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Plant regeneration from embryogenic suspension cultures of oil palm (*Elaeis guineensis* Jacq.)

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Abstract. Suspension cultures of oil palm (*Elaeis guineensis* Jacq.) were established in a medium with 80 or 100 mg.l^{-1} 2,4-dichlorophenoxyacetic acid and 1 g.l^{-1} activated charcoal, from calli producing embryogenic cells and protruding proembryos. The suspension was composed of meristematic clumps, breaking away and giving rise to new smaller aggregates. Under the best conditions, the initial weight increased about 4 fold in one month. Embryo differentiation was achieved when plating the clumps either directly, or after a phase in hormone-free liquid medium. In the second case, early maturation occurred in liquid medium. Secondary embryogenesis was reduced, and up to 18.1 % embryos formed shoots. Rooted plants were sucessfully transferred to soil.

Abbreviations

BAP = 6-benzylaminopurine; NAA = α-naphthaleneacetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid.

Introduction

Given the great economic value of oil palm (*Elaeis guineensis* Jacq.), micropropagation of this perennial allogamous monocotyledon, exclusively propagated by seeds, has been studied for about twenty years.

Numerous successes with regeneration *via* somatic embryogenesis have been reported (see the review by Brackpool *et al.* 1986). For several years, the process developed by ORSTOM-IRHO/CIRAD, based on the culture of unexpanded leaf fragments (Pannetier *et al.* 1981), has been introduced on pre-industrial scale in different countries (Côte d'Ivoire, France, Indonesia, Malaysia) and thousands of true-to-type plants are in the field (Duval *et al.* 1988).

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The first results confirm theoretical expectations in terms of both the value and homogeneity of the clones (Durand Gasselin *et al.* 1990).

The micropropagation technique is carried out on agar for all stages except rooting. This involves constraints linked to solid medium, and a dissection step to isolate ramets from the polyembryonic masses.

In order to improve the process and for a better understanding of the mechanisms involved in oil palm somatic embryogenesis, investigations on suspension cultures were initiated. This report describes the first establishment and maintenance of regenerable embryogenic suspension cultures from oil palm leaf-derived calli.

Material and methods

The basal medium used for all stages contained Murashige and Skoog's macroelements modified by Rabéchault and Martin (1976), Nitsch's microelements (1969), Morel and Wetmore's vitamins (1951) and sodium ascorbate (100 mg.l^{-1}). The pH was adjusted to 5.0 prior to addition of activated charcoal or agar, and autoclaved (30 min at 110 °C).

Initiation and proliferation of embryogenic suspension cultures : The embryogenic calli used to initiate the suspensions were chosen from the collection of nodular calli produced from immature leaflet fragments of adult trees as described by Hanower and Pannetier (1982). The culture medium was composed of basal medium complemented with 2 % glucose, adenine sulfate (30 mg.l^{-1}), BAP (1 mg.l^{-1}), and activated charcoal (1 g.l^{-1}). 2,4-D was added to the medium at 80 mg.l^{-1} (= medium M 80), 100 mg.l^{-1} (= M 100), 150 mg.l^{-1} (= M 150), or 200 mg.l^{-1} (= M 200) to initiate the suspension. Each callus (approximately 500 mg) was placed in a 100 ml Erlenmeyer flask containing 20 ml of medium. The flasks were covered by polyether caps and plastic film. Culture conditions were : temperature 27 ± 1°C; light intensity 50 μE.m^{-2}.sec^{-1}; light/dark photoperiod

12:12-h, agitation 90 rpm. The medium was renewed every 4 to 6 weeks. At each transfer, biomass was weighed and residual callus fragments were removed until a suspension was obtained. Different inoculum densities (0.05 to 0.5 g) and 2,4-D concentrations 25 mg.l^{-1} (= M 25), 50 mg.l-1 (= M 50) or M 100 were tested to monitor proliferation.

