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Establishment of oil palm cell suspensions and plant regeneration

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Abstract

Primary globular callus from immature zygotic embryos and friable embryogenic tissue derived from mature zygotic embryos were used to establish suspension cultures. Callus cultures were established either on modified Y3 or MS medium containing 475–500 μM 2,4-D or 250 μM picloram and 0.3% (w/v) activated charcoal. Suspension cultures of both cell lines were established in modified Y3 medium containing 10 μM 2,4-D. The establishment of cell suspensions from friable embryogenic tissue took only 2 months, in contrast with suspensions from primary globular callus which took 3–5 months to establish. Embryo differentiation was observed only in cell suspensions derived from the friable embryogenic tissue after plating aliquots on regeneration medium. Germinated embryos were recovered and plantlets were successfully established under greenhouse conditions.

Abbreviations: CET – compact embryogenic tissue, FET – friable embryogenic tissue, CIM – callus induction medium, PGC – primary globular callus, 2,3-D – 2,4-dichlorphenoxyacetic acid Y3 – Eeuwens' medium, MS – Murashige & Skoog medium, PVP-40 – polyvinylpyrrolidone, KM – Kao & Michayluk vitamins, ABA – abscisic acid

Introduction

Oil palm does not produce axillary or basal shoots and micropropagation of this species has utilized the somatic embryogenesis process resulting from solid cultures of young leaf or root explants (Jones 1984; Pennetier, et al. 1981). Somatic embryos and plantlets have been produced from mixed cultures of compact and semi-friable embryogenic calli (Rabechault & Martin 1976; Ahee, et al. 1981). Up to now, there is no information on the induc[illegible] multiplicati[illegible] of friable embryogenic cell l[illegible] palm, des[illegible] their importance for the production of high frequ[illegible] cy [illegible] genic cell suspensions as in other monocot species (Prioli & Söndahl 1989).

The availability of high quality embryogenic suspensions derived from mature elite palm explants will lead to the utilization of more efficient micropropagation methods by raising differentiating embryos in large batch cultures. Oil palm embryo regeneration from meristematic clumps maintained in liquid culture was reported (Touchet, et al. 1991). Here, we describe methods for isolation and long-term maintenance of a friable embryogenic tissue (FET) which serves as the inoculum to establish embryogenic suspensions leading to the production of somatic embryos and plantlets.

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The plant material used in this research was collected at the Cocoa Research Center (CEPLAC), Bahia State, Brazil. Mature and immature zygotic embryos of *Eleais guineensis* (Jacq.) were collected from "Tenera" hybrid plants derived from crosses between "Dura" and

Table 1. Solid and liquid culture media used for oil palm cell proliferation and embryo regeneration.

Callus Medium for Immature Embryos	Callus Induction Medium for Mature Embryos (CIM)	Suspension Medium (SM)	Regeneration Medium (RM)
Y3 salts (1.0 ×)	MS macro salts (0.5 ×)	Y3 salts (1.0 ×)	Y3 salts (1.0 ×)
	MS micro salts (1.0 ×)		
		NH_4Cl (267.5 mg/l)	
Y3 vitamins (1.0 ×)	Cysteine (500 mg/l)	Cysteine (500 mg/l)	Y3 vitamins (1.0 ×)
	Inositol (100 mg/l)	Nicotinic Acid (10.0 mg/l)	
Inositol (100 mg/l)	Nicotinic Acid (0.5 mg/l)	Thiamine (2.5 mg/l)	Inositol (100 mg/l)
	Thiamine (2.5 mg/l)	Pyridoxine (0.8 mg/l)	
Charcoal (0.3%)	Pyridoxine (0.8 mg/l)	KM Vitamins (1.0 ×)	sucrose (2%)
PVP-40 (0.5%)	Biotin (0.02 mg/l)	Inositol (100 mg/l)	
2,4-D (500 µM)	Pantothenic Acid (0.2 mg/l)	Ascorbic Acid (250 mg/l)	NAA (15 µM)
Cysteine (500 mg/l)	glycine (2.0 mg/l)	sucrose (1.5%)	
sucrose (3.0%)	PVP-40 (0.5%)	glucose (0.5%)	ABA (2 µM)
	sucrose (3.0%)	2,4-D (or Picloram) (10 µM)	
Gelrite (0.2%)	charcoal (0.3%)	pH (6.0)	Gelrite (0.2%)
	2,4-D (475 µM)		
pH (6.0)	Picloram (250 µM)		pH (6.0)
	Gelrite (0.2%)		
	pH (6.0)		

