

# HISTOLOGICAL ANALYSIS OF *In Vitro* CULTURED COCONUT ENDOSPERM

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## ABSTRACT

Coconut is a very important plant for the livelihood of people in tropical countries. It is also used as an icon of tropical region. Coconut fruit is very heavy and can cause injuries if the fruit falls down and hits somebody who happens to be underneath a coconut tree. In order to avoid the accident, the coconut fruits have to be regularly cut off. Coconut tree originated from *in vitro* cultured endosperm is a triploid plant that produces seedless fruit (without endocarp). Coconut fruit without endocarp is not heavy. The objective of this study was to investigate plant regeneration of fresh and *in vitro* cultured coconut endosperms. The fresh and developed *in vitro* cultured coconut endosperms were observed using histological analysis. Solid endosperm of seven month-old postanthesis coconut from "Samoan Dwarf" cultivar was freshly picked up and cultured *in vitro* on modified Branton & Blake formula. Histological study of fresh coconut endosperm showed that the endosperm consisted of parenchyma cells, which were relatively uniform in shape and size, with some nuclei consisted of 1 – 5 nucleoli. Three month-old calli of *in vitro* grown coconut endosperm in semi solid media showed that its cells varied in shape and size, characterized by high nucleus to cytoplasm ratio, high starch, protein and lipid contents which underwent many divisions. Seven month-old calli of *in vitro* grown coconut endosperm in liquid media showed embryogenic cells which resembled proembryos. Fourteen month-old bud-like structure of coconut endosperm in semi solid media showed a meristematic layer, tunica-carpus structure, cortex-like region and tracheids of xylem. These results indicated that the bud-like structure was an early stage of shoot bud formation in coconut endosperm. This is the first report of early stage of shoot bud formation occurring on coconut endosperm cultured *in vitro*.

**Keywords:** Bud-like structure, calli, coconut endosperm, cultured *in vitro*, histological analysis

## INTRODUCTION

Coconut is one of the most important palm crops in the world because all parts of coconut plant are very useful for humankind. Coconut is planted by 10 million small-holder farmers on 12 million ha of land in the world (Nguyen *et al.* 2015). The decrease of coconut production is mainly caused by palm senescence and diseases; therefore, there is a great demand of coconut plant having high productivity and resistance to diseases (Sandoval-Cancino *et al.* 2016). Coconut tree is a tall tree which fruit weighs about 1.44 kg. Coconut fruit consists of exocarp, mesocarp and endocarp parts. The exocarp and mesocarp parts make up the husk of coconut fruit which is very light in weight. The endocarp part (seed) of

coconut is heavy and contains shell, nut, embryo and water. Endosperm is a triploid tissue. Coconut plant originated from endosperm is a triploid plant and produces seedless fruits. Seedless fruit of coconut does not have endocarp and thus, it is not heavy. Coconut tree should be maintained by regularly cutting off the fruits, which is quite costly. Therefore, triploid coconut tree will be more economical to maintain.

Coconut (*Cocos nucifera* L.) is a long-lived plant with 3 - 5 years juvenile phase. Coconut is generally cross-pollinated and very heterozygous (Sukamto 2011a). Studies on the development of coconut cultured *in vitro* was reported for the past sixty years, mostly using embryos (immature unselected plants). Coconut remains a recalcitrant species that reflected mainly in the slowness of *in vitro* morphogenesis. There is a difficulty to

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monitor the ability of the meristematic cells of coconut to actively divide; coconut also has heterogeneous response of various explants (Sandoval *et al.* 2003; Fernando 2010). Before their differentiations are visually recognizable, many changes occur at the cellular and tissue levels. Cells could undergo a series of orderly divisions, to form callus, proembryo, embryo and bud-like structure. Cell aggregation consists of small, isodiametric, thin-walled, micro-vacuolated cells, highly basophilic, densely staining nuclei and cytoplasm (Thorpe & Murashige 1970; Reinert *et al.* 1977). They may also contain numerous starch grains or lipid deposits (Thorpe & Murashige 1970; Ross *et al.* 1973; Villalobos *et al.* 1985; Arnold & Hakman 1988; Kanchanapoom & Tinnongjig 2001; Liu 2013), and produce proembryos that may develop to become embryos, organs or vascular tissues (Reinert *et al.* 1977; Thorpe 1978).

