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SOMATIC EMBRYOGENESIS FROM CELL SUSPENSION CULTURES OF OIL PALM (*ELAEIS GUINEENSIS* JACQ.)

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ABSTRACT

*Zygotic embryos of oil palm (*Elaeis guineensis *Jacq.) were excised and cultured on callus induction medium (Eeuwens, 1976; 1978) containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Two months-old calli derived from embryos were transferred to liquid callus induction medium without 2,4-D to establish fine suspension cultures. The establishment of cell cultures were obtained in 2-3 months. Cell suspensions were then subcultured to several regeneration media and embryoid differentiation occurred either in regeneration medium supplemented with 20% (v/v) coconut water or 0.01 mg/l α-naphthaleneacetic acid (NAA). However, no plantlet development was recovered. These embryoids were fixed, sectioned, stained and examined microscopically. The results revealed their bipolar structure consisted of shoot and root meristem, procambial cells and protodermal layer.*

INTRODUCTION

To date clonal propagation of oil palm is common[1-5]. Most of plant regeneration were produced through callus cultures which is a time-consuming process. Hence the production costs were encountered at the commercial production scale. Under this circumstance, there is a high potential for suspension culture application in mass propagation of an elite oil palm tree. An established system in which somatic embryogenesis occurs at high frequency can facilitate the large scale propagation and provides tools for tree improvement programs. Induction of somatic embryogenesis from suspension cultures of oil palm has recently been reported by several research groups[6-8].

In this paper, a method for establishing cell suspension and somatic embryogenesis through suspension cultures from oil palm zygotic-derived callus was described.

MATERIALS AND METHODS

Plant material and initiation of callus

Mature *Tenera* seeds were collected and surface sterilized as described by Chourykaew and Kanchanapoom[9]. The culture medium contained Eeuwens[10-11] inorganic salts, with (mg/l) Na_2EDTA 37; $FeSO_4 \cdot 7H_2O$ 13.9; myo-inositol 100; thiamine-HCl 0.5; pyredoxine-HCl 0.05; nicotinic acid 0.05; glutamine 100; arginine 100; asparagine 100; sucrose 45,000 served as basal medium (designated callus induction medium, CIM). Callus was initiated from mature zygotic embryos cultured on CIM and supplemented with 2 mg/l 2,4-D. The pH of all media was adjusted to 5.6 prior to the addition of 0.2% Gelrite (Merck & Co., Kelco Division, NJ, USA) and autoclaved at 121°C for 20 min.

Induction of cell suspension

For establishment of suspension culture, approximately 5 gram fresh weight of 2 months-old callus were used. The fast growing nodular calli were transferred to 125 ml Erlenmeyer flask containing 25 ml of liquid CIM either without 2,4-D or supplemented with 0.2 mg/l 2,4-D. The inoculum was incubated on a rotary shaker with continuous shaking at 100 rpm. The cell suspensions were weekly subcultured to prevent the suspension from becoming brownish. Cells were sieved through 860 μm and 520 μm mesh stainless steel screen for 1 month and those retained on 520 μm pore size were collected and stable suspensions were obtained 2-3 months after initiation in liquid medium.

Regeneration of somatic embryos

Cells cultured in CIM without 2,4-D were transferred to a number of different regeneration media (RM) including :

(1) CIM + 20% (v/v) coconut water

(2) CIM + 0.01 mg/l NAA

(3) CIM + 0.2 mg/l 2,4-D + 20% (v/v) coconut water

Cells were incubated in these media for a period of 7, 14, 21, 28 and 35 days. At the end of each period, cells were transferred to the same media devoid of amino acid mixture (AAM) and successively subcultured every 5 days for 15 days. Then all the samples were transferred to the media lacking growth regulators (GR) and subcultured every 5 days on these media for 1 month, whatever the experimental procedure was. The diagram of these experiments is shown in Fig. 1.

