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Full Length Research Paper

Initial requirements for embryogenic calluses initiation in thin cell layers explants from immature female oil palm inflorescences

Rodrigo da Silva Guedes[1], Tatiane Loureiro da Silva[2], Zanderluce Gomes Luis[3] and Jonny Everson Scherwinski-Pereira[4]*

[1]IDARON – Agência de Defesa Sanitária Agrossilvopastoril do Estado de Rondônia, Porto Velho (Rondônia)-78900903-Brazil.
[2]PPGBIOTEC, Programa de Pós Graduação em Biotecnologia, Department of Biology, Universidade Federal do Amazonas, Manaus (Amazonas)-69077000-Brazil.
[3]POSBOT, Programa de Pós Graduação em Botânica, Institute of Biology, Universidade de Brasília (Distrito Federal)-70910900-Brazil.
[4]Embrapa Recursos Genéticos e Biotecnologia, Brasília (Distrito Federal)-70770917-Brazil.

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This study highlights procedures for embryogenic calluses induction from immature female inflorescences of oil palm using thin-cell-layers explants (TCL). In three experiments, the ability of calluses induction were examined and identified through different types of basal media, position of the TCL explants in the rachillae and concentrations of 2,4-D and types of antioxidants added into the medium. Samples of embryogenic calluses obtained were isolated and transversal and longitudinal cross sections were obtained and stained for observations in light microscopy. The results achieved suggest that immature female inflorescences of oil palm can be reverted from the floral state to the embryogenic vegetative state and are excellent alternative sources of explants for the induction of somatic embryogenesis. In general, 225 to 450 µM of 2,4-D are required to induce embryogenic callus in explants composed of immature oil palm inflorescences and the composition formed by salts and vitamins of MS medium provides superior results than Y3 medium. The activated charcoal at concentration of 3.0 g l^{-1} is the most indicated antioxidant for preventing the oxidation of floral oil palm explants and its presence can be considered essential for the formation of embryogenic callus.

Key words: *Elaeis guineensis*, somatic embryogenesis, micropropagation, floral explants, morphogenesis, agroenergy.

INTRODUCTION

Generating power using alternative models via the production of biodiesel extracted from species such as the oil palm (*Elaeis guineensis* Jacq.) is very promising and meets the objectives of the Brazilian Biofuels Program. Palm oil is particularly important because it is one of the most productive oleaginous species known, yielding 4 to 6 tons of oil/ha when adult. Furthermore, this species is the second largest source of vegetable oil, after soybean (Jaligot et al., 2000).

The oil palm is a perennial cross-pollinating oleaginous monocotyledon, mostly cultivated in tropical regions of Latin America, Southeast Asia and Africa. It originally comes from the Northwest region of Africa (Guinea-Bissau) and belongs to the Arecaceae family. Another important species of oil palm, this time of American origin, is *Elaeis oleifera* (H.B.K), common in the Amazon rainforest of Brazil, where it is known as the “Caiaué” palm. Despite having some desirable agronomic characteristics, such as its small size and resistance to lethal yellowing and *Fusarium E. oleifera* (H.B.K.) it is not considered economically viable when compared to *E.*

*Corresponding author. E-mail: jonny@cenargen.embrapa.br. Tel: +556134484796.

guineensis (Jacq.) as its oil yield is lower. However, E. oleifera is often indicated and used in improvement programs for the species (Moretzsohn et al., 2002) since, despite their geographically distant centers of origin; these two species are compatible for crossing and can generate fertile hybrids. But as it is a typical mono-cotyledonous species, oil palm has a single growth apex, preventing its vegetative propagation by conventional means (Jaligot et al., 2000). In genetic improvement programs for the species present in Brazil, the multiplication of genotypes occurs exclusively by means of seeds, a fact which, in the absence of adequate cloning by conventional methods, makes it virtually impossible to obtain uniform cultures and perpetuate the characteristics in an individual of high selected genetic value (Rival et al., 1998; Scherwinski-Pereira et al., 2010). Therefore, there are concerns over the multiplication of selected genotypes which are already phenotyped when adult, because as well as the possibility of obtaining heterogeneous individuals, multiplication by seeds requires a relatively long period until new plants are formed (Perera et al., 2007).

