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Somatic embryogenesis from immature zygotic embryos of oil palm

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Abstract

Immature zygotic embryos at different developmental stages were used for callus induction and regeneration studies. Immature embryos excised from fruits 77, 91, 100, 114, 128, 140 and 193 days after pollination and mature embryos were cultured on modified Y3 medium containing 500 mg l^{-1} cysteine, 0.5% (w/v) PVP-40, 500 μM 2,4-D and 0.3% (w/v) charcoal. Compact embryogenic tissue began differentiating directly from embryo explants after 2 weeks of culture. The percentage of embryos forming compact embryogenic tissue ranged from 28.6% for 91-day-old embryos to 0% for 140-day-old and older embryos. Friable embryogenic tissue was observed in callus cultures derived from 100-day-old embryos. Although both compact and friable embryogenic tissues were successfully isolated, normal embryo and plantlet development was observed only from friable embryogenic tissue.

Abbreviations: ABA – abscisic acid, 2,4-D – 2,4-dichlorophenoxyacetic acid, NAA – naphthaleneacetic acid, PVP – polyvinylpyrollidone

Introduction

Tissue culture of oil palm (*Elaeis guineensis* Jacq.) has been undertaken mainly for the purpose of clonal propagation. Initial studies relied on mature zygotic embryos (Rabechault et al. 1970; Smith & Thomas 1973; Nwankwo & Krikorian 1983) and seedlings as explants (Rabechault et al. 1972; Jones 1974; Rabechault & Martin 1976). These studies have been useful in developing regeneration protocols that were further tested with explants derived from mature plants, including young leaves and roots (Ahee et al. 1981; Pannetier et al. 1981). Embryo explants are convenient because fruits are readily available, have a high degree of physiological uniformity, and can be shipped long distances.

Although immature embryos have been extensively utilized in cereal tissue culture (Green & Phillips 1975; Shillito et al. 1989; Prioli & Söndahl 1989), no reference is available regarding the use of this type of explant for oil palm. Culture of immature zygotic embryos at various stages of development could lead to unique culture responses in comparison with mature zygotic embryos. This paper describes callus induction, differentiation of compact and friable embryogenic tissues, embryo germination and plant regeneration from immature zygotic embryos of oil palm.

Materials and methods

Plant material

The plant material used in this research was collected at CEPLAC (Cocoa Research Center),

