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ORIGINAL PAPER

An improved protocol for somatic embryogenesis and plant regeneration in macaw palm (*Acrocomia aculeata*) from mature zygotic embryos

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Abstract An improved protocol for plant regeneration via somatic embryogenesis was developed using mature macaw palm (*Acrocomia aculeata*) zygotic embryos as initial explant. For induction of the embryogenic callus (EC), two basic media (BM) were tested consisting of Murashige and Skoog and Eeuwens (Y3) salts with 30 g L^{-1} sucrose, 0.5 g L^{-1} glutamine and 2.5 g L^{-1} Phytagel. The 3,6-dichloro-2-methoxybenzoic acid (dicamba), 4-amino-3,5,6-trichloro-picolinic acid (picloram) and 2,4-dichlorophenoxyacetic acid (2,4-D) auxins were added to the culture media at concentrations of 0, 1.5 or 3.0 mg L^{-1}. After 240 days, the embryogenic calli were transferred to the respective BM media with auxin concentrations reduced to 0.5 or 1.0 mg L^{-1} in order to differentiate the somatic embryos (SEs). Plant regeneration was performed on the BM media without growth regulators. Embryogenic calli were observed after 180 days of culture and in all treatments with auxin. The Y3 medium showed the best EC formation results (60.8 %). These calli showed yellowish coloration, compact consistency and nodular aspect. After 60 days in differentiation medium, SEs were verified in different stages of development. Histological analysis showed that the SEs were formed from a nodular EC. The SEs generally presented unicellular origin with suspensor formation, and at the end of development, bipolar embryos were observed. The plant regeneration frequency reached levels up to 31.9 % when using induction medium consisting of Y3 associated to 1.5 mg L^{-1} of 2,4-D and the subsequent auxin reduction to 0.5 mg L^{-1} in the differentiation stage. Regenerated plants showed normal development, with root and aerial part growth.

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Keywords Arecaceae · Morphogenesis · Zygotic embryos · Auxin · Somatic embryos · Plant regeneration

Abbreviations

BM	Basic media
Dicamba	3,6-Dichloro-2-methoxybenzoic acid
EC	Embryogenic callus
MS	Murashige and Skoog (1962)
Picloram	4-Amino-3,5,6-trichloro-picolinic acid
SE(s)	Somatic embryo(s)
Y3	Eeuwens (1976)
ZE	Zygotic embryos
2,4-D	2,4-Dichlorophenoxyacetic acid

Introduction

Macaw palm [*Acrocomia aculeata* (Jacq.) Lodd. Martius ex] is a palm tree belonging to the family Arecaceae that can reach over 15 feet tall (Tassaro 1996). This species is native to the savannas and open woodlands of Tropical and Subtropical America, occurring in many anthropic areas (Clement et al. 2005). In Brazil, it is considered one of the most dispersed palms, with occurrence of natural populations in practically the whole territory (Lorenzi 2006).

This palm may have oil production up to 5,000 kg per ha/year (Tickel 2000), a figure that resembles the best oil palm genotypes (*Elaeis guineensis* Jacq.) currently planted (Vargas 2007). These factors characterize it as one of the most productive vegetable oil producing species in the world, with the advantage that it can be exploited for a period of over 50 years

(Teixeira 2005). Besides the oil production potential, this species stands out for having a wide geographic distribution and can be grown under varied edaphoclimatic conditions (Motta et al. 2002) that would be unviable for oleaginous palms such as the African oil palm, and coconut palm (Arkcoll 1990).

However, the propagation of the macaw palm occurs exclusively by seed, and this technique has disadvantages such as slow germination, which can take from 1 to 2 years (Lorenzi 2006). Furthermore, this species does not form tillers, preventing its vegetative multiplication by conventional methods (Moura et al. 2009).

