem and limited the conversion of primary somatic embryos into normal plantlets.

In this study, somatic embryogenesis from embryo culture of oil palm and origin of embryoids are described. Somatic embryogenesis is an ideal method of regeneration after genetic transformation if its origin is single cell. Histological knowledge concerning ontogeny of oil palm embryoids can provide important information for improving the somatic embryogenesis process for this crop. Thus, it would be necessary to confirm the origin of embryoids by histological analysis.

Acknowledgements

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