Abbreviations: Y3 salts (Eeuwens 1976; 1978), 2,4-D (2,4-dichlorophenoxyacetic acid), MS salts (Murashige & Skoog 1962), PVP-40 (polyvinylpyrrolidone), KM Vitamins (Kao & Michayluk 1975), ABA (abscisic acid)

"Pisifera" plants. The pollen source was the "Pisifera" tree, No. 2114, while the "Dura" plants were selected from a "Deli Dura" population introduced from Malaysia.

Culture procedures

Callus lines were induced from mature zygotic embryos cultured on a modified MS medium (Murashige and Skoog, 1962; Table 1) or from immature zygotic embryos cultivated on Y3 basal medium (Eeuwens 1976, 1978). All media were sterilized by autoclaving and the pH after autoclaving was 6.0.

Primary globular callus derived from immature embryos was inoculated into Y3 liquid medium supplemented with KM vitamins (Kao and Michayluk, 1975) to establish suspension cultures (Table 1). Before inoculation into liquid medium, sectors of oxidized tissues, especially parenchymatous and epidermal remnants, were removed. Calli were then chopped thoroughly with a razor blade in a plastic petri dish containing 5 ml of suspension medium.

Several lines of FET were isolated from mature embryo callus after 4–6 months of periodic subculture on callus induction medium (CIM). Suspension cultures were established by placing FET in 50 ml of the Suspension Medium (SM; Table 1) contained in 125 ml Erlenmeyer flasks. Cultures were grown in the dark at 26–27°C on a gyratory shaker at 140 rpm. Rapidly growing cell suspensions were maintained through a bi-weekly subculture regime in which 75% of the old medium was exchanged with fresh medium.

The regeneration capacity of FET suspension cultures was studied after transfer to solid or liquid Regeneration Medium (RM; Table 1).

Growth determination

Ten culture replicates in 125 Erlenmeyer flasks were randomly selected for calculating fresh and dry weight ratios. Growth curves were based on fresh weights of cell suspensions measured by the cell clumps collected on 520 µm screens. Cells were then transferred to a petri dish and weighed. Petri dishes containing fresh

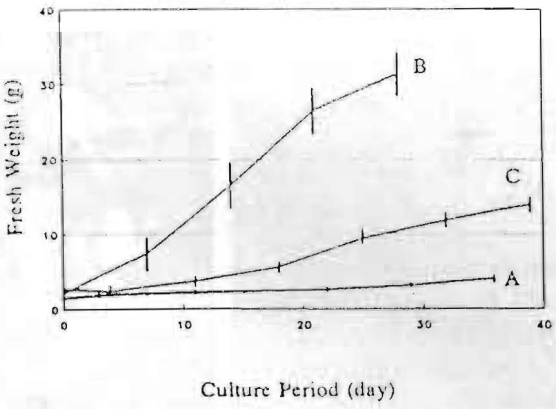

Fig. 1.

Comparative growth of cell suspensions of oil palm (*Elaeis guineesnis* Jacq.) cultured on 50 ml of modified Y3 medium containing 10 µM 2,4-D.

(A) Cell suspensions derived from Primary Globular Callus (PGC). Each point represents the average fresh weight from eight flasks.

(B) Growth of PGC-fine cell suspensions. PGC cell suspensions (A) were plated on solid medium and fast growing colonies were used to initiate the PGC-fine cell suspensions. Each point represents the average of three flasks.

(C) Cell suspensions derived from FET.
Each point represents the average of five flasks.

weight samples were then placed in a 60° C oven for 24 hours and weighed after being cooled in a desiccator.

Analysis of fresh and dry weight growth curves indicated that the use of fresh weight to evaluate cell growth in oil palm suspensions is as precise as dry weight (r^2 = 0.993; data not shown). Whenever possible, assessments of growth were made using fresh weight measurements. Growth rates and doubling times of suspension lines were evaluated according to Singer (1986).