In this context, somatic embryogenesis has emerged as an excellent alternative for mass clonal propagation or to accelerate genetic improvement programs through hybrid cloning of this species (Moura et al. 2009). In this technique, differentiated or undifferentiated somatic cells become competent and determined to follow morphogenetic routes and develop somatic embryos (SEs) (Gueye et al. 2009) without the occurrence of gamete fusion. The competence is in most cases induced by a hormonal treatment, generally auxins in the presence or not of a cytokinin. However, the embryogenic process can be influenced by a substantial number of other factors such as the plant genotype, culture medium, size and type of explant, degree of tissue differentiation, besides nutritional and physiological state of the explant (Fehér et al. 2003). As a general rule, younger tissues, such as zygotic embryos (ZE), are convenient because its use facilitates the detailed study of various factors involved in culture establishment and somatic embryogenesis induction (Teixeira et al. 1993; Abdullah et al. 2005; Scherwinski-Pereira et al. 2012). In addition, in a genetic improvement program, cloning from ZE can accelerate culture selection cycles. This is especially true *in backcross generations*, where each embryo represents a unique genotype, for permitting the replication of experiments in different locations or conditions, reinforcing the representation of the environment throughout the multiple steps of the selection process (Angelo et al. 2009; Balzon et al. 2013).

To date, only one study has described macaw palm somatic embryogenesis from ZE (Moura et al. 2009). However, the results obtained demonstrate deficiencies in the maturation and development phases of the SEs, with rare cases of regeneration and high frequency of abnormal plants. Thus, in this paper we propose an improved protocol for somatic embryogenesis and plant regeneration from ZE of the macaw palm.

Materials and methods

Plant material

Zygotic embryos obtained from macaw palm (*A. aculeata*) seeds were used as the explant source. The seeds were extracted from the fruits at their physiological mature stage, characterized by a yellowish mesocarp with soft consistency. The fruits were collected from natural populations located near the municipality of Sobradinho, a town of Distrito Federal located in the vicinity of Brasília, Brazil (15°35′24.8″S; 47°53′46.7″WO).

After collection, the fruits were manually processed to remove the epicarp and stored for 15 days in a forced-air oven at 35 °C for drying. Then the core material was broken up with the aid of a manual lathe for removal of the seeds.

The disinfection of the seeds was conducted in a laminar flow hood by immersion in 70 % ethanol (v/v) for 3 min, followed by immersion in sodium hypochlorite solution (NaOCl) (2.5 % active chlorine) for 30 min. Three successive rinses were carried out in sterile distilled water. The seeds were then sectioned and the embryos extracted with the aid of a scalpel, dental pliers and fine point tweezers.

For the macaw palm somatic embryogenesis studies, the experiments were divided into four phases: callus induction; somatic embryo differentiation; synchronization and maturation of SEs and plant regeneration. The components of the culture media used in the different stages of macaw palm somatic embryogenesis can be seen in Table 1.

Callus induction

For the induction phase, ZE were inoculated in two culture media, known as basal media (BM). The first medium was composed of MS (Murashige and Skoog 1962) salts and the second medium by Y3 (Eeuwens 1976) salts. The source of iron and vitamins were maintained according to the original concentration of MS. The medium was supplemented with 30 g L^{-1} sucrose and 0.5 g L^{-1} glutamine and the auxins dicamba, picloram and 2,4-D were added at concentrations of 0.0, 1.5 or 3.0 mg L^{-1} (Table 1).

All media used in this study were solidified with 2.5 g L^{-1} Phytagel (Sigma, St. Louis, MO) and the pH was adjusted to 5.8 ± 0.1 before Phytagel was added. The media were autoclaved at 121 °C for 20 min and at 1.5 atm of pressure.

During the induction phase, the explants were cultivated in Petri dishes (15 × 90 mm) and stored in a dark growth room at 25 ± 2 °C for 240 days. The subcultures were performed monthly and after 90 and 180 days the percentage of explants forming primary calli and embryogenic calli was determined, respectively. The determination of the calli percentage was achieved by through the quantity of explants containing a callus divided by the total number of explants per plate and multiplied by 100.