Few studies were conducted on coconut endosperm culture, which only produced low callus and failed to develop further morphogenesis (Fisher & Tsai 1978; Bhalla-Sarin & Bagga 1983; Kumar *et al.* 1985). Recent improvements have been achieved using endosperm explants and they are succeeded to obtain quicker and higher callus formation, also embryo-like structures (Sukamto 2011b). The calli formed an embryo structure after 21 weeks of culture and grew to become bud-like structure after 14 month-old of culture, showing three protuberances on the top. This study was aimed at understanding morphogenesis at cellular and tissue levels using histology of fresh coconut endosperm and *in vitro* cultured coconut endosperm.

## MATERIALS AND METHODS

### Plant Material

Solid endosperm of seven month-old postanthesis coconut from “Samoan Dwarf” cultivar was picked up freshly from coconut palm trees in Oahu Island, Hawaii. The study was conducted at Tissue Culture Laboratory, Horticulture Department of Hawaii University at Manoa, USA. The fresh cuts of solid endosperm were used as control of plant materials for histological analysis. The solid endosperms were

aseptically cored with cork borer and scooped with a sterile spoon inside a laminar air flow cabinet. Cylindrical endosperm having 8 mm diameter and 4 mm thickness, used as explants, were grown on various media treatments. The endosperm formed callus after seven weeks of culture. Three month-old calli cultured in semi solid medium, seven month-old calli cultured in liquid medium and 14 month-old bud-like structure (10 x 8 mm in size) cultured in semi solid medium, were used for histological analysis.

### Culture Media

The culture media were a modification of Branton and Blake formula (Branton & Blake 1986) with addition of 10 mg/L putresine, 2.50 g/L activated charcoal (AC), 1.70 g/L phytigel, 0,  $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$ ,  $10^{-3}$  M 2,4-dichlorophenoxyacetic acid (2,4-D) or 4-amino-3,5,6-trichloropicolinic acid (Picloram) combined with  $10^{-5}$  M 6-benzylaminopurine (BA) at 16 WOC. The pH of the media was adjusted to 5.70 before being autoclaved. The media were poured into 2.5 x 15 cm test tubes (having volume of 14 mL) and were autoclaved at 121 °C and 1 kg/cm<sup>2</sup> for 15 minutes. Media were stored for one week before being used. Single explant was placed in a test tube with the uncut surface upright. Cultures were incubated at approximately 31 °C in a dark room.

### Data Interpretation

Fresh solid endosperm were thinly cut with a sharp and clean razor blade, stained with toluidine blue, then covered with a cover glass. The specimens were examined and photographed using Zeiss photomicroscope. Plant materials of calli and bud-like structure derived from *in vitro* cultured coconut endosperm were obtained using protocol developed previously by Sukamto (2011b).

Several three month-old calli cultured in semi solid medium were cut with a sharp and clean razor blade and put inside a petri dish containing a small volume of glutaraldehyde fixative (2.75% w/v). Thin tissue sections (1 – 2 mm) were vacuumed two times; 1 hour for each vacuum session. Tissues were washed three times with Sorensen phosphate buffer for 10 minutes each time and stored in refrigerator overnight. The tissues were fixed with 2% osmium tetroxide and

washed three times with 0.2 M Sorensen phosphate buffer at pH 7 – 7.4 for 20 minutes each time. Specimens were dehydrated in a graded series of ethanol concentrations (10, 20, 30, 40, 50, 60 and 70%) for 15 minutes in each ethanol concentration. Specimens were immersed overnight in 70% ethanol inside capped vials which were stored in refrigerator. The specimens were furthermore dehydrated with graded series of ethanol concentrations (70, 80, 90, 95 and 100%) for 30 minutes in each ethanol concentration. The dehydrated specimens were transferred into capped vials containing 5 mL of 100% ethanol and 1 mL historesin (HR), without using any transition solutions and were mixed by swirling. These specimens were then stored overnight at room temperature. HR was added 3 mL in day 1, 3 mL in day 2 and 4 mL in day 3. Upon completing those steps, the specimens underwent two times changes of 100% HR at 5 mL each time. The specimens were then sectioned with a rotary microtome at 5 – 10  $\mu$ m.