Regeneration : Either nodules were transferred to basal medium supplemented with 3% sucrose, for early development (1 or 2 cycles), then plated on the same medium with 0.8 % agar onto Petri dishes (9 cm diameter), or the suspension was directly plated. Embryos were subcultured on the same medium until conversion. Rooted plants were sent to Côte d'Ivoire for soil transfer.

Histology : The technique used was described by Schwendiman *et al.* (1990). The sections (3.5 μm) were double-stained by the Schiff-periodic acid reaction and naphtol blue black.

Results and discussion

1) Initiation of the suspension

Description of the initial calli

Friable embryogenic callus with small (< 1 mm) white nodules, slightly connected to the rest of the beige callus was used to initiate the suspension. (Fig. 1).

Histological examination showed that the nodules were small compact aggregates located at the periphery of the parenchymatous callus tissue, with highly vacuolated cells (Fig. 2).

The aggregates were composed of typical meristematic cells containing soluble proteins and sometimes starch granules. They had a round prominent nucleus, and a dense cytoplasm with small vacuoles. These embryogenic masses were dividing actively. Single embryogenic cells and small proembryos, enclosed by a thick cell wall, were also observed bordering on the parenchyma.

Influence of the 2,4-D concentration on embryogenic suspension initiation

From the first day of culture in a stirred medium, some of the embryogenic masses started to detach from the mother callus tissue.

The first suspensions were obtained after 2 subcultures in M 80 and M 100, and their number increased with time (Table 1).

In initial calli, some embryogenic masses were quiescent or already tending towards degeneration, as described on agar medium by Schwendiman *et al.* (1988 and 1990). This physiological heterogeneity could explain the variability of the time taken to obtain a suspension.

The release was stopped on the second cycle in M 200, and on the third one for M 150. In both cases, the 2,4-D not adsorbed on charcoal seemed too high for suspension establishment.

Table 1. Embryogenic masses released (EM) from callus cultured in M 80, M 100, M 150 and M 200 and suspension establishment (S).

Cycle	1		2		3		4		5		6	
	EM	S	EM	S	EM	S	EM	S	EM	S	EM	S
M 80	4/10	0/10	4/8	0/8	3/7	3/7	2/7	4/7	1/7	5/7	0/8	6/7
M 100	4/10	0/10	6/9	0/9	6/9	2/9	4/9	3/9	2/9	5/9	0/8	7/8
M 150	3/10	0/10	4/7	0/7	0/6	0/6	0/6	0/6	•			
M 200	2/10	0/10	0/7	0/7	0/5	0/5	0/5	0/5	•			

Each value represents the ratio : competent calli/calli conserved.
Callus elimination was due to browning or infection.
* calli grown in M 150 and M 200 were eliminated after 4 cycles.

Ebert and Taylor (1990) reported that 2,4-D was adsorbed at about 98% in a liquid medium very similar to M 100. We presume that 2,4-D adsorption is not linear, and free 2,4-D was probably much higher in M 150 and M 200 than in M 100.

High concentrations of 2,4-D have already been reported to slow down the proliferation of white poplar suspension (Park and Son 1988), to inhibit proliferation of soybean suspension (Finer and Nagasawa 1988), or to promote non-embryogenic tissue growth on Chinese yam suspension (Nagasawa and Finer 1989).

2) Proliferation of the suspension

Histology

Histological studies showed that the proliferating embryogenic aggregates did not have an epidermis and were composed of meristematic cells in active division (Fig. 3). They initiated adventious nodules, separated from each other by fragmentation lines, due to a distinct cell wall, thicker in comparison to internal cell walls. Fragmentation lines were also seen by Tisserat and DeMason (1980), to demarcate each meristematic locus on date palm.

When protruding, the nodules were released in the medium by breakage of the larger ones, due to agitation. All steps of the cycle took place at the same time in each flask, which means that at all times, all the various sizes of nodules could be seen.