In general, among the various techniques for cloning plants *in vitro*, somatic embryogenesis is undoubtedly the most scientifically pursued by researchers seeking to clone palm trees *in vitro.* Somatic embryogenesis has the advantage that it is induced from different types of explants (propagules), the immature inflorescences being among the most promising, due to the high number of meristem flowers per inflorescence and the fact that they can be obtained from mature plants without causing major damage to the mother plant. Furthermore, immature inflorescences are usually protected by spathes, virtually eliminating the need for the disinfestations of the explants.

This study sought to indicate the initial requirements for embryogenic callus initiation in thin-cell-layers explants of immature female palm oil inflorescences cultured *in vitro*.

MATERIALS AND METHODS

Influence of types of culture medium and growth regulators

The plant material used consisted of immature female inflorescences of oil palm mother plants *E. guineensis*, type 'Dura', with spathes measuring 20 to 30 cm, with each rachillae used as a source of explants measuring about 5 to 8 cm. After collection, the inflorescences, still protected by spathes, were subjected to a process of sterilization in 70% alcohol for 5 min and 1.0 to 1.25% sodium hypochlorite for 30 min, followed by three washes in sterile distilled water. After sterilization, the spathes were removed; the rachillae were separated from the inflorescences and transversely sectioned in 1.0 to 2.0-mm slices, resulting in thin cell layer tissues (TCL) from immature female inflorescences of oil palm, using tweezers and a scalpel. The modified culture media MS (Murashige and Skoog, 1962) and Y3 (Eewens, 1976) evaluated in this experiment were added with 30 g l^{-1} sucrose, 500 mg l^{-1} glutamine, 3.0 g l^{-1} activated charcoal, combined with 450 µM of 2,4-D and different concentrations of N^6-benzylaminopurine (BAP) (0.25 and 50 µM). The explants were inoculated in a laminar flow chamber and maintained in darkness. Evaluations were performed for up to 42 weeks, when the following responses were evaluated: percentage of explants with embryogenic responses, number of nodular formations per explant, and fresh mass of embryogenic calluses. The study design used was completely randomized, with five repetitions, with each plot consisting of six explants.

Influence of thin-cell-layer explant position and 2,4-D concentrations

In this experiment, the source of explants was the same as that used previously. This time however, the explants TCL were extracted from the apical region and basal rachillae, seeking to evaluate the influence of the position of the explant in the induction of embryogenic callus. The sections had the same characteristics as in the previous test and the culture medium used was MS plus 3.0 g l^{-1} of activated charcoal. 2,4-D was added at different concentrations (0, 225, 450 and 675 µM) also to evaluate its effect on the production of embryogenic callus. After up to 42 weeks, the induction and formation of embryogenic calluses in explants was observed. The experimental design used was completely randomized, with each treatment consisting of four repetitions and six explants per plot.

Effect of antioxidant substances and 2,4-D

TCL explants from rachillae were inoculated on MS culture medium supplemented with 450 µM of 2,4-D. After up to 42 weeks, the quantity of embryogenic callus formed was calculated and the number of dead explants expressed as percentages. Treatments consisted of a 2 × 3 factorial scheme, with two concentrations of PVP-40 (Polyvinylpyrrolidone) (0 and 1.0 g l^{-1}) and three concentrations of activated charcoal (0.0, 1.5 and 3.0 g l^{-1}). The experimental design was completely randomized, with five repetitions and five explants per plot.

In all the experiments, the explants were inoculated in flasks with capacity of 250 ml, containing 40 ml of culture medium and maintained in darkness at 25 ± 2°C temperature. The pH of the culture medium was adjusted to 5.8 ± 0.1 before adding 2.5 g l^{-1} Phytagel (Merck®) and subsequently autoclaved at 121°C and 1.3 atm pressure for 15 min. The data collected were analyzed using the statistical analysis program Sanest (Zonta and Machado, 1984) and the averages were compared by the Tukey test at 5% probability. The data, expressed as percentages (x), were transformed according to arcsine $(x/100)^{0.5}$.