Several seven month-old calli cultured in liquid medium were directly stained as samples. Cell suspensions were transferred using pipette onto a glass slide. The sample was stained using a few drops of 0.25 – 0.5% potassium iodide-iodine (IKI) and was then covered with a cover glass (Berlyn & Miksche 1976). The specimens were examined and photographed with a Zeiss photomicroscope.

An embryo structure of the 21 week-old culture grew to become bud-like structure after 14 month-old culture. The bud-like structure was cut longitudinally into three parts which were immediately fixed in a 50 mL mixture of 1% acrolein, 2% glutaraldehyde, 2% para formaldehyde and 0.05 M sodium cacodylate buffer at pH 7.6. This specimen was vacuumed twice for 2 days each time. After this process, the fixative agent was decanted; the tissue was washed three times with 0.05 M cacodylate buffer for 30 minutes each time and then stored in the refrigerator for three days. The specimens were transferred to peel-a-way molds containing embedding medium. The containers were then closed to exclude oxygen and vacuumed overnight. Polymerized blocks were glued on wood blocks. The embedded samples were then sectioned with a rotary microtome at 5 – 10  $\mu$ m. The sections were stained with 1% toluidine blue,

a combination of toluidine blue and acid fuchsin, Feulgen-fast green stained DNA and counterstained against light green, or Periodic Acid Schiff (PAS) stained polysaccharides. Acid fuchsin stained most cells components, especially mitochondria; toluidine blue was used as a counter stain; PAS was used to stain carbohydrates (Gurr 1965).

## RESULTS AND DISCUSSION

### Fresh Coconut Endosperm and Calli Cultured in Semi Solid Medium

Fresh coconut endosperm from young coconut fruit consisted of parenchyma cells, which were relatively uniform in shape and size (Fig. 1a). Its nuclei had one to five nucleoli (Fig. 1a, 1b).

The similar result was reported by Popielarska-Konieczna *et al.* (2013) in cereal endosperm. After being cultured *in vitro*, the cells grew into various shape and size. The results of this study showed that calli of the three month-old culture had darker cytoplasm at the right side compared to those at the left side (Fig. 1c). Similar result was reported by Fernando *et al.* (2003) in coconut plumule cultured *in vitro* and by Popielarska-Konieczna *et al.* (2013) in cereal endosperm cultured *in vitro*. The dark areas of cytoplasm contained accumulation of lipid bodies and dense cytoplasm (Fig. 1d). This result agreed with Ceniza *et al.* (1992) who studied fatty acids in callus of *in vitro* cultured coconut endosperm. The majority shape of lipid bodies were rod-like shape and the others were droplet/globular shape, which were similar to study results obtained by Liu (2013) on ripe chili pepper fruits. This study also showed that intercellular spaces of callus tissues were connected with fibrous and reticular structures. Similar result was reported by Popielarska *et al.* (2006) in endosperm-derived callus of kiwifruit. In regards to the light area of cytoplasm, the study showed that the cytoplasm had fewer lipid bodies and more frequent cell divisions in many planes; some nucleoli appeared long (Fig. 1e). Formation of four-cell proembryo structures developed from coconut endosperm culture (Fig. 1f) was similar to proembryo formed by segmenting division of coconut inflorescence culture (Verdeil *et al.* 1994).

