HISTOLOGICAL STUDIES ON CALLOGENESIS OF COCOA

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Introduction

Clonal propagation of superior genotypes through *in vitro* propagation technique is one of the identified thrust areas of cocoa biotechnology. Ontogeny of somatic embryo ontogenesis from floral buds revealed that they were of multicellular origin (Alemanno *et al.,* 1996). Two distinct ontogenic pathways were reported in cocoa; multicellular origin of embryos from staminodes and unicellular origin of embryos from cotyledons. The present study was undertaken to locate the origin of callus induction in staminode, leaf and cotyledons.

Materials and Methods

Materials used were leaf, staminode and cotyledons from Malaysian clone Jerangau Red Axil. Explants were sterilized and inoculated to callus induction media.

Culture condition

Explants were inoculated to DKW (Driver Kuniyuki Walnut 1984) basal medium supplemented with 2,4-D 2mg/l and TDZ 25µl (from 0.2mg/ml) and MS medium (Murashige and Skoog) supplemented with 0.5mg/l of NAA and 0.5mg/l of BAP. Initially cultures were incubated in dark, where temperature and RH are maintained at 27±1°C and 80% respectively. Observations were taken periodically for browning, contamination, callusing and growth of the explants.

Histology study

The samples of fresh plant materials (staminode fresh tissue and callus, leaf fresh tissue and callus and cotyledon and callus) were fixed in standard fixative Carnoy's 'B' fluid (Chloroform-30ml; absolute alcohol-60 ml; glacial acetic acid-10 ml) to localize RNA, DNA, proteins and polysaccharides. Fixed materials were dehydrated serially using alcohol and butanol, which replaces the water in the material. A mixture of paraffin wax and bee's wax of melting point 58-60°C was used for infiltration and embedding. Melted paraffin along with the explants was poured into the mould to prepare paraffin block for section cutting.

Microtome sectioning

After cooling, the edge of the paraffin block was trimmed to form a cube and fixed on wooden blocks. Uniformly thin sections of 10μm thickness were cut using Leica RM 2145 rotary microtome.

Affixing the sections on slide

Appropriate portions of the paraffin ribbons were cut into convenient length and floated over 3% gelatin over the slide. Then the slides were placed over a warming plate, maintained at nearly 45°C to facilitate stretching of the ribbon.

Deparaffinisation and dehydration of the sections

Slides were deparaffinised using xylene. Subsequent dehydration was done with butanol and alcohol. Then they were transferred to 100% alcohol and subjected to histochemical staining either directly or after hydration depending on the stain used.

Staining procedure: (1) Periodic acid Schiff's reagent

The slides were deparaffinised and dehydrated. The sections were hydrated by dipping in distilled water. These were placed in 1% periodic acid solution for 15 minutes. Then washed in water for four to five minutes and were placed in Schiff's reagent for 15 minutes. Subsequently they were dipped in water for 10 minutes.

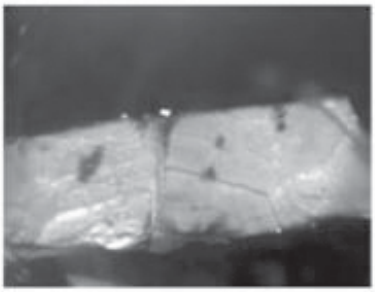


Fig 1.

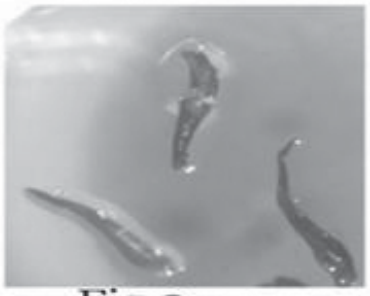
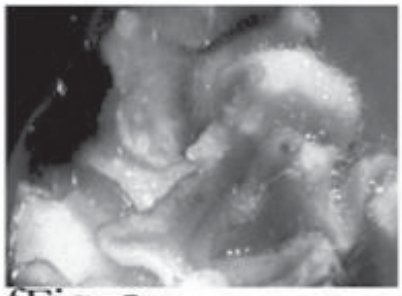


Fig 2.



