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Somatic embryogenesis from zygotic embryos and thin cell layers (TCLs) of Brazilian oil palm (*Elaeis guineensis × Elaeis oleifera*)

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Oil palm hybrid BRS Manicoré is important for plantations in the north of Brazil, as it is resistant to fatal yellowing and is compact. Seed germination is slow and reduced, so somatic embryogenesis is a promising alternative for its propagation. Two kinds of starting explants were used: Zygotic embryos (ZE) and thin cell layers (TCL) from the base of seedling aerial parts. Two culture medium formulae were compared (Y3 and modified MS), and several growth regulators (2,4-D, Picloram, BA, 2iP), putrescine and activated charcoal (AC) were used during the different stages. Somatic embryos (SE) were obtained from ZE-derived embryogenic masses cultured on Y3 medium with or without 2,4-D (9 µM) + 1000 µM putrescine and 40% were converted into seedlings in the Y3 medium supplemented with 2 g.L^{-1} AC and without growth regulators, under light. SE developed in 49 to 53% TCL-derived calli and 50% of them were converted into seedlings in the same conditions described for ZE calli. The yield of both processes was compared, showing the superiority of TCL explants for SE production.

Key words: Y3 culture medium, clonal propagation, putrescine, thin cell layer.

INTRODUCTION

Among palms cultivated for oil production, oil palm (*Elaeis guineensis*) is the most productive, with yields higher than 25 tons of bunches per ha per year (SUFRAMA, 2003). Two types of oils are extracted from the fruits: Oil palm removed from the pulp and oil palm kernel obtained from the endosperm. They are used in the manufacturing of cosmetics, the oleochemical industry, food and animal feed, and in the preparation of commercial foods (SUFRAMA, 2003). The oil palm industry suffers from pests and diseases and slow seed germination (Pádua et al., 2013). In order to solve some limitations, such as disease sensitivity, the interspecific hybrid BRS Manicoré was created in 2010 by the breeding program of EMBRAPA (Brazilian Agricultural Research Corporation), resulting from crossing *E. guineensis* (African) with *Elaeis oleifera* (Amazonian)

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Table 1. Media used in the first experiment of somatic embryogenesis of hybrid oil palm BRS Manicoré.

1st experiment	Salts and organic compounds	PGR*	Concentration (µM)	AC** (g.L-1)
Callus induction	mMS or	2,4-D	0 – 500	2
Medium	Y3	Picloram	0 – 500	2
	mMS or	2,4-D	100	2
		2-iP	7.9	2
	Y3	2,4-D	100	2
1st subculture		BA	7.9	2
		Picloram	100	2
		2-iP	7.9	2
		Picloram	100	2
		BA	7.9	2
2d subculture	Y3	2,4-D	0, 9 or 27	0.5
		Putrescine	1000	
Maturation and conversion medium	Y3	-	-	2

*PGR: Plant Growth Regulator; ** AC: activated charcoal; mMS: Murashige and Skoog medium (1962) with 500 mg.L-1 cysteine; Y3: Eeuwens medium (1976).

(Collares, 2011). It is resistant to fatal yellowing and the stipe has a reduced vertical growth (Conceição and Muller, 2000; Cunha and Lopes, 2010). The fruits of this cultivar have a high oil and unsaturated fatty acid content, which enables high quality biodiesel to be produced from its oil (Collares, 2011).

The conventional method of multiplication of palm tree hybrid is by seeds, but their germination rate is low (30%) (Angelo et al., 2007), due to the abortion of the embryo inside the seed (Alves et al., 2011). The process is also very slow and irregular, and various degrees of dormancy may exist. These aspects constitute a bottleneck for seedling production.

Somatic embryogenesis is a process whereby somatic cells differentiate into somatic embryos (SE) (George et al., 2008). It has innumerable applications for plant vegetative propagation and is a promising alternative for the production of oil palm saplings on a large scale.

In the case of *E. guineensis*, several types of explants were already used to initiate somatic embryogenesis process: Mature or immature zygotic embryos (Balzon et al., 2013; Silva et al., 2014), cell suspensions from immature leaf segments (De Touchet et al., 1991), leaf segments (Pádua et al., 2013) and immature male inflorescence (Jayanthi et al., 2015).