Differentiation of somatic embryos

The calli obtained from induction phase were sub-cultivated to somatic embryo differentiation medium. The

Table 1 Composition of culture media and periods (days) used in the macaw palm (*A. aculeata*) somatic embryogenesis process

Components	Calli induction (240 days)	Somatic embryo differentiation (90 days)	Synchronization and maturation (60 days)	Plant regeneration (>35 days)
Culture medium	MS or Y3	MS or Y3	MS or Y3	MS or Y3
Vitamins	MS	MS	MS	MS
Glutamine (mg L^{-1})	500	500	500	500
Dicamba (mg L^{-1})	0; 1.5 or 3.0	0.5 or 1.0	–	–
Picloram (mg L^{-1})	0; 1.5 or 3.0	0.5 or 1.0	–	–
2,4-D (mg L^{-1})	0; 1.5 or 3.0	0.5 or 1.0	–	–
Sacarose (%)	3.0	3.0	4.5	3.0
Phytagel (g L^{-1})	2.5	2.5	2.5	2.5
Activated charcoal (g L^{-1})	–	–	–	2.5

MS Murashige and Skoog medium (1962), *Y3* Eeuwens (1976), *Dicamba* 3,6-dichloro-2-methoxybenzoic acid, *Picloram* 4-amino-3,5,6-trichloropicolinic acid, *2,4-D* 2,4-dichlorophenoxyacetic acid

medium consisted of a reduction in the each type of auxin concentrations used for induction (1.5 or 3.0 mg L^{-1}) to 0.5 or 1.0 mg L^{-1}, preserving the BM of origin, totalizing four treatments.

At this stage, the embryogenic aggregates were cultivated in Magenta GA-7 culture flasks (Sigma Chemical Co., St. Louis, USA) containing 45 mL culture medium and kept in the dark in a growth room at 25 ± 2 °C for 90 days. The subcultures were performed every 45 days, and for every subculture the number of SE per explant was determined.

Synchronization and maturation of somatic embryos

After 90 days in differentiation medium, the remaining calli and SEs were transferred to their respective BM, with added MS vitamins, 45 g L^{-1} sucrose and 0.5 g L^{-1} glutamine. At this stage, the culture media were devoid of growth regulators. The explants remained in the dark for 60 days in a growth room at 25 ± 2 °C.

Plant regeneration

For plant regeneration, torpedo- and cotyledonary-shaped differentiated SEs were cultured in regeneration medium consisting of BM containing 30 g L^{-1} sucrose, 0.5 g L^{-1} glutamine, 2.5 g L^{-1} activated charcoal for about 45 days, until the development of the roots and aerial part. For somatic conversion and plant growth, the cultures were maintained at 25 ± 2 °C at a photosynthetic photon flux of 38 μmol m^{-2} - s^{-1} provided by cool white lamps with a 16-hd^{-1} photoperiod. The plantlets were transferred to medium devoid of activated charcoal for growth and development.

The plants grown under the conditions described above, containing roots, first leaves and more than 5.0 cm in height were selected for acclimatization. These plants were washed with running water to remove culture medium residue and planting was carried out in 300 mL pierced plastic cups containing in a mixture of commercial substrate (Bioplant, Nova Ponte, Brazil) and sand (1:1). After planting, the plastic cups were covered with small individual transparent plastic bags tied with a string, in order to avoid shoot dehydration and kept in an incubator at 25 ± 2 °C for 30 days and were watered with water. Each week, an opening was made in the plastic bag, increasing in size each week, until the plants were acclimated to the environmental conditions (about 14 days). Subsequently, the plants were transferred to the greenhouse.

Histological analysis

For analysis by light microscopy, samples of calli and SEs at different development stages were fixed in an FAA solution (Formaldehyde, acetic acid and ethanol; 1:1:18 v/v) for a minimum of 24 h under vacuum during the first hour, followed by dehydration in ascending alcohol series (70–100 %) and infiltrated in Historesin (Leica' Heidelberg, Germany). Longitudinal and transverse serial cuts (7 μm) were obtained in a manual rotary microtome (Leica, RM2125RT), distended and adhered to microscope slides with water. The sections were stained with Toluidine blue (O' Brien et al. 1964) and mounted with Entellan (Merck, Darmstadt, Germany). Results were registered in a microscope (Motic, BA300) coupled to a digital image capture system.