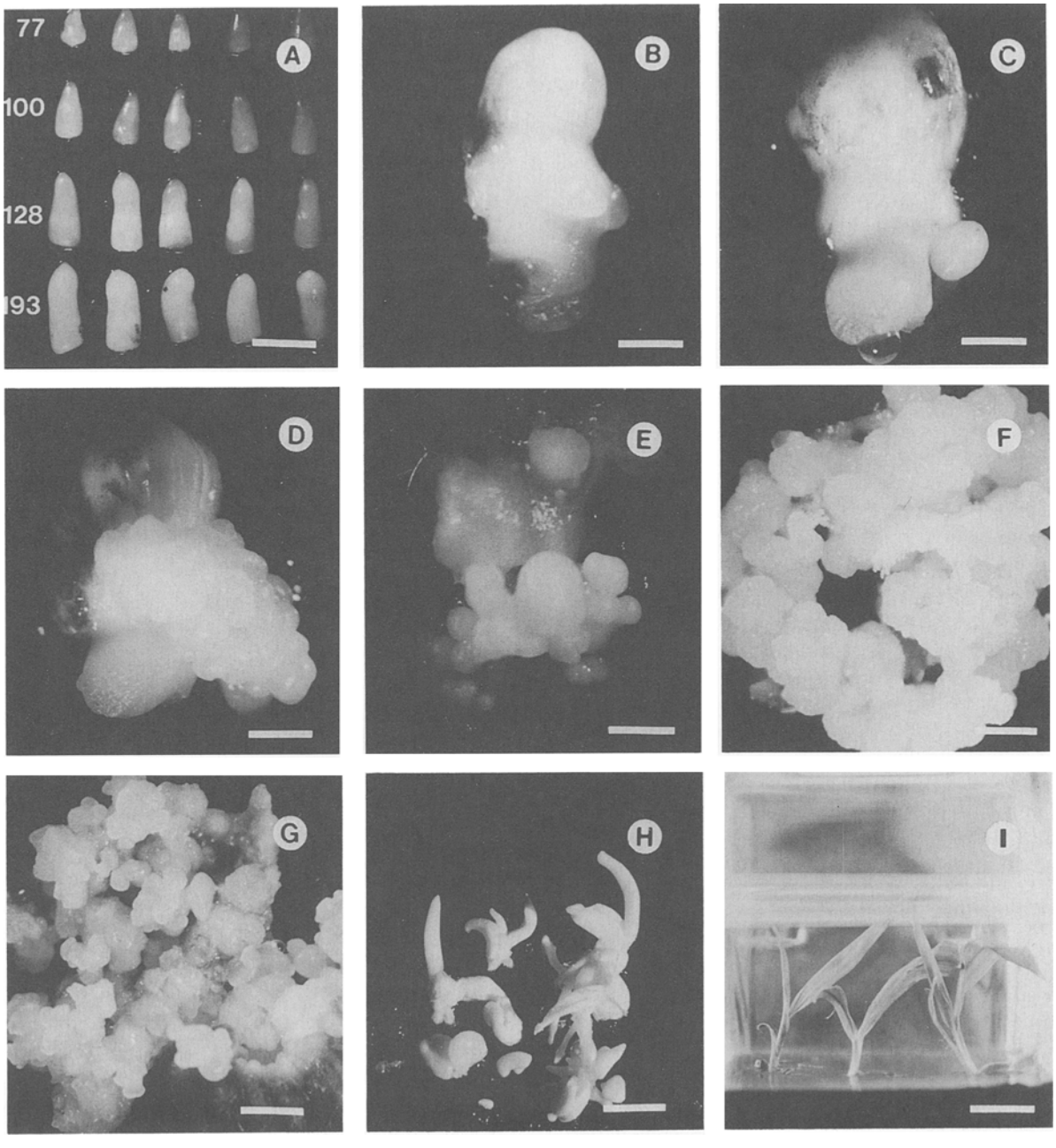


Fig. 1. In vitro response of immature zygotic embryos of oil palm (*Elaeis guineensis* Jacq.) cultured on modified Y3 medium. (*A*) Immature embryos in different stages of development. The numbers represent 77, 100, 128, 193 days after pollination. Bar = 2.25 mm. (*B*) Immature embryo 91 days after pollination after the first week of culture on callus induction medium containing 500 μM 2,4-D and 0.3% activated charcoal. Bar = 2.75 mm. (*C*) Immature embryo 91 days after pollination after 3 weeks of culture on same medium as described in B. Bar = 2.75 mm. (*D*) Immature embryo 91 days after pollination after 12 weeks of culture on callus induction medium. Bar = 2.0 mm. (*E*) Compact embryogenic tissue after 6 weeks of isolation on callus induction medium. Bar = 2.5 mm. (*F*) Callus and embryo-like structures produced on callus induction medium from immature embryos 91 days after pollination. Bar = 2.0 mm. (*G*) Friable embryogenic tissue developed from callus derived from embryos 100 days after pollination after 9 months of culture on callus induction medium. Bar = 2.5 mm. (*H*) Differentiated somatic embryos derived from friable embryogenic callus after 6 weeks of culture on MS medium containing 0.3% (w/v) activated charcoal and no growth regulators. Bar = 1.5 mm. (*I*) Plantlet growth on growth regulator-free MS medium at the first leaf stage of development. Bar = 4.0 mm.