Within micropropagation techniques, thin cell layer (TCL) has the advantage of using explants of reduced size which have a greater contact with the medium than other kinds of explant (Tran Thanh Van, 1980). This technique has been used successfully for peach palm (*Bactris gasipaes*) (Steinmacher et al., 2007), *Acrocomia aculeate* (Padilha et al., 2015) and oil palm (*E.*

guineensis) (Scherwinski-Pereira et al., 2010). This study was developed to address the need for multiplication and production of hybrid oil palm BRS Manicoré, aimed at more rapid production on a scale that meets national demand. A somatic embryogenesis protocol was established from zygotic embryos and TCL using the shoots of *in vitro* grown seedlings.

MATERIALS AND METHODS

Somatic embryogenesis from mature zygotic embryos

Mature fruits were collected 150 days after pollination from mother plants obtained by controlled crossing of selected plants of the hybrid BRS Manicoré (*E. guineensis* x *E. oleifera*), provided by EMBRAPA - Western Amazon, Brazil. Several lots of seeds (every lot comes from one tree) were used, differing among the experiments. The endocarp was removed and almonds washed with mild soap and tap water and immersed in 70% ethanol for 5 min, then in a 10% (v/v) solution of sodium hypochlorite (NaClO) containing 1% Tween-20 for 20 min and rinsed four times with sterile distilled water. The zygotic embryos were isolated and disinfected with commercial NaClO at 2% (v/v) for 5 min, washed in sterile distilled water and inoculated on Petri dishes containing 20 mL of culture medium.

Two experiments were performed; comprising several stages until SEs were converted into seedlings. The composition of media used in the first experiment is indicated in Table 1. Seed lots were: CS428, 736, 1477 and 1681. In a second experiment, the first medium was Y3 without 2,4-D or supplemented with 250, 500 or 750 µM 2,4-D. Seed lots were: CS 47 and 1951. The calli were subcultured on the same media indicated in Table 1. In both experiments, sucrose (30 g.L⁻¹) and agar (Vetec®) (6 g.L⁻¹) were added to all media.

The growth of calli was observed every 30 days and the number

of embryogenic and non-embryogenic calli was recorded after 150 days. After 30 days of first subculture, calli with globular embryos and non-embryogenic calli were counted. Those containing globular and torpedo embryos were transferred to Y3 medium without growth regulators for maturation and conversion into seedlings. After one to three weeks the presence of root and leaf was recorded.

The cultures were maintained in the dark at 25 ± 2°C during the day and 21 ± 2°C by night. During the maturation and conversion stage, they were transferred under fluorescent light (white light) with an irradiance of 40 µmol. m⁻².s⁻¹ and a photoperiod of 16 h.

The experimental design was completely randomized with 10 repetitions (Petri dishes) and five embryos/dish. For the conversion of SE, 616 replications were used with one embryo per tube (15 cm high × 2,5 cm diameter). The variables evaluated were: percentage of formation and consistency of callus, formation of embryogenic callus and SEs. The data were subjected to ANOVA variance analysis, homogeneity by Bartlett´s test and comparison of means by Tukey´s test at 5% probability.

Somatic embryogenesis from leaf TCLs

After 30 to 50 days of *in vitro* culture, seedlings 2 to 6 cm in length were used as a source of explants. The root primordium and cotyledon petiole were removed, leaving the leaf primordia. The primordium was sectioned transversely from the apex to the base into segments approximately 1 mm thick (TCL) which were used as explants. Two experiments were conducted: The first one consisted of three treatments: Y3 medium supplemented or not with 2,4-D (500 or 800 µM) and the second one tested four concentrations of 2.4 -D (250, 500, 800 and 1000 µM) in Y3 medium and a control without 2,4-D. The media were supplemented with 2 g.L⁻¹ AC.

After 90 days, the calli obtained in all treatments of both experiments were sub-cultured in Y3 medium containing 100 µM putrescine and 0.5 g.L⁻¹ AC. Three treatments were compared (0, 50 and 100 µM 2,4-D). The SEs formed on these calli were transferred to Y3 medium supplemented with 2 g.L⁻¹ AC and without growth regulators for embryo maturation and conversion. All media contained 30 g.L⁻¹ sucrose and 2.5 g.L⁻¹ Gelzan. The pH of all culture media was adjusted to 5.8 with NaOH or 0.1 M HCl and the media were autoclaved at 120°C for 20 min. Activated charcoal was added along with Gelzan, after pH adjustment.

