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SOMATIC EMBRYO FORMATION FROM COTYLEDONARY CULTURE OF *THEOBROMA CACAO* L.

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ABSTRACT

Calli were initiated from embryo and cotyledon of Theobroma cacao *L. cultured on MS medium enriched with several concentrations of 2,4-D and NAA. The calli did not produce embryoids either on these media or hormone-free medium. A stable suspension culture was established from these calli and maintained in a MS liquid medium containing 2,4-D. Induction of embryoid was successful when cultured cotyledonary explants on MS medium containing NAA and CW. Embryoid developed into plantlet in the same medium devoid of NAA and CW. Histological analysis revealed that the embryoid was in torpedo stage of development and showed the anatomical organization namely shoot apex, leaf primordia, vascular strand and root apex.*

INTRODUCTION

Clonal propagation using tissue culture technique provides the advantage of reducing the time scale in the bulking-up stage for large supplies of initial parent material. However, the application of micropropagation technique of tropical trees is difficult to be successful. To obtain viable plantlets through organogenesis or embryogenesis in these tree species is rather limited[1]. *Theobroma cacao* L. is one of the most valuable extractable tree in the tropic. The cacao seeds provide the raw material for the manufacture of cocoa and chocolate and for the extraction of cocoa butter. *In vitro* culture of cacao has been successfully initiated from various sources of explants including cambium[2], seedling[3], zygotic embryo[4,5], cotyledon[6,7], flower bud[8], axillary bud[9]. The results from these researchers seem to be inadequate to increase plant propagation efficiency in this plant. In this report, we describe conditions for callus induction, cell suspension culture and somatic embryogenesis from mature cotyledon of *Theobroma cacao* L.

MATERIALS AND METHODS

Plant material

Mature cocoa pods were collected from Changwat Surattanee, Southern Thailand and surface sterilized in 70% ethanol for 15-20 min. The sterilized pods were aseptically cut open with a knife. The mature seeds were then transferred to petri-dishes and the mucilaginous pulp of the seeds was removed. Individual embryos were drawn from the seeds and used as explants since investigators have shown that somatic embryogenesis might be observed in cell culture starting from young embryos.

Initiation and growth of callus

Sterile embryos with cotyledons were cultured on the basal nutrient medium (mg/l) contained Murashige and Skoog[10] salts,with sucrose 30,000; thiamine-HCl 0.1; pyredoxin-HCl 0.5; nicotinic acid 0.5; myo-inositol 100; casein hydrolysate 1,000; 2,4-D 0.01, 0.1, 1.0; NAA 1.0, 1.5, 2.0 and solidified with 0.2% (w/v) Gelrite. The pH of the medium was adjusted to 5.7 prior to the addition of agar and autoclaved 20 min at 1.05 kg/ cm^2. The cultures were incubated at 25±1°C in the dark. Calli were initiated from these explants and subcultured every 4 weeks to maintain optimum growth.

Establishment of cell suspension

Once callus was established, cell suspension were initiated by transfer of 1 gram fresh weight (g. f. wt.) of 3 months-old callus, harvested 15 days after subculture, to 25 ml of liquid MS medium supplemented with 0.5 mg/l 2,4-D in 125 ml Erlenmeyer flask. The inoculum was incubated at 25±1°C on a rotary shaker with continuous shaking at 120 rpm. After 2 months, the suspension was seived through 500μm and 380 μm mesh stainless steel screen. Cells retained on the 380 μm pore size were collected and subcultured every 14 days.

Preparation of material for histological examination

Callus and compact nodular structure were fixed in FAA (formalin : glacial acetic acid: ethanol, 5:5:90, v/v/v). Tissues were dehydrated through an ethanol-butanol series for 48 h and embeded in Paraplast. Specimens were cut to 10 μm thick and stained with safranin and fast green prior to examination by light microscopy.

RESULTS

Callus initiation and growth from embryos and cotyledons were first observed 5-6 weeks after culturing. The level of 2,4-D that promoted the maximum growth of callus was found to be 0.01 mg/l and 2,4-D at 0.1 and 1.0 mg/l resulted in death of explants. The embryos were covered with white to brown compact callus tissue (Fig. 1a). Cotyledon-derived callus was compact and white colored amorphous texture. After 2-3 passages, cotyledonary calli gave rise to adventitious roots without generating shoots or embryoid-like structures (Fig. 1b). Very little callus was initiated on media supplemented with NAA. Embryos produced brownish friable callus, while cotyledons gave white compact callus.