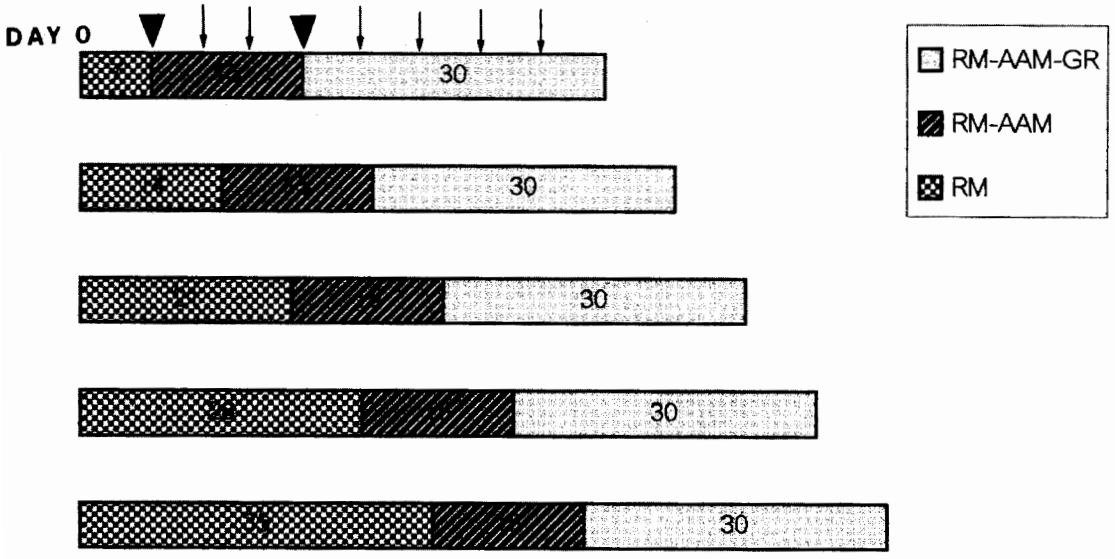

Fig.1 Experimental systems used for the regeneration of oil palm suspension culture. Day 0 : start of culture on regeneration medium. Arrows show successive subculturing every 5 days on each type of medium. Black triangles indicate subculture to different medium.

Culture conditions

Callus and suspension cultures were incubated at 25±2°C with a 16-h photoperiod under an illumination of 20 $\mu molm^{-2}s^{-1}$ provided by Gro-lux lamps. All experiments were carried out at least three times with fifteen cultures per treatment.

Histological studies of embryoids

Embryoids at various stages of development were fixed using FAA solution of 90 ml 50% ethyl alcohol, 5 ml glacial acetic acid and 5 ml formalin solution. Tissues were dehydrated through an ethanol - butanol series for 48 h and embedded in Paraplast. Specimens were cut into 10 μm sections and stained with safranin and fast green prior to examination by light microscope. Histological analysis was carried out on representative samples of the embryoid explants.

RESULTS

Establishment of cell suspensions

During the first phase of culture of the mature zygotic embryos, callus formation was initiated after 45-60 days on solid CIM containing 2 mg/l 2,4-D. The callus was small, compact and creamy yellow (Fig. 2). On day 90, the callus covered the embryo completely and the growth of the callus was rather slow. After completion of this callusing phase, primary calli were transferred to liquid CIM either with reduced 2,4-D content to 0.2 mg/l or CIM without 2,4-D. The rate of multiplication in both media was 2-3 fold the original size in one month. However, cell suspension production in CIM with 0.2 mg/l 2,4-D was slightly compact and did not separate well in liquid medium. While in CIM without 2,4-D the newly formed cells were friable-to-nodular and proliferated successfully upon agitation in culture flask (Fig. 3). The screening through 860 μm mesh screen and cells collected at 520 μm mesh screen helped starting the production of uniform cell aggregates. The cell fraction below 520 μm contained small aggregates with fewer embryogenic cells. Therefore a fine suspension culture was established from these friable embryogenic cells.