The cultures were kept in the dark at 25 ± 2°C during the day and 21 ± 2°C by night for four weeks and then transferred under fluorescent light (white light) with an irradiance of 40 µmol. m⁻².s⁻¹ and a photoperiod of 16 h.

The experimental design was completely randomized with 10 repetitions (Petri dishes) and six explants per dish. For the conversion of SEs, 250 replications were used (tubes) with one embryo per tube. The variables evaluated were: Consistency of callus (friable or compact), rate of callus formation, callus type and formation of SEs. The data were submitted to the same statistical analysis as for experiments using mature zygotic embryos.

RESULTS

Callus and somatic embryos formation from zygotic embryos

After inoculation of fifteen-day-old zygotic embryos on mMS and Y3 culture media, callus formation was observed in embryos cultured in media containing 2,4-D or Picloram (Figure 1A and B). After 30 days of culture, 76% of the explants formed calli on Y3 medium

supplemented with 2,4-D (Table 2), whereas in the media without 2,4-D or Picloram, ZE germinated without callus development (data not shown). After 90 days the formation of calli was observed on the explants that did not previously respond. Friable calli developed in all media and embryogenic calli appeared (Table 2 and

Figure 1C, D, E and F). The mMS medium with 2,4-D and Y3 with Picloram were the most suitable for the formation of friable calli observed in 62.11 and 59.29% of the explants respectively, after 90 days, differing from the other means (Table 2). Considering these results, we chose Y3 as basal medium for further experiments.

When 2,4-D was added to Y3 culture medium at concentrations of 250 and 500 µM, there was a higher percentage of explants forming calli (49 and 62% respectively) than at 750 µM (31%) after 30 days of culture (Table 3). After 90 days, friable calli developed on the media containing 250 and 500 µM (58.7 and 72.1%, respectively) (Table 3).

Calli cultivated for 150 days on mMS or Y3 medium containing 2,4-D or Picloram and transferred to Y3 medium containing only auxin or auxin and cytokinin (BA or 2-iP) showed embryogenic calli (Figure 1G, H and I) irrespective of the type of auxin. The addition of 2iP to 2,4-D or Picloram promoted the formation of embryogenic calli by 34 and 143% respectively, and the combination of BA and 2,4-D or Picloram by 40 and 210% (Table 4).

SEs or pre-globular SE were formed on calli grown for two months on multiplication medium (Y3 with Picloram, 2,4-D + BA or 2iP) (Table 4) and transferred into Y3 medium with or without 2,4-D (9 or 27 µM), and with 1000 µM putrescine (Figure 1G and H). SE formation occurred in all these culture media, regardless of the presence and concentration of auxin (Figure 1G, H and I), but was higher in the media without 2,4-D or with 9 µM (37 and 43% respectively) (Table 5). Each callus formed an average of 25 to 30 embryos after 60 days.

The SEs in the torpedo stage (Figure 1J), transferred to the conversion medium (Y3 without regulators) under light conditions appeared white after one to two weeks (Figure 1J). After two weeks they turned green and the cotyledon petiole started to elongate, as well as the radicle and plumule (Figure 1 K). Some of the embryos were considered abnormal, as they were green but did not emit a root or cotyledon and others had only a root. 40% of SEs were converted into plants,

The process of somatic embryogenesis from zygotic embryos (ZE) was divided into four stages until the conversion of somatic embryos into plants (Table 6).

Callus and somatic embryos formation from TCLs

After 30 days shoot TCLs inoculated in Y3 culture medium supplemented with 500 µM 2,4-D exhibited formation of friable calli around the explant (Figure 2A), reaching 39.45% after 60 days (Table 7). The explants cultivated in the medium containing 800 µM 2,4-D showed slow

Figure 1. Stages of somatic embryogenesis from zygotic embryos of the hybrid oil palm BRS Manicoré (*Elaeis guineensis* × *E. oleifera*) cultured on Y3 medium. (A) Callus on medium containing 500 µM 2,4-D after one month. (B) with 500 µM Picloram after one month. (C) Friable callus on medium with 2,4-D (100 µM) and BA (7.9 µM). (D) Friable callus on medium with 2,4-D (100 µM) + 2iP (7.9 µM). (E) Embryogenic callus in medium + 2,4-D (100 µM) + BA (7.9 µM). (F) with 2,4-D (100 µM) + 2iP (7.9 µM). (G) Embryogenic callus with embryos in torpedo and globular stages in medium with 9 µM 2,4-D + 1000 µM putrescine after 7 months. (H) Somatic embryos in globular and scutellar stages in medium with 1000 µM putrescine. (I) Somatic embryos in growth regulator-free medium after 8 months. (J) In torpedo stage in growth regulator-free medium after 9 months. K) Converted embryo after two weeks in growth regulator-free medium under light. (L) After three weeks. Bars: A, C, F, H, I, J and K = 2 cm. B = 1 cm. D, E, G and L = 3 cm. Source: The author (2014).