Bahia State, Brazil. Immature zygotic embryos of the 'Tenera' hybrid, derived from crosses between 'Dura' and 'Pisifera', were used as starting materials. The pollen source for the hybridization came from a single 'Pisifera' tree (2114). The 'Dura' plants used as females were derived from a Malaysian 'Deli Dura' population.

Embryos were excised from fruits harvested from the following developmental stages: 77, 91, 100, 114, 128, 140, and 193 days after pollination (Fig. 1A, Table 1). Fruit size (length and diameter), fruit fresh weight, and embryo length were determined in order to characterize the various developmental stages (Table 2).

Culture procedure and media

Fruits were cut in half and the endosperm tissues containing the embryos were excised and disinfested in 1.58% (v/v) sodium hypochlorite for 30 min followed by brief rinsing in 96% (v/v) ethanol. Embryos were excised aseptically and inoculated onto callus induction medium in petri dishes consisting of modified Y3 medium (Eeuwens 1976, 1978) supplemented with 500 mg l^{-1} cysteine, 0.5% (w/v) PVP, 0.3% (w.v) activated charcoal, 500 μM 2,4.D, and 0.22% (w/v) Gel-Rite. The pH was adjusted to 5.0 before adding charcoal and equilibrated to approximately 6.0 after autoclaving. Callus induction, callus proliferation and embryogenic tissue maintenance were performed on the same callus induction medium. Callus was subcultured at 3–4 week intervals. All cultures were kept in the dark at 27–29°C.

Embryo differentiation was obtained after transfer onto regeneration medium consisting of callus induction medium lacking activated charcoal and 2,4-D and containing 15 μM NAA and 2 μM ABA. Embryo germination and plantlet development was obtained by culturing somatic embryos on MS (Murashige & Skoog 1962) medium containing 0.3% activated charcoal and no plant growth regulators.

Table 1. Source of *Eleais guineensis* fruits, harvesting dates (days after pollination), and consistency of the fruit endosperm.

'Dura' plants	Harvesting dates (days after pollination)			Endosperm consistency
2036	69 *		–	liquid
2291	77	and	91	very soft to soft
2287	110	and	114	soft to hard
2024	128	and	142	hard
1973	193 **		–	very hard

* Embryo size was less than 0.1 mm (not cultured);
** Presence of oil in the mesocarp (mature fruit).

Table 2. Characterization of oil palm immature fruits and embryos of oil palm (*Elaeis guineensis* Jacq.) at four stages of development. The fruits were harvested 77, 100, 128 and 193 days after pollination.

Days after pollination	Fruit diameter* (cm)	Fruit length (cm)	Fruit weight (g)	Embryo size (mm)
77	2.4	3.4	9.6	1.4
100	2.3	3.4	8.8	2.8
128	2.3	3.4	9.2	3.0
193	3.4	5.1	31.0	3.3

* Twenty-five fruits from each stage of development were evaluated.

Results

During embryo development *in situ*, the increase in fresh weight of fruits from 77 days after pollination (9.62 g) to 193 (31.02 g) was due to elongation and an increase in fruit density (Table 2). The fruit embryos, however, seemed to follow a different pattern of growth. The largest increase in embryo length took place between 77 and 100 days after pollination (1.35–3.0 mm), well before fruit maturation and oil accumulation.

After the first week of culture on callus induction medium, all embryo explants began to swell at the mesocotyl region (Fig. 1B). This swelling continued until the embryos reached approximately 3.0–6.0 mm in diameter. Callus initiation was first observed in the area of the vascular tissues. Embryo explants containing swollen vascular bundles were then subcultured onto fresh callus induction medium for callus proliferation.

The percentage of explants forming callus varied from 55% to 88% for immature embryos, but for embryos collected from mature fruits (193 days after pollination) the percentage was considerably higher (approximately 93%). The

Table 3. In vitro responses of cultured zygotic embryos of oil palm (*Elaeis guineensis* Jacq).