Embryoid formation in liquid culture medium

When suspension cells were transferred to several regeneration media as described in methods, differentiating embryoids were evidenced. In CIM + 20% (v/v) coconut water, rhizogenesis was observed when cells were treated for 7, 14, and 21 days (Fig. 4a, d, g) but declined at 28 and 35 days (Fig. 4j, m). Roots found in all treatments were green in color and some embryoids were found in all treated cells. None of the embryoids in 14-, 21-, 28-, and 35-day treated cells displayed all the characteristics that would make them typical somatic embryos at the end of treatments (Fig. 4f, i, l, o). Only cells incubated in 7-day treatment underwent complete embryogenesis after a total of 42-45 days. A distinct shoot, scutellum and root were observed as shown in Fig. 5, indicating a bipolar structure characteristic of embryos. In addition, embryoids showed different degrees of morphological conformity. Similar observations were also found in cultures of CIM + 0.01 mg/l NAA in all treated cells (Fig. 6). Those occurred in CIM + 0.01 mg/l NAA for 14 days showed very differentiated appearance of embryoid (Fig. 6b). After several subcultures on CIM lacking amino acid mixtures and NAA for 1 month, these embryoids developed shoot, root and haustorium (Fig. 6e). Nevertheless, they remained at this stage and became necrotic after being transferred to solid CIM.

In contrast cultures in CIM + 0.2 mg/l 2,4-D + 20% (v/v) coconut water showed only large cell aggregates, rhizogenesis and no differentiating embryoids were observed. At the end

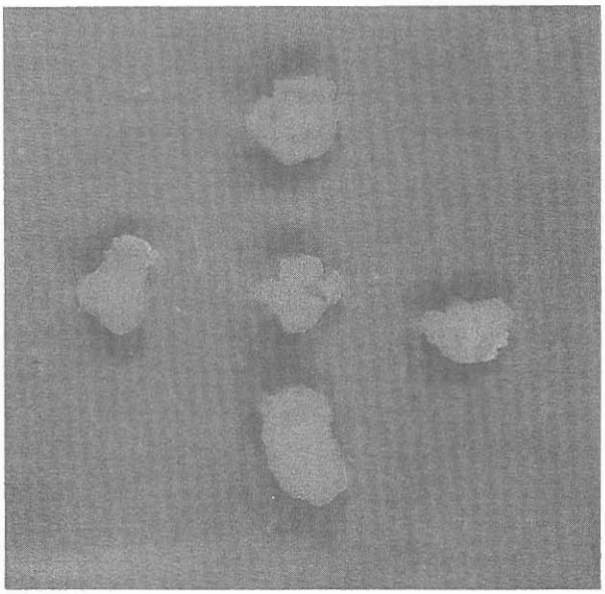

Fig.2 Creamy compact callus cultured on callus induction medium containing 2 mg/l 2,4-D.

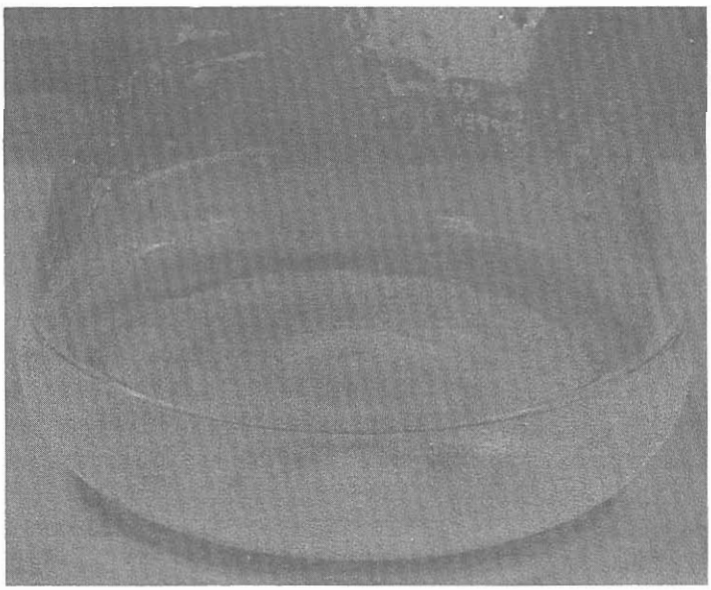

Fig.3 Fine suspension culture derived from compact callus after 3 months of continuous shaking on CIM without 2,4-D.