Results

Cell suspensions were established from two tissue sources: primary globular callus (PGC) and friable embryogenic tissue (FET). The percentage of immature embryo explants yielding PGC varied from 0 to 28.6%, depending upon the stage of embryo development. The frequency of FET induction was highly dependent on the genotype and ranged from 0.6 to 21% (Table 2).

Oxidation and phenolic accumulation in the suspension medium observed in preliminary experiments were eliminated by changing several culture procedures. These included autoclaving the sugar sources in the presence of activated charcoal, addition of ascorbic acid and cysteine to the medium, and selection of donor tissues containing actively growing cells.

Cell suspensions from PGC

A fine cell suspension was established from the PGC after 3 months of repeated subculture (Fig. 1A, B). Although these fine PGC suspensions consisted of small, fast-growing cells, they were unable to differentiate into embryos after plating on regeneration medium.

Cell suspension from FET

Cell suspensions were also successfully established from FET isolated from mature embryo callus. The growth pattern of FET suspensions is shown in Fig. 1C. An increase in fresh weight from 2.6 g to 14.0 g was observed after 39 days of culture. No oxidation or browning was observed in these embryogenic cell suspensions. For these FET cell suspensions, the optimum inoculum size was 1.5 g of cell fresh weight for 50 ml of liquid medium. As the cell density increased, the growth rate decreased and the doubling time (dt) increased exponentially (data not shown).

FET suspension cultures were maintained on 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram. Independent of the auxin source, these cell suspensions displayed similar color and morphology, and they were visually distinguished by the presence of small cell aggregates hereafter described as "Fine cell suspensions" (Fig. 2A, B).