Suspension characteristics

The diameter of the particles in suspension varied from 0.1 mm to 2.5 mm in M 100 and M 80 (Fig. 4).

For an initial seeding varying from 0.1 g to 0.5 g, a mass effect was observed (Table 2).

Table 2. Effect of initial weight (IW) on the final weight (FW) after 1 month in M 100.

IW (g)	0.05-0.1	0.1-0.2	0.2-0.3	0.3-0.4	0.4-0.5
FW/IW	1.81±0.19*	3.83±0.30	3.97±0.34	2.83±0.37	2.81±0.48

* Each value represents the mean ratio of 5 replications ± standard error.

The best inoculum weight was between 0.1 of and 0.3 g, with a weight increase by a factor of around 4 after 1 month.

Concentrations of 2,4-D in M 25 and M 50 were insufficient to maintain nodules at this stage (data not shown). Nodules tended towards differentiation ; weight and size increased and clumps turned yellow.

Sometimes they looked like the haustorial structures developed in hormone-free liquid medium (see below).

On the other hand, in M 100 the suspension established during early experiments has been maintained for over 18 months with no decline of embryogenic potential.

3) Embryo development

In liquid medium

The most advanced form obtained in hormone-free liquid medium, was an embryo with a gemmule and a radicle, which did not develop further and became necrotized after transfer to agar medium.

If conserved for a long time in liquid medium without (at least 1 cycle) or with low auxin concentration (M 50 or M 25: at least 2 cycles), the swollen nodules tended towards haustorium-like structures, sometimes with roots, and remained at this stage.

Histological studies revealed they had lost their embryogenic potential. They showed no cell division, and had enormous amyloplast accumulation around the edges. The centres were vascularized and an epidermis was sometimes visible.

In solid medium

Embryos were obtained:

- either, by direct spreading of proliferating nodules from M 80 or M 100. After a latent period (1 week to 1 month), a stage of multiplication was observed, prior to obtaining embryos. When plating isolated embryos, adventitious embryogenesis was noticed, as in the current process. It was probably due to the remaning effect of the 2,4-D.