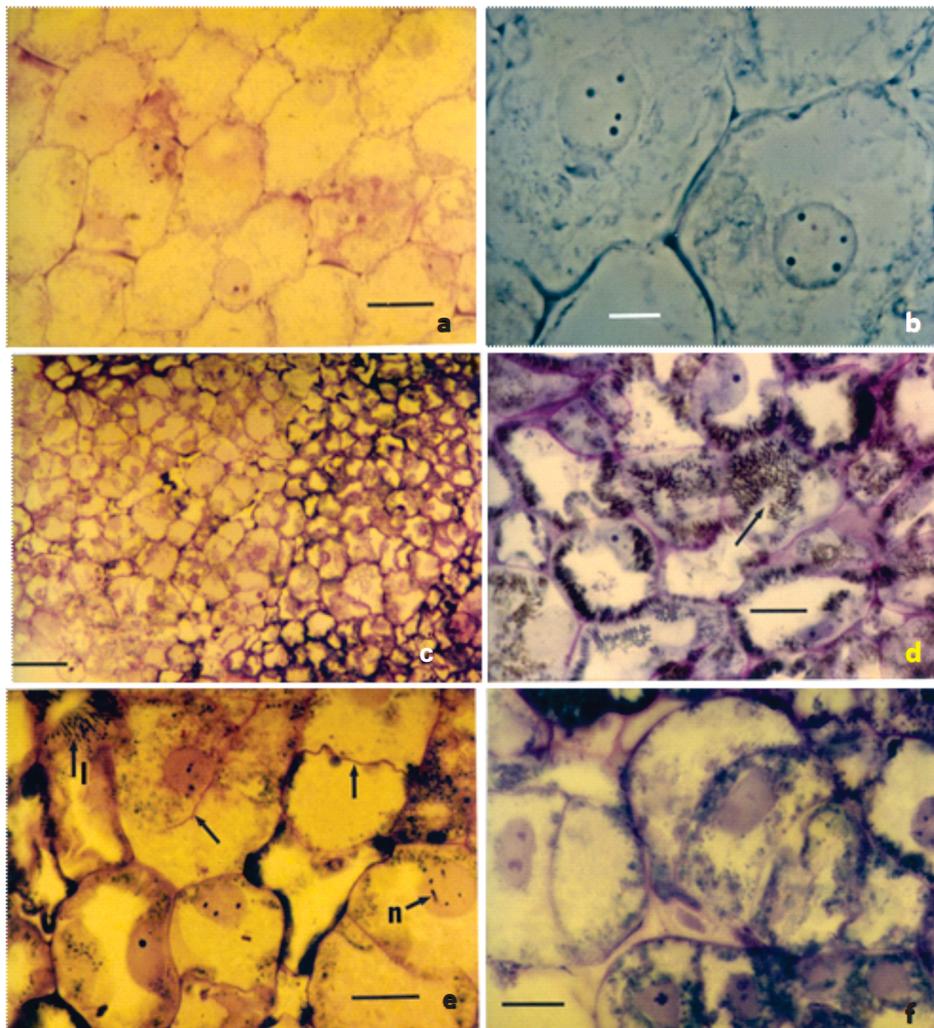


Figure 1 Fresh endosperm cells shows: a). Relatively uniform shape and size (bar = 4  $\mu\text{m}$ ) and b). Nuclei consists of 4 – 5 nucleoli (bar = 10  $\mu\text{m}$ ). Fragmentation of the three month-old calli consists of c). Dark and light areas (bar = 10  $\mu\text{m}$ ); d). Dark area composed of cells with dense cytoplasm and a lot of lipid components (bar = 2  $\mu\text{m}$ ); e). Light area composed of highly vacuolated cells composed of many divisions of cells with few lipid components (l) (bar = 2  $\mu\text{m}$ ); f). Formation of proembryos structures with several number of cells (bar = 20  $\mu\text{m}$ )

### Calli Cultured in Liquid Medium

Seven month-old calli cultured in liquid medium showed development of proembryo structure from coconut endosperm (Fig. 2a).