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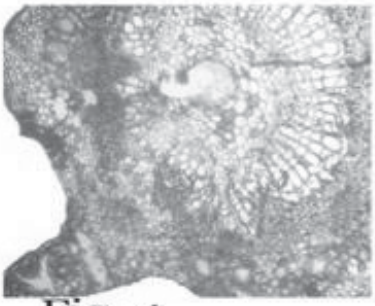
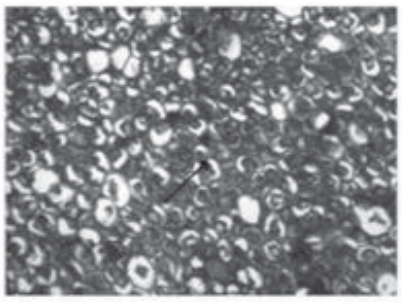


Fig. 4

Fig. 5



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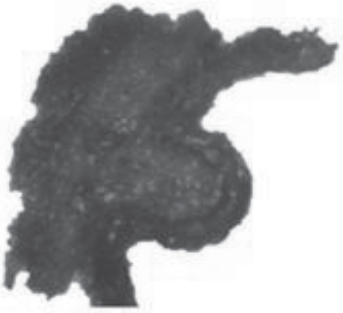

Fig. 7

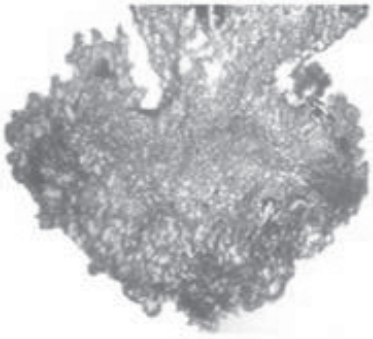

Fig. 8

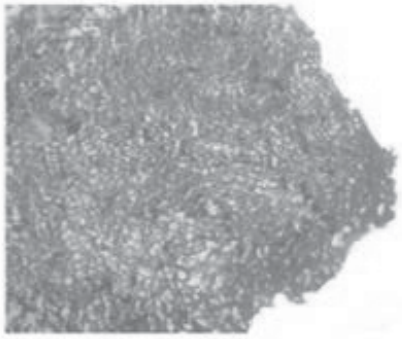

Fig. 9

Fig. 1. Fresh leaf explant, Fig. 2. Staminode, Fig. 3. Cotyledon explant after inoculation, Fig. 4. Transverse section of leaf, Fig. 5. Longitudinal section of staminode, Fig. 6. Localization of nucleic acids in cotyledon explant, Fig. 7. Callus initiation in leaf explant, Fig. 8. Callus initiation from staminode, Fig. 9. Callus initiation from cotyledon.

Alcohol was used to remove excess stain. Then these were dehydrated by passing through butanol, cleared in xylene and mounted in DPX. The sites of polysaccharides and starch granules take red colour and the intensity of the colour was used to measure the amount of polysaccharide present.

Staining procedure: (2) Toluidine blue

Slides were deparaffinised and dehydrated using xylene and butanol. Then the sections were hydrated using distilled water and were placed in 1% Toluidine blue stain for 20-30 minutes. Then the sections were rinsed in water to remove excess stain and dehydrated by passing through butanol, cleared in xylene and mounted in DPX and viewed through Wild Heerburg stereo and Leits Diaplan binocular microscope. The sites of nucleic acid appear dark blue in colour against faintly stained background. The intensity of which is the measure of the amount of RNA or DNA present in tissues.

Results

Cotyledon and staminode callus showed dense amount of polysaccharides and nucleic acids (Fig. 3). In case of cotyledon explants, the calli originated from the peripheral active cells (Fig. 9) and perivascular region. Leaf tissue shows origin of callus from mid rib region (Fig. 7). In case of staminodes, callus originates from the marginal active cells (Fig. 8).

Discussion

Histological studies revealed that cotyledon and staminode collected from fresh tissues were very rich in polysaccharides and nucleic acids. In cotyledon explant callus originated from marginal active cells as well as from perivascular region. However, Schwendimann *et al.* (1998) reported that the callus was formed by the perivascular cells at the veins of young leaf explant of oil palm. However, histological monitoring of somatic embryo ontogenesis from floral buds revealed that the somatic embryos were of multicellular origin and production of somatic embryos after few days of culture initiation, through staminode explants was proved by Alemanno *et al.,* 1997. A large number of cocoa plants have been regenerated from somatic embryos formed from staminodes (Li- Zhijian, 1998). Ontogeny of cocoa reported two distinct ontogenic pathways. Staminodes formed embryos of multicellular origin, while embryos from cotyledons developed primarily from unicellular origin. In staminode marginal active cells undergoes series of division and forms callus. However, compact and globular type of calli developed over the entire staminode. Leaf tissue shows origin of callus from mid rib region as well as from mesophyll cells.

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