Days after pollination	Number of explant	Callus (%)	Oxid. (%)	Germ. (%)	Shoot (%)	CET (%)	Doubling time (days)
77	289	88	0	0	12	14	10
91	91	68	2	0	1	29	–
100	300	81	0	0	0	23	11
114	304	55	8	0	4	9	–
128	356	61	0	0	9	1	12
140	206	74	14	0	9	0	–
193	292	93	0	2	39	0	8

Embryos were excised and cultured for 3 months on modified Y3 medium containing 500 μM 2,4-D and 0.3% (w/v) activated charcoal.
Oxid. – explant oxidation,
Germ. – zygotic embryo germination,
Shoot – seedling without root development as a result of abnormal germination,
CET – compact embryogenic tissue.

doubling time for callus from mature embryos was similar to the growth rate for callus from immature embryo explants (Table 3).

Zygotic embryo germination occurred in the presence of 2,4-D. In the case of mature embryos, up to 2% of the cultures produced normal seedlings. Partial germination (shoot development) was observed in cultures of immature embryos (Table 3). Shoots clearly arose from the embryo apical meristems and formed adventitious buds. Root meristems became disorganized by high 2,4-D concentrations, resulting in callus formation at the base of seedlings.

After 2–3 weeks of primary culture, a small pearly-white compact embryogenic tissue began to emerge at the mesocotyl regions of immature embryos (Fig. 1C). After 3 months in culture, the compact embryogenic tissue was approximately 1 cm in diameter, very compact, and contained many small pearly white structures (Fig. 1D). The surface of the compact embryogenic tissue was shiny, probably due to the presence of an epidermis-like tissue. This tissue was separated from the original explant (Fig. 1D) and subcultured onto callus induction medium, which allowed continuous proliferation of compact embryogenic tissue for at least 10 months. The percentage of explants that produced compact embryogenic tissue varied from 0 to 28.6%, depending on the stage of embryo development (Table 3).

When compact embryogenic tissue was subcultured onto regeneration medium, the pearly white structures differentiated into embryo-like structures (Fig. 1E, F). However, no germination was achieved from these embryo-like structures.

After approximately 9 months in culture on callus induction medium, a friable embryogenic tissue was observed on primary globular callus cultures derived from 100-day-old embryos. This friable embryogenic tissue was composed of small, round, and cytoplasmically dense cells that grew in small clumps (Fig. 1G). Sectors of friable embryogenic tissue were isolated and maintained on callus induction medium for more than 1 year. Normal embryo differentiation has been achieved upon transfer of the friable tissue to regeneration medium (Fig. 1H). Embryo germination and plantlet development were achieved on growth regulator-free MS salts in the presence of charcoal (Fig. 1I).

Discussion

There are several reports of embryogenesis in callus derived from embryos, young leaf and root tissues of oil palm. Defined protocols for the different phases of the process are, however, limited.

The induction of somatic embryogenesis from immature embryos showed a pattern of development distinct from all other reports on oil palm. The developmental stage of the explant determined the type of response obtained *in vitro*.

Direct formation of compact embryogenic tissue was induced in embryos taken 77–128 days after pollination (Fig. 1B, C, D, E). Primary globular callus proliferated from all stages of embryos sampled (Fig. 1F). Although embryogenesis in oil palm cell cultures appears to be more related to the explant source than to cultural conditions, addition of 2,4-D was essential for callus induction and for maintenance of embryogenic tissues.

Although primary cultures from immature embryos followed a pattern of growth somewhat similar to that observed for cultured mature embryos, several aspects were unique to immature embryo explants. Callus initiation was observed from embryos of all stages of development tested; however, embryos from early stages of development showed a tendency to produce less primary globular callus than older embryos. Furthermore, older embryos showed a greater tendency for *in vitro* germination and shoot development, even at high 2,4-D concentrations. These two factors explain, in part, the lower fresh weight doubling time found for older embryo explants. The doubling time (8–12 days) found during callus induction for all embryo stages was probably more related to elongation of explants than to cell proliferation.