development and a transparent gelatinous mass (Figure 2B and C) and only 17.7% of them formed calli (Table 7). After 60 days of culture, the calli were friable (Figure 2D). After 90 days the culture medium supplemented with 500 µM 2,4-D gave the highest percentage of yellow (embryogenic) and translucent (non-embryogenic) calli (47.7 and 32.7%) differing from medium supplemented with 800 µM 2,4-D (12.2 and 14.7% respectively) (Table 7).

The concentration of 2,4-D affected the development of the callus as 30% of the TCLs cultured in the presence of 250 and 500 µM 2,4-D formed calli while no callus was formed or was formed in low percentages at concentrations above 500 µM (2 and 4% respectively for 800 and 1000 µM) (data not shown).

After transfer of calli to Y3 culture media with or without 2,4-D (50 or 100 µM) and putrescine, embryogenic calli containing somatic pro-embryos developed independently of the presence of 2,4-D (49 and 53% respectively) (data not shown) (Figure 2E and F) with 5 to 20 embryos at

Table 2. Formation of callus from zygotic embryos of the hybrid oil palm BRS Manicoré after 30 days in two culture media supplemented with 500 µM auxin and consistency of calli after 90 days.

Culture media	30 days		90 days	
	Formation of callus (% of explants)	Non-responding explants (%)	Friable calli (%)	Compact calli (%)
MS+2,4-D	52.83[a]	47.17[a]	62.11[a]	15.80[b]
MS+Picloram	65.65[a]	34.35[a]	43.00[b]	39.00[a]
Y3+2,4-D	76.32[a]	23.68[a]	44.59[b]	36.40[a]
Y3+Picloram	60.90[a]	39.10[a]	59.29[a]	36.00[a]
LSD	32.6	38.62	7.66	6.82

Means followed by the same letter are not statistically different by Tukey´s test at 5% probability. Callus formation after 90 days: data not shown. LSD = Least significant difference.

Table 3. Formation of callus from zygotic embryos of hybrid oil palm BRS Manicoré in Y3 culture medium supplemented with three concentrations of 2,4-D after 30 days.

Concentration of 2,4-D (µM)	30 days	90 days	
	Formation of callus (% of explants)	Friable callus (%)	Compact callus (%)
Control	0.25[b]	----	----
250	49.10[a]	58.7[a]	41.4[b]
500	62.27[a]	72.1[a]	27.8[b]
750	31.30[ab]	23.7[b]	76.2[a]
LSD	47.22	34.23	33.89

Values are mean values of four seed lots. Means followed by the same letter are not statistically different by Tukey's test at 5% probability. LSD = Least significant difference.

Table 4. Multiplication of callus formed from zygotic embryos of oil palm hybrid BRS Manicoré after 60 days in Y3 culture medium supplemented with 100 µM auxin and 7.9 µM cytokinin.

Plant growth regulators	Embryogenic callus		Non-embryogenic callus	
	(n/N)*	(%)	(n/N)*	(%)
2,4–D	12/31	38.75[b]	19/31	59.87[bc]
PICLORAM	5/31	16.12[c]	26/31	83.875[a]
2,4–D + 2iP	15/29	51.65[a]	14/29	48.30[d]
PICLORAM + 2iP	25/64	39.06[b]	39/64	60.93[b]
2,4–D + BA	20/37	54.06[a]	17/37	45.88[d]
PICLORAM+ BA	28/56	50.00[a]	28/56	50.00[cd]
LSD		9.89		10.44
CV%		5.99		4.55

Means followed by the same letter are not statistically different by Tukey's test at 5% probability. Data not shown for calli without development. Values are averages of two replications. *n = Number of calli, embryogenic or not; N = total number of calli; CV = coefficient of variation; and LSD = least significant difference.

globular and torpedo stage per callus (Figure 2G). Somatic embryos at the torpedo stage were separately converted into plantlets (Figure 2H, I and J).