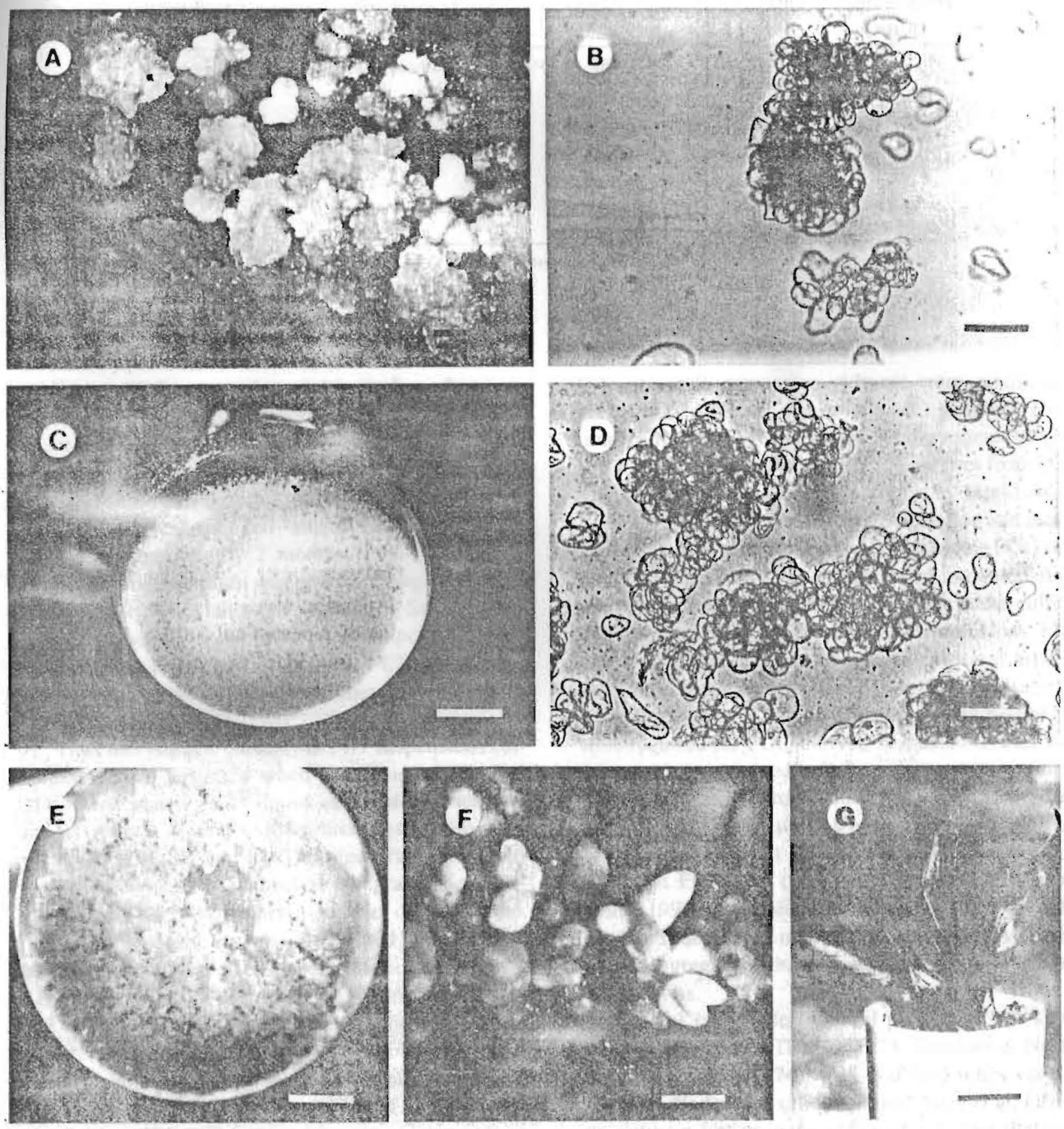

Fig. 2.

Establishment of embryogenic cell suspensions using friable embryogenic tissues (FET) derived from mature zygotic embryos of oil palm (*Elaeis guineensis* Jacq.).

(A) FET isolated from primary callus after 7 months of continuous subculture on modified MS medium containing 250 µM picloram and 0.3% (w/v) activated charcoal. Bar = 2.0 mm.

(B) Illustration of FET cellular aggregates containing round and actively dividing cells measuring 10–20 µm in diameter. Bar = 75 µm

(C) Example of fine FET cell suspensions cultured on modified Y^3 basal medium containing 10 µM 2,4-D after 2 months in liquid medium. Fresh medium was provided twice a week. Bar = 1.3 cm.

Table 2. Frequency of friable embryogenic tissue (FET) induction from callus derived from mature embryos of several oil palm genotypes. The embryos were excised and cultured for 15–26 weeks on BM medium containing 475 µM 2,4-D and 0.3% (w/v) activated charcoal.

Genotype	Period of Culture (Week)	Explant (No.)	(FET) (%)
D-1976	22	502	0.6
D-1986	21	221	0.9
D-2107	15	129	3.4
D-1974	26	558	3.4
D-2036	26	256	14.8
D-2289	26	605	21.0

BM medium: Murashige and Skoog's (1962) half-strength major salts, full-strength minor salts and iron.

The FET lines were successfully multiplied on solid medium by periodic subculture. FET cultures showed a tendency to differentiate into embryos after a few passages on multiplication medium. Subculture of small quantities of FET (ca. 1 g of fresh weight) at 3–4 week intervals was necessary to maintain FET cell lines in active growth, thus avoiding embryo differentiation.

Establishment of cell suspensions from FET required approximately 2 months (Fig. 2C). Initially, the starting inoculum was passed through a 520 µm screen to eliminate large cell aggregates. Such screening led to the production of fine cell suspensions. The embryogenic cells observed in these FET suspensions were round, small (10–20 µm diameter), and densely cytoplasmic (Fig. 2D).

The embryogenic capacity of FET suspensions cultures was demonstrated when suspension cells (Fig. 2D) were plated onto liquid or solid regeneration media. After 4–6 weeks, differentiating embryos were observed (Fig. 2E and F). The regeneration frequency observed was 180 embryos per gram of settled-cell volume or 5,400 embryos per liter of suspension. The majority of the embryos developed into complete and normal plantlets after subculture onto half-strength MS medium devoid of growth regulators and containing 0.3% (w/v) activated charcoal. The presence of arrested and abnormal embryos were observed. Germinated embryos underwent normal plantlet growth and development after being transferred to greenhouse conditions (Fig. 2G).