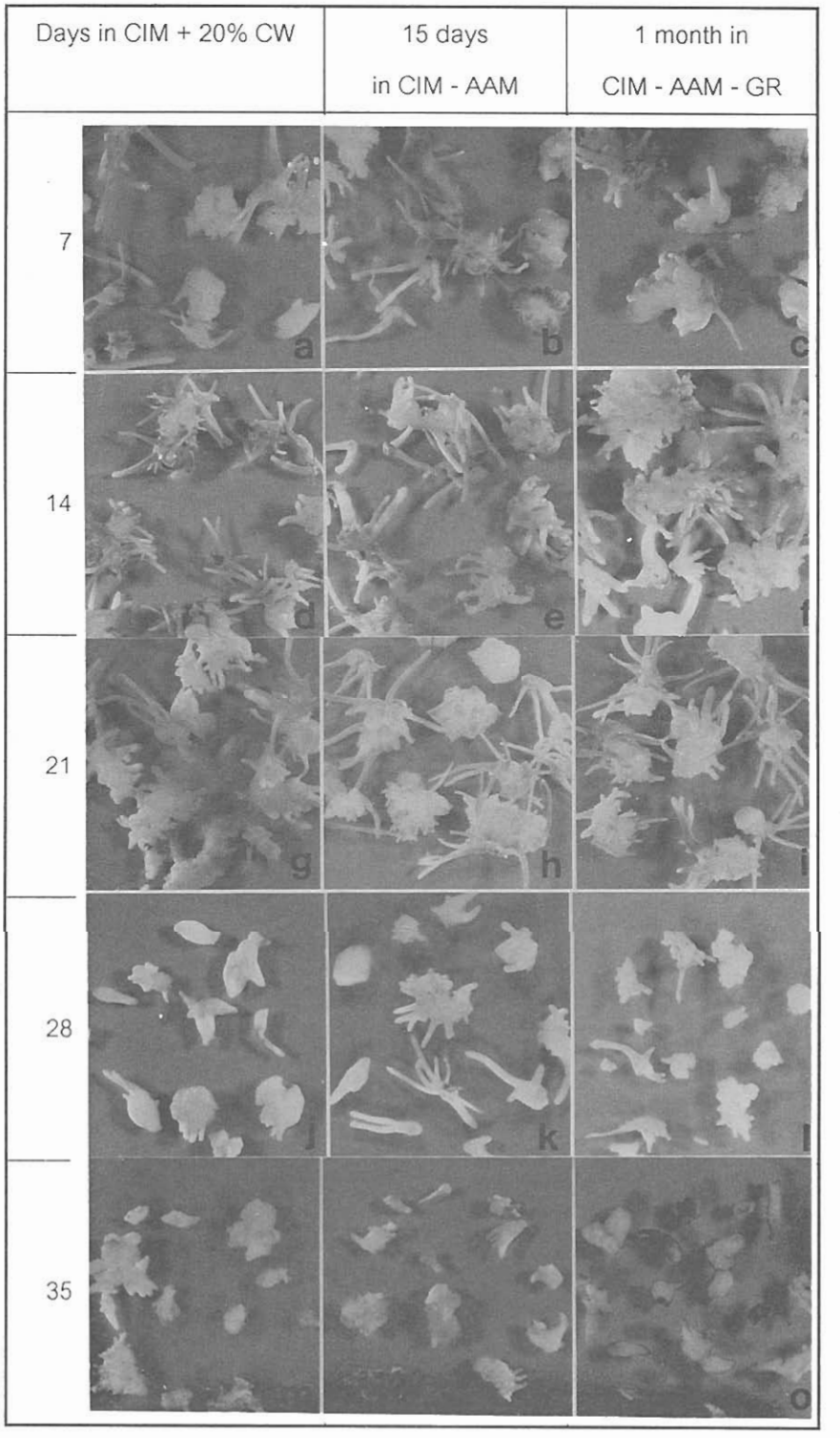

Fig.4 Morphological change of oil palm cell suspension cultured in CIM + 20% coconut water (regeneration medium, RM) for a different period of time (a, d, g, j, m = 7, 14, 21, 28, 35 days, respectively). Cells were then transferred to RM without amino acid (RM-AAM) mixture for 15 days (b, e, h, k, n). Finally cells were transferred to the same medium but lacking 20% coconut water (RM-AAM-GR) for 1 month (c, f, i, l, o).