The proembryo resembled the Embryo-Like Structure (ELS) in soybean anther culture (Rodrigues *et al.* 2005). Proembryo composed of embryogenic cells characterized by high nucleus to cytoplasm ratio, high starch and protein reserves (Fig. 2b, 2c, 2d). This result resembled to embryo and callus formation of pollen grain culture of coconut (Perera *et al.* 2008). Formation of proembryo structures could happen without suspensor (Fig. 2e) or with suspensor (Fig. 2f). Suspensor of proembryo was important to

increase embryo survival on the development of early globular embryos (Paolicchi *et al.* 2003).

### Bud-like Structure Cultured in Semi Solid Medium

An embryo occurred on callus treated with Picloram  $10^{-6}$  M after 21 weeks of culture (Fig. 3a).

The embryo grew to become bud-like structure after 14 months of culture (Fig. 3b). Histology of bud-like structure showed an apical meristem tip between cotyledonary notch, vascular strand and dermal layer (Fig. 3c). This structure was primary meristematic state of apical meristem, tended to organize shoot primordia of coconut embryo development (Guzman *et al.*

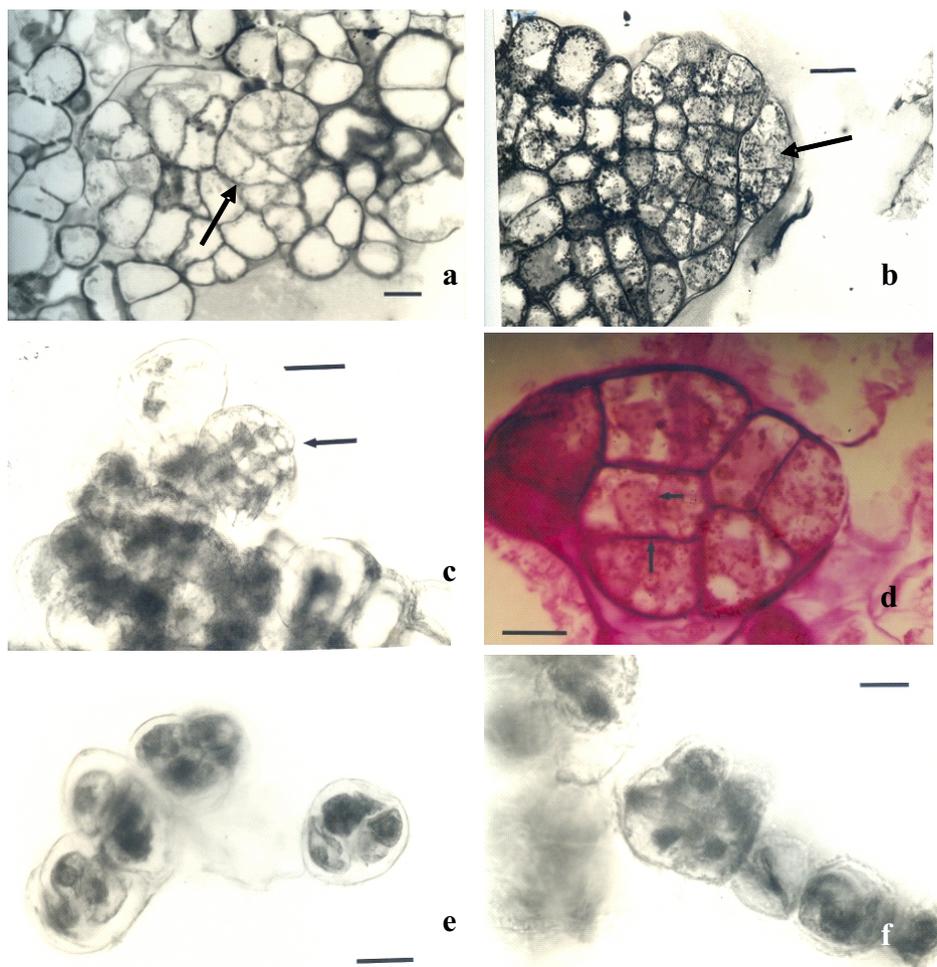


Figure 2 a) Formation of proembryo composed of seven cells (bar = 4  $\mu\text{m}$ ); b). Formation of cells containing starch grains (bar = 4  $\mu\text{m}$ ); c). Formation of proembryo composed of seven month-old calli cultured *in vitro* in liquid medium (bar = 4  $\mu\text{m}$ ); d). Formation of proembryonal cell complex composed of several proembryo units, showing segmented division (bar = 2  $\mu\text{m}$ ); e). Formation of three-celled structures of proembryos cultured in liquid medium (bar = 4  $\mu\text{m}$ ); f). Formation of five-celled structures of proembryo with suspensor in liquid medium (bar = 2  $\mu\text{m}$ )