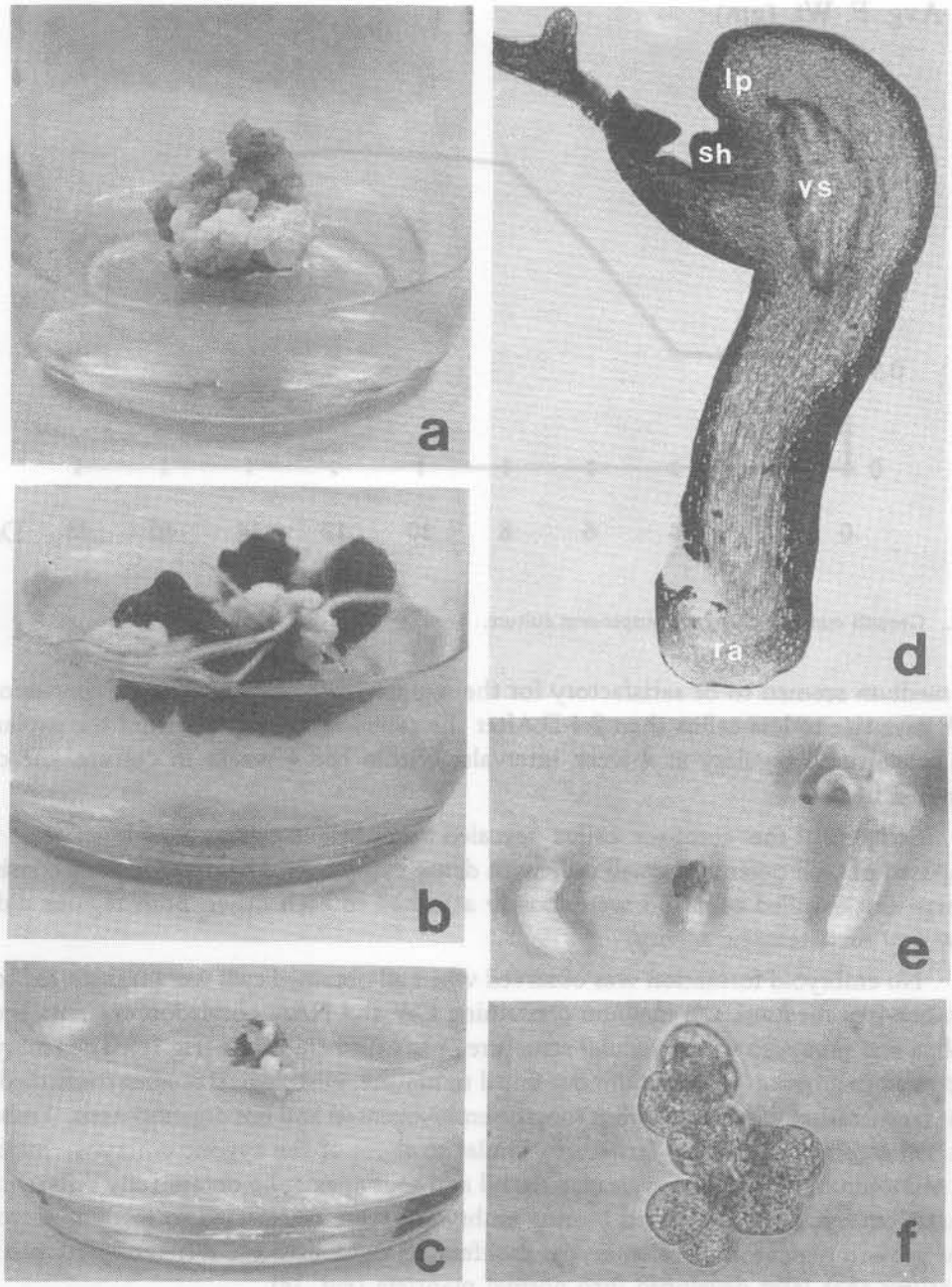

Fig. 1. Compact calli cultured on MS media supplemented with 0.01 mg/l 2,4-D initiated from (a) embryo (b) cotyledon. Note : adventitious roots arose from callus without shoots. (c) Embryoid at torpedo stage from cotyledon explant cultured on MS medium containing CW and NAA. (d) Histology of embryoid showing shoot apex (sh), vascular strand (vs), leaf primordia (lp) and root apex (ra). (e) Developmental stages of embryoid into plantlet. (f) Small and rich, thin-walled cell suspension of cacao.