Approximately 230 SEs were converted into plants on conversion medium (growth regulator-free medium) under light. After two weeks they started to turn green and developed cotyledonary petioles and primary roots (Figure 2I and J). After 45 days, 50 plants out of a total of 230 (21.7%) had root and shoot parts.

The process of somatic embryogenesis from TCLs was divided into three steps until the conversion of SEs into plants (Table 6).

Table 5. Somatic embryo formation from zygotic embryo-derived calli of palm tree hybrid BRS Manicoré after 60 days in Y3 culture medium supplemented with 2,4-D and 1000 µM putrescine.

2,4-D (µM)	Callus with embryos*		Embryogenic callus***		Friable callus	
	(n/N)**	(%)	(n/N)*	(%)	(n/N)*	(%)
0	34/91	37.36[a]	53/91	58.24[a]	4/91	4.39[a]
9	53/121	43.80[a]	56/121	46.28[a]	14/121	11.57[a]
27	19/101	18.81[b]	58/101	57.48[a]	24/101	23.76[a]
LSD		79.74		69.59		39.33
CV%		119.76		65.56		149.71

Means followed by the same letter are not statistically different by Tukey´s test at 5% probability. Values are averages of two replications *In scutellar and torpedo stages. **n = Number of calli with embryos; N = total number of calli; *** with globular embryos; CV = Coefficient of variation; and LSD = least significant difference.

Table 6. Stages of somatic embryogenesis and media composition for palm tree hybrid BRS Manicoré. Basal medium is Y3 (Eeuwens, 1976).

Time (months)	From zygotic embryos	Time (months)	From leaf TCLs
1st - 3d	**Callus induction** 250 µM 2,4-D, 2 g.L⁻¹ AC, 30 g.L⁻¹ sucrose, 6 g.L⁻¹ agar	1st - 3d	**Callus induction** 250 µM 2,4-D, 2 g.L⁻¹ AC, 30 g.L⁻¹ sucrose, 2.5 g.L⁻¹ Gelzan
4th - 5th	**Callus multiplication** 100 µM 2,4-D or Picloram, 7,9 µM BA, 2 g.L⁻¹ AC, 30 g.L⁻¹ sucrose, 6 g.L⁻¹ agar	4th - 6th	**Embryogenic callus and SE formation** 100 µM put, 0,5 g.L⁻¹ AC, 30 g.L⁻¹ sucrose, 2.5 g.L⁻¹ Gelzan
6th - 8th	**Conservation of embryogenic calli and SE** formation 1000 µM put*, 0.5 g.L⁻¹ AC, 30 g.L⁻¹ sucrose, 6 g.L⁻¹ agar	7th - 8th	**Conservation, SE maturation and conversion** 2 g.L⁻¹ AC, 30 g.L⁻¹ sucrose, 2.5 g.L⁻¹ Gelzan
9th - 10th	**Maturation and SE conversion** 2 gL⁻¹ AC, 30 g.L⁻¹ sucrose, 6 g.L⁻¹ agar.		

*Putrescine, **somatic embryo.

Table 7. Formation of callus from shoot TCLs of hybrid oil palm BRS Manicoré cultivated in Y3 culture medium supplemented with 2,4-D after 60 and 90 days.

	60 days		90 days		
2,4-D Concentration (µM)	Callus Formation (%)	Non-responding explants (%)	Yellow Callus (%)	Translucent Callus (%)	Non-responding explants (%)
500	39.45 a	59.65 a	47.73 a	32.76 a	19.50 b
800	17.70 a	82.30 a	12.20 b	14.73 a	73.06 a
LSD	31.38	47.6	32.73	54.57	59.08
CV%	63.49	30.6	31.38	99.0	56.26

Means followed by the same letter are not statistically different by Tukey´s test at 5% probability. Average of four repetitions. CV = coefficient of variation. LSD = Least significant difference.