Histological analyses

Samples of embryogenic calluses obtained after up to 42 weeks of cultivation were isolated and fixed in 50% FAA (Formalin: acetic acid: 50% ethylic alcohol, at a ratio 5: 5: 90), for 24 h in a vacuum. The samples were dehydrated in a graded ethylic series and immersed in methacrylate (Historesin, Leica), prepared according to manufacturer's instructions. Transversal and longitudinal cross sections (7 µm thick) were obtained using a manual microtome. The cross sections obtained were stained with Toluidine blue; observations and photographic documentation were carried out using an Olympus light photo-microscope (Motic BA300).

RESULTS AND DISCUSSION

Influence of types of culture medium and growth regulators

In general, when assessing types of culture medium and

Table 1. Influence of culture media and different concentration of BAP in combination with 2,4-D on the induction of nodular embryogenic callus (EC), fresh callus mass and number of nodular formations (NF) in thin-cell-layers explants from immature female palm oil inflorescences.

Media	Explant with nodular EC (%)				Fresh mass per EC (g)			N° NF per callus		
	BAP (µM)				BAP (µM)			BAP (µM)		
	(0)	(25)	(50)	Average	(0)	(25)	(50)	(0)	(25)	(50)
MS	40.4	68.5	55.6	54.8[a]	0.79	0.81	1.1	29	45	72
Y3	22.7	6.1	11.6	13.5[b]	1.1	0.84	1.1	15	25	ND[†]
F (A: Media): 14.756**						ND			ND	
F (B: BAP): 0.011[ns]						ND			ND	
F (A x B): 1.588[ns]						ND			ND	

Mean values followed by the same lowercase letter within columns are not significantly different according to Tukey's test. ** P < 0.01; ns: not significant. †ND: not determinate. BAP, N^6-benzylaminopurine

BAP concentrations on embryogenic callus formation in immature female inflorescences of oil palm, there were significant effects for explants only for the culture medium factor. The better results were obtained in the formulation of the MS than the Y3 medium, regardless of the BAP concentrations tested, which did not exhibit any significant influence (Table 1). The use of responsive explants in physiological conditions to which allow for following a morphogenic program and an adequate culture medium seems to be essential for palm micropropagation (Blake, 1983). The most widely used culture medium is described in Murashige and Skoog (1962) (MS medium), because most plants react to it favorably. It is classified as a high salt medium in comparison to many other formulations, with high levels of nitrogen, potassium and some of the micronutrients, particularly boron and manganese (Cohen, 1995). However, Eeuwens (1976) reported that Y3 medium was better than MS medium for calluses initiation in coconut due to mineral deficiencies in macro elements such as nitrogen (ammonium), potassium and in micro elements as iron and molybdenum. In the literature, only the study conducted by Teixeira et al. (1994), which used inflorescences as a source of explants, tests the efficiency of the MS and Y3 media in one of the steps in the induction protocol of somatic embryogenesis in oil palm. In their experiment, they also observed that the MS medium supplemented with activated charcoal and 2,4-D provided the best results for calluses induction.

For fresh mass and number of embryogenic nodular formations formed for each explant, there was an improvement trend in the responses for these variables, with an increase in BAP concentrations, especially in the MS medium. No study was found in the literature on the influence of BAP in the induction of somatic embryogenesis in immature inflorescences of oil palm. However, the induction of embryogenic callus has been extensively reported in the literature by a combination of treatments with auxins and cytokinins (Nath and Buragohain, 2005). Jain et al. (2005) used hybrid inflorescences from *Cynodon* sp. to induce embryogenic callus with morphogenic competence in MS medium, adding 4.0 mg l^{-1} of 2,4-D and 0.01 to 1.5 mg l^{-1} of BAP and obtaining a clear increase in fresh mass of callus with the increase of BAP concentration in the medium.