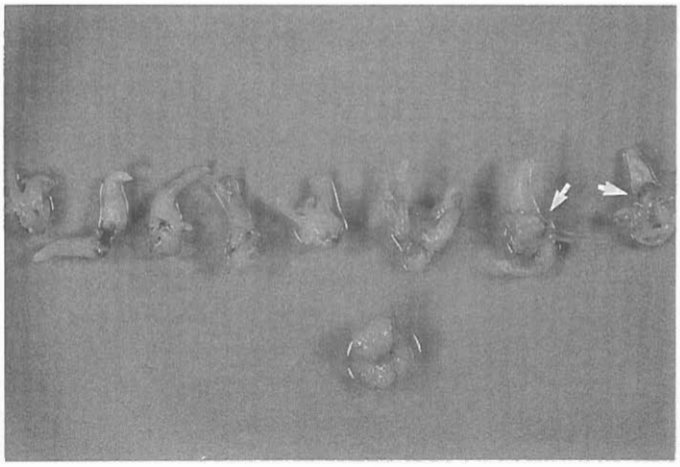

Fig.5 Embryoid formation derived from cell suspension cultured in CIM + 20% coconut water showing different degrees of morphological conformity and bipolar structure of embryoid (arrows).

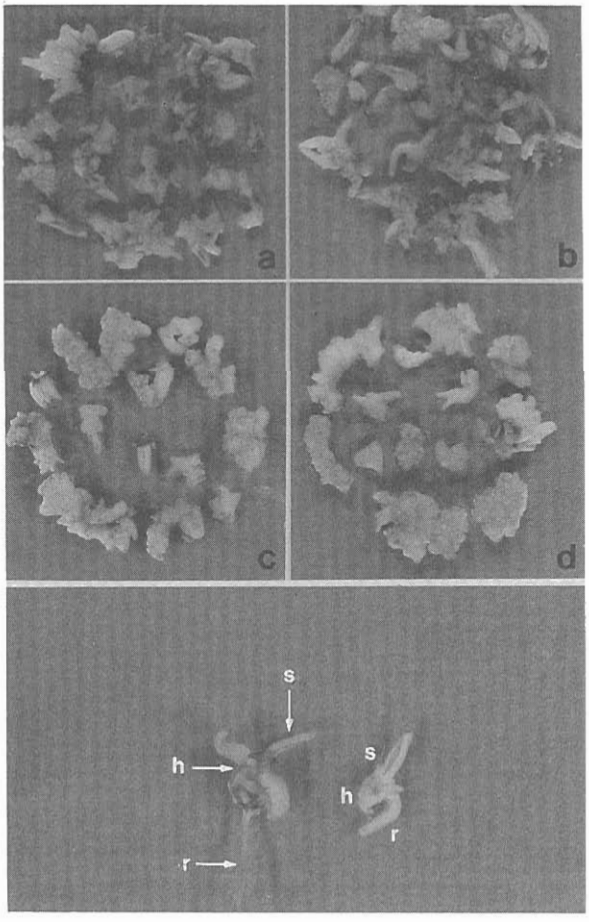

Fig.6 Embryoid formation derived from cell suspension cultured in CIM + 0.01 mg/l NAA (a, b, c, d = 7, 14, 21, 28 days, respectively). (e) shoot, root and haustorium regeneration after cultured in the same medium without amino acid mixture and NAA for 1 month. (s = shoot ; r = root ; h = haustorium)