Discussion

Oil palm cell suspension cultures were established using two different donor tissues: primary globular callus (PGC) and friable embryogenic tissue (FET). Establishment of suspension cultures from PGC required the alternation of cultures in liquid-solid-liquid phases in order to eliminate oxidation and maximize growth in the liquid phase. After the fine PGC suspensions were established, they failed to demonstrate embryo regeneration capacity under the same culture conditions utilized for the fine FET suspensions. FET embryogenic suspensions differed in several aspects from PGC suspension cultures. The FET cell lines led to establishment of fine cell suspensions in half of the time required for PGC-derived suspensions. FET cell suspensions contained cellular aggregates composed of round, densely cytoplasmic cells which were small in diameter (10–20 µm). Conversely, cells of PGC suspensions were not isodiametric and were larger in size than FET cells (20–40 µm in length). Touchet et al. (1991) described the establishment of oil palm liquid cultures from meristematic clumps giving rise to cell aggregates, which resembled our PGC-derived suspensions.

Several reports describe oil palm callus as slow-growing (Smith and Thomas 1973; Turnham & Northcote 1982; Jones 1974), with doubling times varying from 30–40 days on solid medium. Ahee et al. (1981) described a fast growing callus of oil palm that pro-

(D) Isolated cellular aggregates from embryogenic cell suspensions, measuring from 20 to 40 µm in diameter. Bar = 75 µm.
(E) Somatic embryos at the globular stage after 6 weeks of culture on modified liquid Y3 medium containing 15 µM ANA and 2 µM ABA. Bar = 1.1 cm.
(F) Differentiated embryos derived from embryogenic cell suspensions after 5 weeks of plating on modified Y3 medium containing 0.3% (w/v) activated charcoal. Bar = 3.0 mm.
(G) Established plantlets after 1 month of transfer to soil mix under 80% shading at greenhouse. Bar = 2.5 cm.

duced somatic embryos when subcultured onto regeneration medium, but no growth rates were provided. In the present work, FET cultures had rapid growth on solid charcoal medium with a doubling time of 23 days. Inoculation of FET into liquid medium resulted in a substantial increase in growth rate, with doubling times as short as 7 days. Therefore, by using suitable cell lines and optimal culture conditions, high rates of growth can be obtained for oil palm cells.

A critical factor affecting the establishment of embryogenic cell suspensions is the amount of inoculum per flask. The growth rate decreased logarithmically with an increase in fresh weight per flask. In liquid medium, with frequent subcultures, nutrient depletion does not appear to be the main cause of poor growth when cell density increases. Other factors, including oxygen deprivation, increases in carbon dioxide level, secondary product accumulation and changes in pH may be involved.

Oxidation and phenolic accumulation were frequently observed in callus lines of oil palm maintained on solid medium without charcoal. However, oxidation was not observed in our fast-growing FET liquid cultures. This differential growth reaction in liquid phase is probably due to better nutrient absorption by the cells resulting in rapid cell growth. Aparavatjrut and Blake (1977) suggested that tissue oxidation could be controlled by using a nutritionally balanced medium. Furthermore, frequent medium replenishment significantly reduced the levels of phenolics and other toxic compounds.

The isolation of friable embryogenic calli in cereals (Type II callus) has allowed the establishment of long-term solid and liquid cultures (Green 1982; Prioli & Söndahl 1989; Wang et al. 1989). Type II callus described for cereals is similar in morphology and growth characteristics to the FET lines described here. The availability of suspension cultures with high regeneration capacity has been critical for repeated protoplast isolation and regeneration in important monocot species, including maize (Prioli & Söndahl 1989) and rice (Wang et al. 1989). Similar to the success in plant regeneration from embryogenic suspensions in other monocots, the oil palm protocols described here will facilitate the application of more advanced technologies for this crop, such as protoplast culture, *in vitro* selection and transformation.

Although compact embryogenic tissue (CET) has been used for micropropagation purposes in callus cultures of oil palm (Pannetier et al. 1981), an alternative route for micropropagation is indicated by the present work. It is proposed that the isolation and maintenance of FET cell lines on solid media and the establishment of FET liquid cultures would lead to the production of very large numbers of somatic embryos.

This report describes protocols for establishment of oil palm embryogenic cell suspensions from zygotic embryo tissues and the recovery of plantlets under greenhouse conditions. The production of FET cell suspensions from somatic explants of mature trees will be crucial for the utilization of this process for large scale clonal propagation of elite oil palm individuals. Such FET liquid cultures could be carried out in bioreactor vessels to increase the efficiency of somatic embryo production and, thus, reducing the unit cost of each resulting plantlets.

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