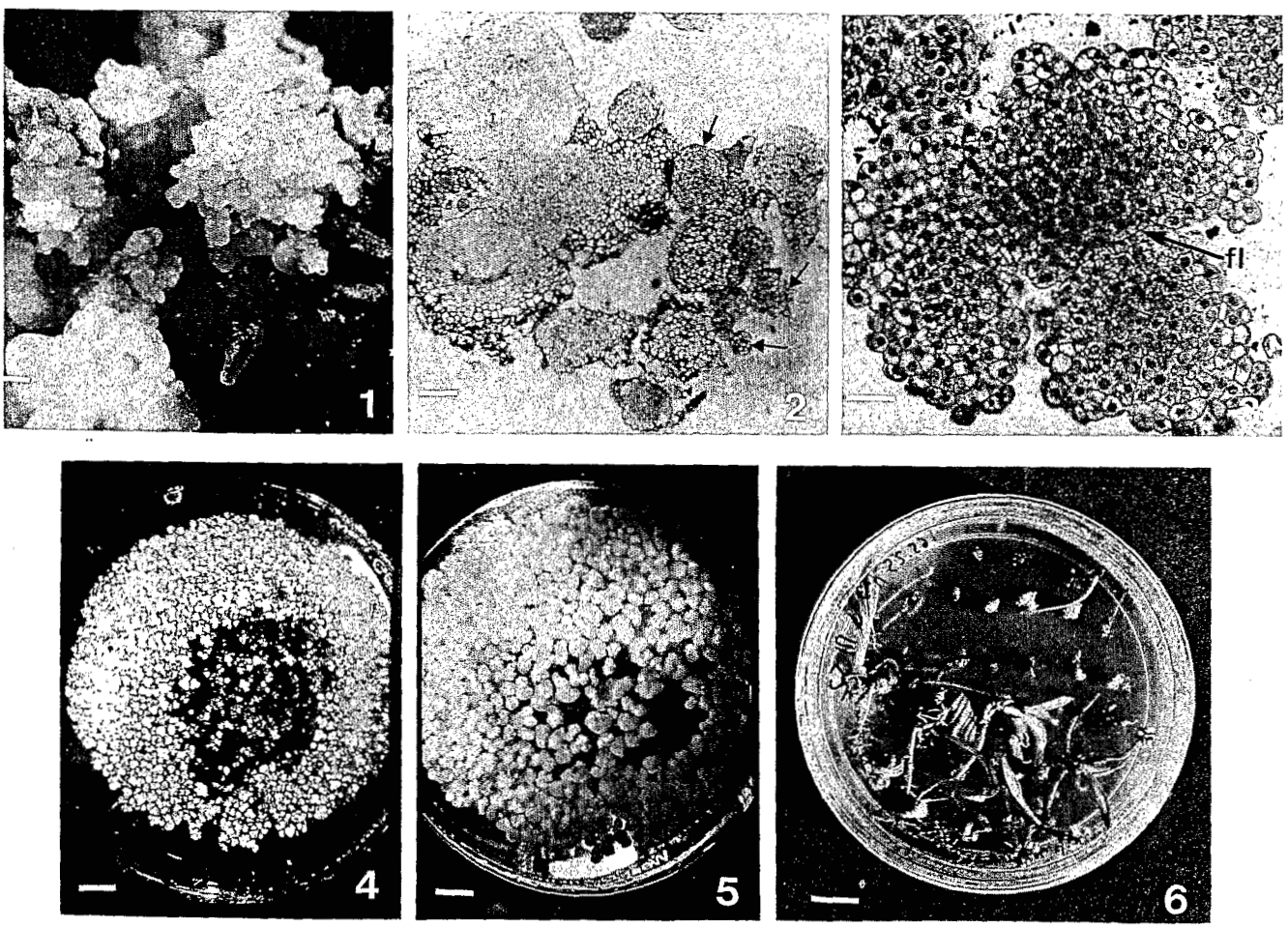

Fig 1 to 6 : Oil palm regeneration from embryogenic suspension culture. (1) Embryogenic callus with friable nodules (bar=1 mm). (2) Embryogenic masses (arrows) at the periphery of the parenchymatous callus (bar=100 μm). (3) Formation of adventive nodules by breakage; fl=fragmentation line (bar=50 μm). (4) Embryogenic suspension in proliferation medium (bar=5 mm). (5) Synchronous maturation in hormone-free liquid medium (bar=5 mm). (6) Shoot and root regeneration after plating (bar=5 mm).

- or from synchronously maturing nodules, at an early stage of differentiation, in a liquid medium deprived of hormone. The stage was determined visually before plating : clumps were pale yellow or white, maximum 3 mm, with one or several lobes (Fig 5). Under these conditions, multiplication was greatly reduced, and one nodule usually produced one to three embryos.

4) Plant Regeneration

Embryos developed in one of two ways:

- a root formed, in which case the cauline pole was inhibited. Inhibition of shoot pole development by early root formation is a problem encountered for several plants : *Digitalis* (Arrillaga *et al.* 1987), coconut (Verdeil *et al.* 1989), and banana (Novak *et al.* 1989).
- a shoot and then sometimes a root formed; the first shoots were visible after 4 to 6 weeks of culture. The percentage of shoots per dish varied from 1.8 % to 18.1 %. They were usually isolated from one another, or slightly connected at the root pole (Fig. 6). This is a major advantage in comparison with the standard process, where the shoots have to be separated manually before rooting. Rooted plants obtained from these embryos have been successfully transferred to soil.

The embryogenic suspension system described is a first step in the development of large scale oil palm propagation using bioreactors and artificial seed technology. Before full application of the system, clonal fidelity has to be checked. Factors reponsible for secondary embryogenesis as well as maturation of the embryos should also be investigated.