DISCUSSION

Both auxins 2,4-D and Picloram were efficient in inducing somatic embryogenesis from zygotic embryos of hybrid oil palm (BRS Manicoré). By culturing zygotic embryos of Tenera oil palm in N6 medium (Chu et al., 1975) supplemented with 9 µM 2,4-D, Dicamba or Picloram, Thuzar et al. (2011) were able to obtain 32, 17 and

Figure 2. Stages of somatic embryogenesis from shoot TCLs of hybrid oil palm BRS Manicoré cultured on Y3 medium. (A) With 500 µM 2,4-D after one month. (B) and (C) With 800 uM 2,4-D after one month. (D) Embryogenic callus on medium with 100 uM 2,4-D + 100 uM putrescine after 5 months. (E) On medium with 50 uM 2,4-D + 100 uM putrescine after 5 months. (F) Globular embryos on growth regulator-free medium after 8 months. (G) Somatic embryo in growth regulator-free medium after 8 months and 10 days. (H) Somatic embryo individualized after 9 months. (I) Embryo converted into seedling after 14 days in growth regulator-free Y3 medium. (J) Seedling after 21 days. Bars: A, B and C = 1 cm. D, E, F and G = 1.5 cm. H, I and J = 2 cm. Source: the author (2014).

21.5% of embryogenic calli, respectively. These were smaller values than those obtained in the Y3 medium supplemented with 2,4-D or Picloram in the present study (76.3 and 60.9%).

For this hybrid, concentrations of 2,4-D or Picloram between 250 and 500 µM were suitable for initiation of somatic embryogenesis when the auxin was added to mMS or Y3 culture medium. Muniran et al. (2008) cultivated zygotic embryos of *E. guineensis* "Dura" and observed that Y3, MS and N6 media were effective for callus formation when supplemented with 11 µM 2,4-D, obtaining respectively 98, 80 and 82% of explants forming calli. It should be noted that although our hybrid cultivar needs a particular concentration and type of auxin, other cultivars or genotypes may require other concentrations. The concentrations of 250 or 500 µM used in this study were effective but for varieties SJ-165

and SJ-167 of *E. guineensis* × *E. oleifera* concentrations of 375 and 625 µM 2,4-D were necessary for callus formation in zygotic embryos cultured in half-strength MS culture medium (Alves et al., 2011). Similar responses were obtained by Silva et al. (2012) for different genotypes of *E. guineensis* in MS culture medium containing 450 µM Picloram. Those authors showed that two of those genotypes had high potential for the formation of embryogenic calli and differentiation into SEs.

The combination of low concentrations of auxin (2,4-D or Picloram) with a cytokinin (BA or 2iP) is necessary for the multiplication of the calli. In this study, 100 µM 2,4-D or Picloram, combined with 7.9 µM BA or 2iP, led to callus proliferation in 50 to 54% of explants. Similar results were obtained for *E. guineensis* genotypes BRS C2328: On MS medium containing 0.6 µM ANA and 12.3

µM 2iP, 48% of the calli were embryogenic and 52.5% of them produced SEs (Silva et al., 2012). Likewise, in *Acrocomia aculeata*, Padilha et al. (2015) observed friable calli in 53% of TCLs cultivated in Y3 culture medium supplemented with 75 µM Picloram and 12.5 µM 2iP. These results show the importance of auxin/cytokinin balance for embryogenic callus multiplication.

The project presented here depicts the formation of SEs on media without 2,4-D or with a low concentration of 2,4-D (9 µM) and in the presence of putrescine (1 µM), with an average of 25 to 30 embryos per callus. The effect of polyamines on growth, differentiation and senescence of tissues cultured *in vitro* has already been described by Kaur Sawhney et al. (1985), indicating a causal connection between polyamine titer and cellular activity and differentiation. The positive effect of putrescine on the SE process has been observed by Rajesh et al. (2003) who obtained somatic embryogenesis in palm tree calli cultivated in modified MS medium containing 1 µM putrescine and 0.045 µM 2,4-D. This effect was also verified for other species, such as *Araucaria angustifolia* (Silveira et al., 2006), *Ocotea catharinensis* (Santa-Catarina et al., 2007), *Citrus sinensis* (Wu et al., 2009) and *Saccharum officinarum* (Reis et al., 2015). Moreover, the latter authors showed that addition of putrescine to the culture medium induced proteomic changes in six classes of proteins previously associated with somatic embryogenesis process. In tissue culture of several species, addition of polyamines or their biosynthesis inhibitors to culture medium demonstrated their role in somatic embryogenesis regulation and the importance of the putrescine/spermidine ratio (Kakkar et al., 2000). However, their requirement for tissue culture may be not universal and varies according to the stage of embryo development (Kakkar et al., 2000; Bais and Ravishankar, 2002). In our case, putrescine applied alone positively affected somatic embryogenesis but spermidine was not tested. The internal level of these polyamines in the calli should be measured in order to understand the way they influence this process.