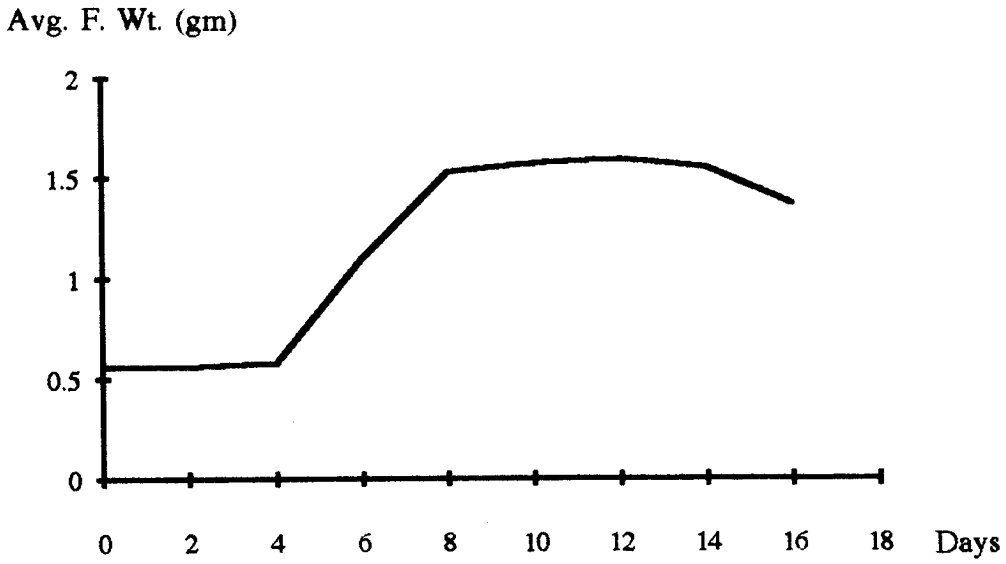

Fig.2. Growth curve of cacao cell suspension culture.

MS medium seemed to be satisfactory for the maintenance of callus culture continuously. NAA gave rise to less callus than 2.4-D.After the callus was separated from the explant, it was subcultured regulary at 4-week intervals. Within the 4 weeks in culture, the callus remained fresh.

Sections of the compact callus revealed two distinct regions. The inner areas were composed of well organized small cells with dense cytoplasm. The outer regions consisting of large thick-walled cells and were loosely attached to each other. Both regions did not show any meristematic acivity.

No embryoid formation was observed when all obtained calli were transferred to MS hormone-free medium. On medium containing CW and NAA, cotyledon explants became swollen and produced white nodular structure or so called embryoid (Fig.1c). The embryoids continued to grow on this medium but failed to mature. Histological studies confirmed that plant regeneration was derived from somatic embryogenesis and not organogenesis. Embryoid (Fig. 1d) showed anatomical structures similar to those of the zygotic embryos, including leaf primordium, shoot apex, vascular strand and root apex. To obtain fully differentiated somatic embryos, the cotyledon bearing embryoids were transferred to medium devoid of plant growth regulators. The embryoid proliferated vigorously and differentiated into leafy structures that later developed into normal plantlets (Fig. 1e).

The calli in MS liquid medium supplemented with 0.5 mg/l 2,4-D readily disaggregated into small clumps and proliferated sucessfully upon agitation in culture flask. During the first 4-6 weeks the suspension was decanted every 5-7 days, discarding all the contents except the cell clumps to prevent the suspension from becoming brownish. The growth curve of suspension cultures is shown in Fig. 2. The cell number increased 3-fold in 14 days;

therefore, subculture was carried out every 14 days. If this was not done, the color of cell suspension would be changed to dark brown, and the growth rate was slow and finally declined.