Influence of explant position and 2,4-D concentrations

On assessing the effect of the position of explant in combination with different concentrations of 2,4-D in MS culture medium, it was found that on average, the best results for percentage of embryogenic explants were obtained in a culture medium with 225 µM of 2,4-D regardless of the position of explant in the rachillae (Table 2). On the other hand, concentrations over 225 µM negatively influenced the induction of explants with embryogenic characteristics. Teixeira et al. (1994) found that after five months of culture, the induction of somatic embryos in immature oil palm inflorescences of the *pisifera* variety occurred at a concentration of 500 µM of 2,4-D. However, as reported by Steinmacher et al. (2007), comparatively to the Picloram and Dicamba the 2,4-D did not induce a notable embryogenic response in TCL explants from inflorescences of peach palm (*Bactris gasipaes*), but the use of a pre-treatment with 2,4-D (200 µM) in liquid MS culture medium increased the embryogenic capacity and diminished the development of flower buds.

These results are in agreement with those of Vasil (1987), who cites that in tissue culture from monocotyledons, it is essential that the source of explant to be used is composed of meristematic cells and the explant that best satisfies this premise seems to be the inflorescences, due to the presence of numerous meristematic portions in their tissues. Verdeil et al. (1994) used four immature inflorescences (younger If1, If2) and (older If3,

Table 2. Influence of the position of the TLC explant on the rachillae (basal and apical) and different concentrations of 2,4-D in culture medium on the formation of nodular embryogenic callus in thin-cell-layer explants from immature female oil palm inflorescences.

Position of the TLC explant on the rachillae	Explant with nodular embryogenic callus (%)			
	2,4-D (µM)			
	0	225	450	675
Apical	1.1	43.8	35.0	0.0
Basal	0.0	54.3	4.1	0.0
Average	0.5^{C}	49.0^{A}	19.5^{B}	0.0^{C}
F (A: Explant position): 1.708^{ns}				
F (B: 2,4-D concentrations): 19.341**				
F (A x B): 0.142^{ns}				

Mean values followed by the same uppercase letter within line are not significantly different according to Tukey's test. ** P < 0.01; ns: not significant. 2, 4-D, 2, 4-dichlorophenoxyacetic acid.

Table 3. Influence of activated charcoal and PVP-40 in the embryogenic callus formation in thin-cell-layer explants from immature female oil palm inflorescences.

Activated charcoal ($g.l^{-1}$)	Explant with nodular embryogenic callus (%)			Dead explant (%)		
	With PVP	Without PVP	Average	With PVP	Without PVP	Average
0.0	0.0	0.0	0.0^{b}	100.0	100.0	100.0^{b}
1.5	0.0	0.0	0.0^{b}	100.0	96.2	98.1^{b}
3.0	56.5	30.9	43.7^{a}	0.0	0.0	0.0^{a}
F (A: With and without PVP): 1.043^{ns}				1.000^{ns}		
F (B: Activated charcoal): 31.565**				240.999**		
F (A x B): 1.043^{ns}				1.000^{ns}		

Mean values followed by the same lowercase letter within columns is not significantly different according to Tukey's test. ** P < 0.01; ns: not significant. PVP, Polyvinylpyrrolidone

lf4) from each of the elite coconut hybrids tested (PB 111, PB 121 and PB 131) and concluded that 2 to 2.5 mg l^{-1} activated charcoal and 99.55 to 271.5 µM 2,4-D were necessary to induce calluses, due to the variable sensitivity among the genotypes.