1978). It resembled shoot bud proliferating on surface of *Pinus eldarica* (Wagley *et al.* 1987) and revealed a well-structured shoot meristem towards whole coconut plant (Verdeil *et al.* 1994). There were tracheids of xylem, inside of vascular strand and lipid droplets in the bud-like structure (Fig. 3d). Similar lipid droplets were reported by Kanchanapoom & Tinnongjig (2001) in embryoid cells of oil palm and Liu (2013) in plastoglobular shape of chili pepper fruits.

In this study, somatic embryogenesis occurred on the cultured coconut endosperm. Many protuberances were visible in the mantle, one of which resembled early stage of proembryo (Fig. 3e). Similar structure was reported by Dawayati *et al.* (2012) in embryonic callus of date palm. The peripheral cells, especially the

protuberances, differed from cells in the adjacent internal region. The mantle cells were smaller, had denser cytoplasm and had prominent dark nuclei. This result was similar to cells of leaf and hypocotyl cultures of *Torenia fournieri* and *Anagallis arvensis*, which destined to become embryos, buds and shoot apices (Reinert *et al.* 1977). This study revealed that the protuberance had one layered tunica covering a large group of irregularly arranged cells which could be the corpus (Fig. 3f). This was found in regenerated shoot bud, which was similar to organogenesis in leaf-derived callus of black pepper (Sujatha *et al.* 2003) and endosperm-derived callus of kiwifruit (Popielarska *et al.* 2006). This tunica-corpora resembled bud meristem in *Foeniculum vulgare* callus (Anzidei *et al.* 1996).