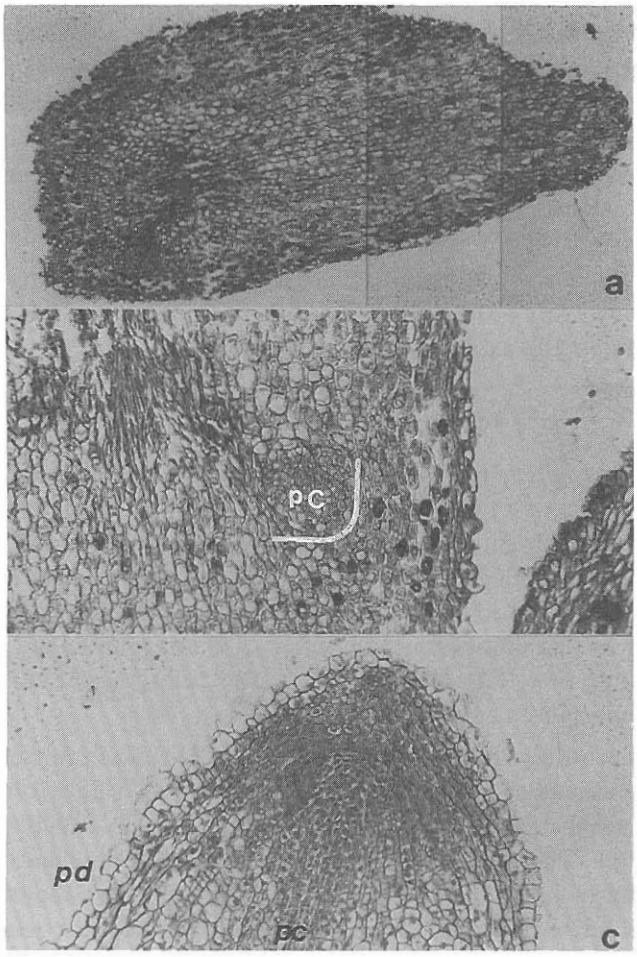

Fig.7 Anatomical characteristics of embryoids cultured in CIM + 20% coconut water. (a) longitudinal section through the embryoid, (b) transverse section of procambial cells as densely stained individual bundles, (c) longitudinal section of root apex showing meristematic cells with dense cytoplasm. (pc = procambial cells; pd = protodermal cells)

of the experiments these aggregates became brownish and no persistent growth or further differentiation took place.

Histology of embryoid

Histological monitoring of embryoids cultured in CIM supplemented with 20% (v/v) coconut water revealed that well defined polarity was apparent by the organization of shoot and root meristem at one end of the embryoid (Fig. 7a). Procambial cells were evidenced as densely stained individual bundles in transverse section (Fig. 7b). Protodermal cells were tabular in outline and formed distinct layer around the surface of root pole region which mainly consisted of typical meristematic cells that had a dense cytoplasm enclosed a round nucleus (Fig. 7c). Further divisions of the bipolar structure gave rise to cylindrical embryoids which resembled to the zygotic embryo and consisted of numerous polyphenolic cells. Shoot and root elongation were observed simultaneously when embryoids were transferred to medium devoid of amino acid mixtures and coconut water.

DISCUSSION

The friability of callus which favors callus dispersion in liquid medium depends on various parameters i.e. genetic factors, type of explants and culture media. Considering media components, plant growth regulators are responsible for modification of callus texture. In general, friability is obtained when auxin and cytokinin concentrations are reduced or omitted. The absence of 2,4-D in liquid CIM thus efficiently resulted in callus friability of oil palm cell suspension cultures. Inoculation of callus into liquid medium resulted in a substantial increase in cell growth suggesting the effectiveness of physico-chemical culture conditions towards the establishment of suspension cultures.