Effect of antioxidant substances and 2,4-D

The evaluation of the influence of activated charcoal and PVP-40 on the embryogenic potential of immature female inflorescences of oil palm revealed that on average, 43.7% of explants showed reversion of the floral state to the embryogenic vegetative state after up to 42 weeks of culture (Table 3). This result was only observed in MS culture medium supplemented with 3.0 g l^{-1} activated charcoal, independent of whether the medium was supplemented with PVP-40. Activated charcoal is a component which, while often being added to culture mediums to adsorb toxic phenolic compounds into the explants, can promote the adsorption of other components of the culture medium, such as plant growth regulators, influencing the pattern of development of the crops (Van Winkle et al., 2003). Thus, it is possible that the responses of the explants at the higher concentration of activated charcoal were due to the more pronounced adsorption of 2,4-D by this antioxidant, reducing or eliminating its phytotoxic effects, as seen in the other treatments, where the rate of conversion of inflorescences to the embryogenic vegetative state was zero, with 100% death of the explants. The adsorption of 2,4-D in the culture medium by activated charcoal was also reported in the studies of Ebert and Taylor (1990) and Verdeil and Buffard-Morel (1995), who affirm that the concentration of 2,4-D available to the explants in cultivation is unclear when activated charcoal is present in the culture medium. Kanchanapoom and Domyoas (1999) noted the differentiation of oil palm embryoids when they were transferred to the MS medium free of growth regulators and with 0.5 g l^{-1} of activated charcoal for 5 weeks. Guerra and Handro (1998) concluded that the establishment of viable cultures and their gradual

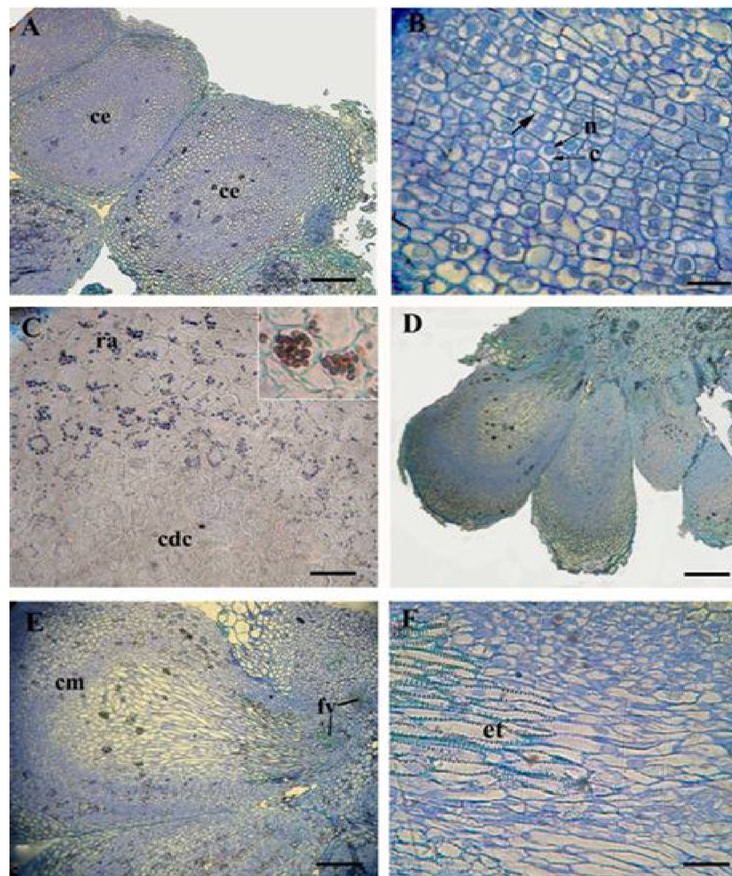

Figure 1. Transversal and longitudinal cross sections of embryogenic calluses originating from thin-cell-layer explants of immature female oil palm inflorescences grown on MS culture medium with 450 µM 2,4-D, after up to 42 weeks of cultivation: A, Formation of nodular embryogenic callus (ce); B, region with meristematic cells, characterized by a large nucleus (n) and dense cytoplasm (c) and demonstrating intense cell division (arrow); C, detail of the region of cells with an accumulation of starch grains (ra), close to centers with intensive cell division (cdc); D, root primordia; E, detail of root primordia originating from the vascular bundle (fv) containing meristematic cells (cm); F, region of the root primordia with differentiated tracheary elements (et). Bars: A and D - 20 µm; B - 100 µm; C - 50 µm; E and F - 20 µm.

development arising from leaf explants, zygotic embryos and inflorescences of *Euterpe* takes place only in solid medium containing activated charcoal.