Oil palm callus is classified as slow growing. Smith & Thomas (1973) found that primary callus derived from shoot apices of oil palm doubled in weight every 42 days. On the other hand, fast growing callus selected by Hanower & Pannetier (1982) from young leaf tissues exhibited a doubling time of 10–15 days. Therefore, callus lines derived from different explant sources and submitted to different culture treatments have different growth rates.

Phenolic oxidation and browning is a serious problem in the tissue culture of palms (Eeuwens & Blake 1977; Ammar & Benbadis 1977; Reynolds & Murashige 1979; Jones 1974; Nwankwo & Krikorian 1983). In several instances, addition of activated charcoal has minimized oxidation, provided the concentration of auxin (2,4-D) was increased to suitable levels. In the present work, activated charcoal was required in order to obtain high quality primary callus cultures. To produce callus proliferation, the concentration of auxin had to be increased to 50 times the normal level when in the presence of activated charcoal. Charcoal adsorbs toxic phenolic compounds and their oxidation products (quinones) that are released by the explants (Reuveni & Lilien-Kipnis 1974; Ziv & Halevy 1983).

The induction of compact embryogenic tissue was observed in cultures of immature embryo explants with little or no callus proliferation. Stereomicroscope observations indicated that this tissue was initiated from the outer cell layers of mesocotyl. Similar results were found by Guerra & Handro (1988) in cultures of immature embryos of *Euterpe edulis*, another palm species.

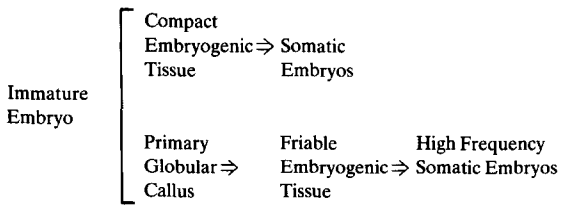
The induction of somatic embryogenesis in palms is not well understood. In general, callus induction is considered a prerequisite for somatic embryogenesis (Rabechault & Martin 1976) and auxin is required for callus induction and proliferation (Nwankwo & Krikorian 1983). After successive subcultures callus cells acquire the capability for embryogenesis. This has been observed both at high and at low auxin concentrations (Turnham & Northcote 1982). In some cases, the reduction or omission of auxin is necessary for embryo development (Reynolds & Murashige 1979; Tisserat 1979). In other cases, changes in auxin source as well as the addition of a cytokinin seems to be helpful (Rabechault & Martin 1976). However, Jones (1974) suggested that embryogenesis in oil palm cell cultures is a function of explant source rather than of culture medium and culture conditions.

In the present studies, induction of compact embryogenic tissue was observed, with little or no primary callus formation, as early as 2 weeks after inoculation. This pattern of embryogenesis is somewhat similar to the direct embryogenic pathway in coffee, as reviewed by Sharp et al. (1980).

In contrast to mature embryo explants, immature embryos produced the compact embryogenic tissue during primary culture without passing through a callus phase. Alternatively, the primary globular callus can be formed much later in the culture process, followed by the production of a friable embryogenic tissue. The primary globular callus induced from immature embryo explants did not produce low frequency somatic embryos, as observed in cultures derived from mature embryos (J.B. Teixeira, M.R. Söndahl

and E.G. Kirby, unpublished data). The friable embryogenic tissue isolated from immature embryo culture was similar to other embryogenic systems previously described for dicots (Street 1979; Söndahl et al. 1979) and type II callus in monocots (Green 1982; Armstrong & Green 1985) and led to production of high frequency somatic embryos.

Somatic embryogenesis from immature embryo explants of oil palm can be represented as follows:



Somatic embryos induced from immature embryo explants can be derived either from compact embryogenic tissue or from friable embryogenic tissue. However, somatic embryos from compact embryogenic tissue can be obtained more quickly from immature embryos than from mature embryo explants. The distinct regeneration behavior leading to various culture types clearly demonstrates the effect of developmental stages of immature embryos explants upon specific *in vitro* responses.

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