The high concentration of 2,4-D (2 mg/l on solid CIM) was probably essential to foster embryogenic initial. Kuijpers *et al.*[12] reported that embryogenic callus formation in cucumber increased to 40% by initial culture on high concentration of 2,4-D. Regeneration started during which the tissue became competent to respond to 2,4-D. For development of somatic embryos, 2,4-D had to be removed. In oil palm, high concentration of 2,4-D allowed cells to grow but did not favor differentiation. Subsequent transfer to the regeneration medium devoid of 2,4-D and supplemented with coconut water or NAA was a prelude to embryogenesis development. The substitution of 2,4-D to coconut water or NAA seems to enhance differentiation since tiny embryo-like structures of varying sizes were present. In rice suspension culture, Ozawa and Komamine[13] reported that the highest frequency of differentiation was observed in half N6 medium supplemented with 0.01 mg/l NAA, 0.1 mg/l 4-PU and 3% sucrose. The pattern of culture on several successive media to produce somatic embryos in oil palm is comparable to other plant systems in which the phenomenon has been obtained[14].

Apart from growth regulators, the embryogenic event of the suspension culture appeared to be closely related to the timing of subcultures. Michaux-Ferriere and Carron[15] reported that unrenewed medium MH1 for 40 days neither enables *Hevea brasiliensis* cell suspensions to conserve their meristematic features nor enables pre-embryogenic cells to become true embryogenic cells. Typical embryogenic cells formed when medium MH 1 was renewed once during the first phase of culture. Proembryos developed when the calli were subcultured on medium MH 3 10-15 days later. In contrast to rice cell suspension culture[13] the interval between subculturing was the most important variable obtaining a high frequency of rice regeneration. Subculturing at intervals of 3 days induced regeneration at high frequency, while subculturing

at intervals of 7 days did not result in any embryogenesis at all. In case of oil palm, prolonged culture in regeneration medium (more than 7 days in CIM + 20% coconut water and more than 14 days in CIM + 0.01 mg/l NAA)) did not result in embryoid development.

All the structures common to zygotic embryo were observed in the oil palm embryoids. Some embryoids appeared to be similar to those described by DeMason[16] in the palm *Washingtonia filifera* and Tisserat and DeMason[17] in the date palm *Phoenix dactylifera* L. The procambial strands resembled that of *Washingtonia* and *Phoenix* in that the strands were organized in bundles and centrally located along the embryoids.

The cell suspension system described here would be ideal for oil palm growing in the long run. The results suggested that embryogenesis continued to develop if subculturing was carried out when they were in a specific physiological state. However, an improvement of culture conditions and duration of culture is still required in order to obtain oil palm capable of germinating.

ACKNOWLEDGMENT

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บทคัดย่อ

เพาะเลี้ยงไซโกติกเอ็มบริโอของปาล์มน้ำมันในอาหารชักนำแคลลัสสูตรของ Eeuwens (1976; 1978) ที่มี 2,4 dichlorophenoxyacetic acid (2,4-D) เข้มข้น 2 มิลลิกรัมต่อลิตร นำแคลลัสอายุ 2 เดือนที่ได้จากเอ็มบริโอไปชักนำเซลล์แขวนลอย โดยเลี้ยงในอาหารเหลวสูตรเดิมที่ไม่มี 2,4-D เพื่อให้ได้เซลล์แขวนลอยที่มีขนาดละเอียดภายใน 2-3 เดือน จากนั้นย้ายเซลล์แขวนลอยไปยังอาหารที่ชักนำให้เกิดต้นหลายๆ ชนิด พบว่าเกิดเอ็มบริออยด์ในอาหารเหลวที่มีน้ำมะพร้าว 20% (ปริมาตรต่อปริมาตร) หรือ α-naphthaleneacetic acid (NAA) เข้มข้น 0.01 มิลลิกรัมต่อลิตร แต่ไม่พบการเจริญเป็นต้นอ่อน เมื่อนำเอ็มบริออยด์มาฟิกซ์ ตัดชิ้นส่วน ย้อมสี และศึกษาภายใต้กล้องจุลทรรศน์ พบว่าเกิดโครงสร้างไบโพลาร์ที่มีทั้งเนื้อเยื่อเจริญปลายยอด เนื้อเยื่อเจริญปลายราก เซลล์โพรแคมเบียล และชั้นโพรโทเดอร์มา