Cells in suspension cultures were comprised of isodiametric, thin-walled cells, small and rich in starch grains (Fig.1f). When allow to grow for more than 14 days in the same medium, the starch grains present in the cells started disappearing, the cytoplasm became empty and the wall thickened. Six days-old cells, after each transfer, were suitable to be used as a source for protoplast isolation for future experiments.

DISCUSSION

2,4-D is generally known to play an essential role in both callus formation and plant regeneration. The presence of 2,4-D in the medium was essential for cacao callus initiation and proliferation but not plant regeneration. Subculture of cacao calli did not result in developing embryoids. Tsai and Kinsella[6] also described yellow callus from cotyledons of zygotic embryos but embryogenesis was not observed. In contrast to Kononowicz *et al.*[7] who reported the spontaneous production of somatic embryos from callus on hormone-free medium for 2 clones of cacao. In particular morphogenesis is generally achieved when calli grown in the presence of 2,4-D are transferred to medium lacking or substantially lower in 2,4-D. Such embryogenesis was never evident in our experiments.

In the culture of cotyledon segments reported here, somatic embryos occurred without the intervening callus stage. Such direct embryogenesis as in this case has been described by Pence *et al.*[5]. The cotyledon segments cultured on MS medium at low 2,4-D level (0.01 mg/l) did not produce embryoids, but formed numerous roots instead. This suggests that the existence of 2,4-D at over a certain level is necessary for the segments to produce embryoids. CW in combination with NAA was able to regenerate embryoids probably attributed to the cytokinin-like substances in CW as reported by many researchers. The absence of plant growth regulators appeared to be critical for plantlet formation. This similarity was also observed in *Ranunculus serbicus* Vis[11] when embryoids from MS media containing NAA and BA were transferred onto hormone-free medium, 80% embryoids developed into plantlets.

The fresh weight of the suspension cells after 14 days of culture showed about 3-fold increase when compared to that of the initial inoculum. This compares unfavourably with 20- fold in 14 days for cell suspensions originally derived from immature cotyledon[6].Our results indicated that 2,4-D alone was much less effective for the proliferation of cell growth and 2,4-D in combination with kinetin was more effective for stimulating growth of suspension as described by Tsai and Kinsella[6]. Although these cells were nonembryogenic, they were speculated to be in the transition phase between being embryogenic and nonembryogenic since microscopic observation revealed that they were characterized by small cells with dense cytoplasm and numerous starch grains.

Somatic embryogenesis from cotyledon could provide a simple, alternative method for propagating this plant. Further improvement of cacao suspension culture may generate potential for massive vegetative propagation of superior trees in this species.

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Abbreviations:

MS	-	Murashige and Skoog
NAA	-	α–naphthaleneacetic acid
2,4-D	-	2,4- dichlorophenoxeacetic acid
CW	-	coconut water

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บทคัดย่อ

การเพาะเลี้ยงเอ็มบริโอ และใบเลี้ยงของโกโก้บนอาหารสูตร MS ที่มี 2,4-D และ NAA ความเข้มข้นต่างๆ สามารถชักนำให้เกิดแคลลัส แคลลัสที่ได้ไม่สามารถเกิดเอ็มบริออยด์เมื่อเลี้ยงต่อไปบนอาหารเหล่านี้ หรือบนอาหารที่ไม่มีสารควบคุมการเจริญเติบโตของพืช จากแคลลัสเหล่านี้สามารถชักนำให้เกิดเซลล์ซัสเพนชั่นได้ดีเมื่อเลี้ยงในอาหารเหลวสูตร MS ที่มี 2,4-D เมื่อเพาะเลี้ยงชิ้นส่วนใบเลี้ยงบนอาหารสูตร MS ที่มี NAA และน้ำมะพร้าว สามารถทำให้เกิดเอ็มบริออยด์ได้ และเอ็มบริออยด์ได้พัฒนาเป็นต้นโกโก้ที่สมบูรณ์ เมื่อย้ายเอ็มบริออยด์ไปเลี้ยงบนอาหารสูตรเดิมแต่ไม่มี NAA และ น้ำมะพร้าว จากการศึกษาทางกายวิภาคของเอ็มบริออยด์ พบว่า เอ็มบริออยด์อยู่ในระยะตอร์ปิโด และมีส่วนที่จะเจริญพัฒนาไปเป็นยอด ใบ กลุ่มท่อลำเลียงและราก