In the case of PVP, also regarded as an antioxidant, the addition of this substance to the medium did not promote any difference in the responses of immature floral explants of oil palm, although, Pasqual et al. (2002) verified the beneficial effects of PVP when added to the culture medium on calluses formation in the coffee plant (*Coffea arabica* L.). Similar results to those obtained in this work were observed by Sáenz et al. (2005) in the coconut palm who, testing a protocol for callus formation from plumule explants in the presence of 2,4-D and PVP, found that approximately 43% of the explants cultured with 1 µM of 2,4-D formed embryogenic calluses after four months, regardless of the presence of PVP in the culture medium.

Histological analyses of embryogenic calluses

Histological analysis showed embryogenic calluses originating from female inflorescences after up to 42 weeks of cultivation. These calluses, in general, originate from tissues near the vascular bundle, probably from the procambial cells or adjacent cells. In the cross sections, it was observed that the calluses developed individualized nodular structures (Figure 1a) with small cells, juxtaposed, with a large nucleus, containing two to three prominent nucleoli, dense cytoplasm, arranged concentri-

cally and in intense cell division (Figure 1b). However, at this stage, these nodular calluses still have no procambium or characteristic protoderm. Nodular calluses induced from perivascular cells were also observed by Schwendiman et al. (1988) in leaf explants of *E. guineensis.*

In the outer region of the nodular callus there were large cells containing starch, visualized using toluidine blue staining (Figure 1c). The starch grains accumulated primarily in cells close to sites of intense cell division, especially those with less dense cytoplasm, whereas no accumulation of starch grains was observed in sites with intense cell division. Kanchanapoom and Domyoas (1999) also detected the accumulation of starch in calluses and in bipolar embryoids of *E. guineensis*, indicating that starch accompanies the formation of somatic embryos in this species. This is due to the fact that embryogenesis is a morphogenic process that requires a high amount of energy and the catabolism of starch produces intermediate glycolytic compounds that provide the ATP needed for cell metabolism (Martin et al., 2000). Additionally, the starch in embryogenic cells of calluses of *Gentiana punctata* L. was related to a source of energy for intense cell division and subsequent development of embryos (Mikula et al., 2004). According to Silveira et al. (2004), the level of this molecule may change depending on the phase of embryo growth, since cell division and differentiation require large amounts of carbon and ATP.

The embryogenic calluses also gave rise to structures similar to root primordia, characterized by the absence of protoderm, elongated shape, the presence of tracheary elements and the fact that they remain connected to the source explant (Figure 1d). The formation of root primordia during the regeneration process is probably due to the imbalance of auxin concentrations present in the culture medium. In explants of *Solanum melongena,* the same cell type of vascular origin led to the formation of somatic embryos and root primordia (Tarré et al., 2004). In the periphery of the root primordia, there were meristematic cells undergoing intense division (Figure 1e), while at the base and near the vascular bundle of origin, the presence of procambium and tracheary elements was observed, showing a degree of lignification with thickened walls in a spiral form (Figure 1f). According to Guerra and Handro (1998), unlike the process of organogenesis where the regenerated propagules are connected to the explant of origin, there is no connection in those that result from the process of somatic embryogenesis.The results achieved here suggest that TCL explants from immature female inflorescences of oil palm are a promising source of explants for induction of embryogenic calluses, the first step to obtaining somatic embryogenesis. Callus initiation require high concentrations of auxin 2,4-D (450 µM) and the composition formed by salts and vitamins of MS medium added with 3.0 g l activated charcoal provide the best results. The activated charcoal is the most indicated antioxidant for preventing the oxidation of floral oil palm explants and its presence can be considered essential for the formation of embryogenic calluses.

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