This technique enables the production of individualized embryos in synchronous growth, with both root and shoot poles, compared to the routine process with polyembryonic cultures and compulsory rooting treatment.

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References

Arrillaga I, Brisa MC, Segura J (1987) Somatic embryogenesis from hypocotyl callus cultures of *Digitalis obscura* L. Plant Cell Reports, 6:223-226.

Brackpool AL, Branton RL, Blake J (1986) Regeneration in palms. *In* : Cell culture and somatic cell genetics of plants. vol 3 Academic press pp 207-222.

Corley RHV, Barrett JN, Jones LH (1977) Vegetative propagation of oil palm via tissue culture. Oil Palm News 22:2-7

Durand Gasselin T, Le Guen V, Konan K, Duval Y (1990) Oil palm (*Elaeis guineensis* Jacq.) plantations in Côte d'Ivoire obtained through *in vitro* culture. First results. Oléagineux 45:1-11.

Duval Y, Durand Gasselin T, Konan K, Pannetier C (1988) *In vitro* vegetative propagation of oil palm (*Elaeis guineensis* Jacq.) Strategy and results. Oléagineux 43:39-47

Ebert A, Taylor HF (1990) Assessment of the changes of 2,4-dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. Plant Cell Tissue Org Cult 20:165-172

Finer JJ, Nagasawa A (1988) Development of an embryogenic suspension culture of soyabean (*Glycine max* Merill.). Plant Cell Tissue Org Cult 15:125-136

Hanower J, Pannetier C (1982) *In vitro* vegetative propagation of the oil palm, *Elaeis guineensis* Jacq., pp 745-746. *In* Plant Tissue Culture ed A Fujiwara, Maruzen, Tokyo

Morel G, Wetmore RH (1951) Tissue culture of monocotyledons. Am J Bot 38:141-143

Nagasawa A, Finer JJ (1989) Plant regeneration from embryogenic suspension cultures of Chinese yam (*Dioscorea opposita* Thumb.). Plant Science 60:263-271

Nitsch JP (1969) Experimental androgenesis in *Nicotiana.* Phytomorphology 19:389-404

Novak FJ, Afza R, Van Duren M, Perea-Dallos M, Conger BV, Tang Xiaolang (1989) Somatic embryogenesis and plant regeneration in suspension cultures of dessert (AA and AAA) and cooking (ABB) bananas (*Musa* spp). Biotechnology 7:154-159

Pannetier C, Arthuis P, Liévoux D (1981) Neoformation of young *Elaeis guineensis* plant from primary calluses obtained on leaf fragments cultured *in vitro*. Oléagineux 36:119-122

Park YG, Son SH (1988) Regeneration of plantlets from cell suspension culture derived callus of white poplar *Populus alba* L. Plant Cell Reports 7:567-570

Rabéchault H, Martin JP (1976) Multiplication végétative du palmier à huile (*Elaeis guineensis* Jacq.) à l'aide de cultures de tissus foliaires. C R Acad Sci Paris 238:1735-1737

Schwendiman J, Pannetier C, Michaux-Ferrière N (1988) Histology of somatic embryogenesis from leaf explants of the oil palm *Elaeis guineensis*. Ann Bot 62:43-52

Schwendiman J, Pannetier C, Michaux-Ferrière N (1990) Histology of embryogenic formations during *in vitro* culture of oil palm *Elaeis guineensis* Jacq. Oléagineux 45:409-418

Tisserat B, DeMason DA (1980) A histological study of development of adventive embryos in organ cultures of *Phoenix dactylifera* L. Ann Bot 46:465-472

Verdeil JL, Buffard-Morel J, Pannetier C (1989) Somatic embryogenesis of coconut (*Cocos nucifera* L.) from leaf and inflorescence tissues : research findings and prospects. Oléagineux 44:403-411