The formation of SEs in the absence of 2,4-D is very important as it avoids problems of somaclonal variation. This disturbance was defined as "a phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones, namely plants derived from any form of cell culture involving the use of somatic plant cells" (Schaeffer, 1990). In Tenera oil palm, SEs were also obtained when the embryogenic calli were transferred to N6 maturation medium supplemented with 0.45 µM 2,4-D, 1.8 µM putrescine, 0.5 g.L^{-1} hydrolyzed casein and 2 g.L^{-1} AC (Thuzar et al., 2011).

For the conversion of somatic embryos into plants, plant growth regulator-free medium was efficient (Figure 1I and 2F-G). The plants produced shoots and roots and these were more developed than the roots of plants from *in vitro* germination of zygotic embryos. This may be because these somatic embryos are in contact with auxin from the beginning of callus induction and the roots develop after having absorbed a sufficient amount of auxin from the media of previous stages. Teixeira et al. (1994) also observed the regeneration and conversion of SEs in palm plants on MS medium devoid of growth regulators.

TCL technique using the base of the aerial part of seedlings grown *in vitro* is suitable for callus induction and obtaining SEs of the hybrid oil palm BRS Manicoré. This technique stood out from the works of Tran Thanh Van (1973) which considered that "the cells in TCL systems are literally reprogrammed in order to express all patterns of differentiation" (Tran Thanh Van, 2003). This system is considered efficient for the regeneration of embryos in different species, including palm trees (Samosir et al., 1998; Nhut et al., 2003; Steinmacher et al., 2007). The use of small explants is also advantageous as they have greater contact with the culture medium and can absorb the nutrients of the medium more easily (Fehér et al., 2003).

When cultivated in a medium containing 2,4-D (250 and 500 µM) TCLs formed calli in 33.1 and 31.8% of the explants but none when cultured in a 2,4-D-free medium. Similar results were obtained by Pádua et al. (2013) who observed callus induction in leaf explants of *E. guineensis* var. Tenera, grown in Y3 culture medium with Picloram or 2,4-D (9 µM) and no callus formation in auxin-free media. The pioneer work of Skoog and Miller (1965) showed that a proper balance between auxin and cytokinin in the medium stimulates callus formation in carrot tissues. However, internal balance of our explant is not known. In many cases, a strong auxin applied exogenously induces callogenesis without a need for cytokinin addition.

For the formation of embryogenic calli and SEs, calli need less exposure to auxin if it is combined with a cytokinin or putrescine. In this study, SEs were obtained in the culture medium with 2,4-D as well as in the medium without 2,4-D, both containing 100 µM putrescine. SE were also obtained in *Euterpe edulis*, using a combination of auxin and cytokinin (0.54 µM NAA and 9.8 µM 2-iP) (Guerra and Handro, 1998).

In this research, the process of somatic embryogenesis from TCL of hybrid oil palm BRS Manicoré was more effective than from ZE due to the fact that one plant provides five to eight explants to be used in the formation of callus. The cost of the technique is also approximately 20% lower because the process is reduced to three steps until SEs are converted into plants and the plant growth regulators (auxin, cytokinin and putrescine) are used in two phases only instead of three (Table 6). This means a smaller number of transfers to a new medium and lower labor costs. The calli derived from ZE provided one to five more embryos than those from TCL, since they remained two months in a culture medium containing cytokinin and auxin, whereas the TCLs were transferred directly to a

medium containing putrescine. However, TCL technique is still better, since an average of 30 to 50 SEs are formed from a single plant (five explants) whereas 3 to 4 ZE would be required to obtain the same number of ES.

In conclusion, protocols for somatic embryogenesis of hybrid *E. guineensis x E. oleifera* were described for the first time with two kinds of starting explants. In summary, for callus induction from ZE or TCLs, the use of 2,4-D at 250 µM is necessary. The combination of 100 µM auxin with a cytokinin is efficient for multiplication when calli are derived from ZE or with putrescine in TCL-formed calli. Embryogenic calli and SEs are formed in media without 2,4-D or with putrescine (100 or 1000 µM). SEs are converted into complete plantlets in Y3 culture medium devoid of plant growth regulators. Different auxin types, such as naphthaleneacetic acid, 2,4-D and Picloram, as well as concentrations should be adapted for each species or genotype/cultivar.

Conflicts of interest

The authors have not declared any conflict of interest.

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