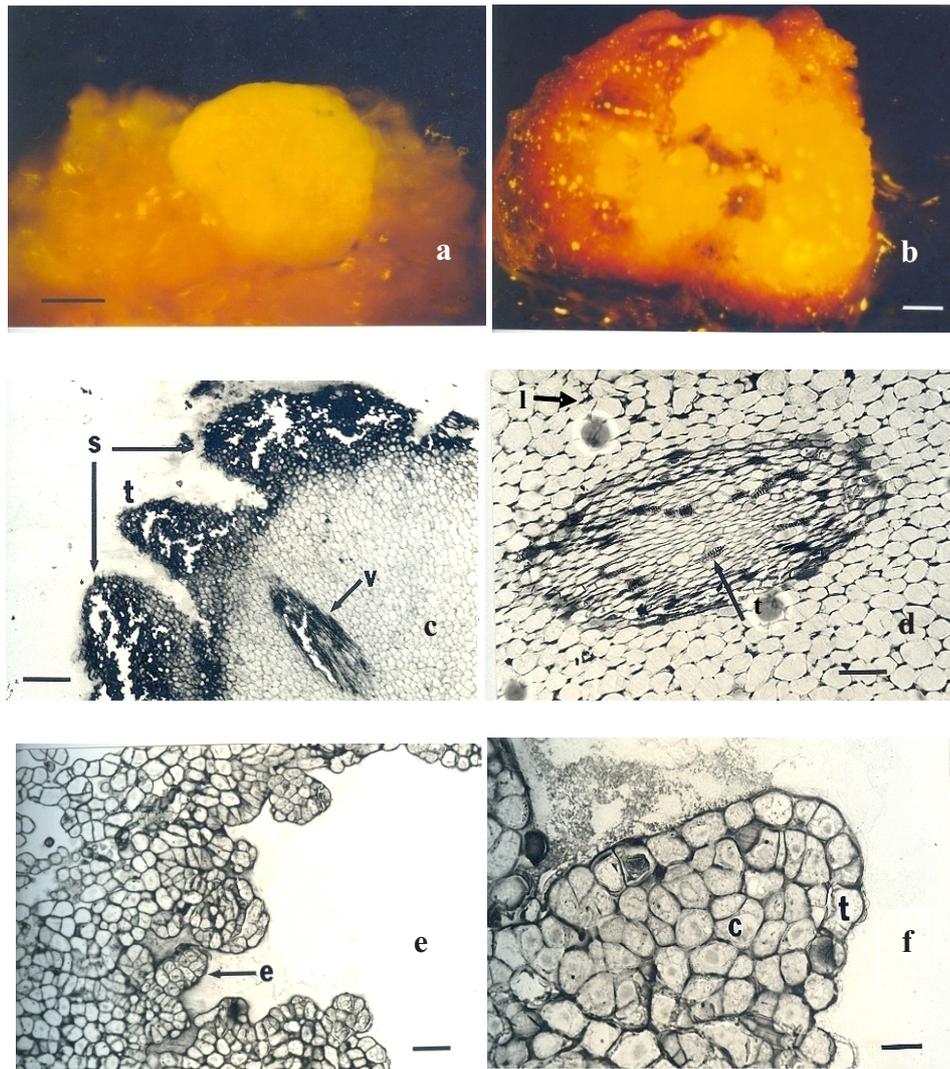


Figure 3 a). Embryo structure occurred after 21 weeks of cultured (bar = 1 mm); b). Embryo grew to become bud-like structure (bar = 1 mm) after 14 months of culture; c). Cross section of the bud-like structure showing cotyledonary notch (s) and vascular strand (v) toward apical meristem (t) (bar = 30  $\mu\text{m}$ ); d). Enlargement of vascular strand showing parenchymatous cells, tracheids (t) of xylem and lipid droplet (l) (bar = 10  $\mu\text{m}$ ); e). Somatic embryogenesis on meristematic area showed globular proembryo (e) (bar = 10  $\mu\text{m}$ ); f). Protoderm layer of tunica (t) and corpus (c) in meristematic area (bar = 50  $\mu\text{m}$ )

Histological analysis of bud-like structure of coconut endosperm showed clearly that there were a meristematic layer with a dermal layer, cortex-like region and central vascular tissue. It was likely that shoot organogenesis occurred in the endosperm calli of coconut. This is potentially important because caulogenesis has not been reported yet in coconut endosperm culture. These results are encouraging and warrant further studies to get plant regeneration. The plant is traditionally propagated by seed and is generally cross pollinated. Therefore, micropropagation is needed in order to get the best genotype with abundant offspring.

The micropropagation can also support the easy exchange of coconut germplasm. Although coconut micropropagation from various tissues had been attempted, it was mostly hindered by embryo tissue that was not true to type (Fernando & Gamage 2000; Sukendah & Cedo 2005; Ake *et al.* 2007; Saenz *et al.* 2010; Koffi *et al.* 2013; Muhammed *et al.* 2013). It is more difficult to have successful tissue culture of adult coconut explants, especially on inflorescence (Blake & Eeuwens 1980; Sandoval-Cancino *et al.* 2016), stem apice (Blake & Eeuwens 1982) and leaf (Raju *et al.* 1984; Buffard-Morel *et al.* 1992; Verdeil *et al.* 1994).

## CONCLUSIONS

Solid endosperm of seven month-old postanthesis coconut could produce calli, proembryos and bud-like structure. Histological analysis showed that the calli composed of highly vacuolated cells and small cells with dense cytoplasm, high nucleus to cytoplasm ratio, high starch, protein and lipid. Calli cells underwent many divisions and formed proembryo structures. The proembryo structure grew to become bud-like structure, which showed cotyledonary notch, tunica-carpus structure and central vascular strand. These results indicated that an early stage of shoot bud occurred in *in vitro* cultured coconut endosperm.

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