For scanning electron microscopy, samples were fixed in Karnovisk (Johansen 1940) for 24 h, rinsed in sodium cacodylate buffer (0.05 M, pH 7.1) and post-fixed in 2 % osmium tetroxide and sodium cacodylate buffer for 3 h. They were then washed three times in the same fixation buffer, dehydrated in an ethanol series (25–100 %), dried to critical point with CO_2 and metallized with gold–

palladium. The observations and image capture were performed in a Zeiss DSM 962 scanning electron microscope.

Statistical analysis

At all stages, the experiments were performed in a randomized design. In the induction phase, treatments consisted of five replicates containing five explants. In the somatic embryo differentiation, each callus was considered one repetition and at least ten calli were evaluated per treatment. The data were subjected to analysis of variance (ANOVA), and means were compared by the Scott–Knott test at 5 % probability, with the aid of the Sisvar statistical analysis program (Ferreira 2011). Data expressed as percentages were previously processed according to arcsin $(x/100)^{0.5}$. All experiments were repeated at least twice.

Results and discussion

Calli induction

For calli induction, ZE was extracted from seeds that showed whitish coloration, with the proximal region narrower than distal region (cotyledon) and measured about 5 mm long and 1 mm wide. They were inoculated into culture medium for callus induction (Table 1; Fig. 1a, b). After 1 week of culture, it was found that the embryos responded to all treatments tested with auxins. The response was characterized by swelling of the ZE that, in general, doubled in size.

Except for the control treatments, it was found that after 15 days of culture, ZE exhibited a widening of the mesocotyl region and acquired a yellowish coloration. At this stage, the explants were about four times their original size, regardless of the type and concentration of auxin tested, although this mesocotyl enlargement was more evident in the explants inoculated in culture media containing picloram (Fig. 1c).

In this study, somatic embryogenesis followed an indirect model. Calli formation started after 30 days of culture in induction medium and occurred mainly in the distal region and the ZE mesocotyl (Fig. 1c–e). The calli formed in these two regions showed morphological differences: the calli formed in the mesocotyl were compact and dark yellow color, whereas those in the distal region had a brittle aspect with a whitish appearance and mostly occurred in areas in contact with the culture medium (Fig. 1c, e). Cultures from the control treatments showed elongation of the cotyledons followed by 100 % embryo germination (Fig. 1f).

Morphological differences were also observed in the ZE response pattern to the auxins applied. In general, the ZE inoculated into medium containing picloram, independent of the BM and concentration, showed growth about two times higher than those inoculated in medium with 2,4-D (Fig. 1d) and dicamba (Fig. 1e). Furthermore, in the picloram medium, the primary callus formation initiated from the mesocotyl region, but only after the swelling and the increase, of at least three times, of the initial ZE size. As for the treatments with 2,4-D and dicamba, although the embryos had also shown swelling before the beginning of calli emergence, this increase smaller, in addition to an earlier calli formation.

When comparing the effect of these three auxins on the oil palm *E. guineensis*, Thuzar et al. (2011) found better results in the formation of embryogenic calli when using 2,4-D. However, Moura et al. (2009), on testing the effect of 2,4-D and picloram in macaw palm observed no differences between auxins on calli formation. However, according to these authors, the differences became evident in the differentiation phase of the SE with the best results obtained in media with picloram.

In the present work, explant oxidation was discrete or nearly zero and, when observed, were in those areas of explant contact with the culture medium. These results contrast those found by Moura et al. (2009), who report that the calli oxidized and lost prolificacy when cultured in medium devoid of activated charcoal.

The percentage of primary calli formed was analyzed after 90 days of culture. During this period, all treatments containing auxin formed primary calli. For dicamba, the lowest concentration (1.5 mg L^{-1}) was the most effective for inducing primary calli, with 75 % in MS media. At higher concentration (3.0 mg L^{-1}), the explants tended to have mild oxidation and few calli formations (25–30 %). In treatments containing picloram, 30 % and 50 % of the explants presented calli formation on MS media and Y3, respectively. The auxin 2,4-D presented the best results, with an average of calli formation over 30 % in MS medium, and 60 % in Y3 medium (Table 2).

On average, when analyzing the influence of the basic medium on the production of primary calli, no significant differences were found (Table 2). In *E. guineensis*, Thuzar et al. (2011) observed that during the calli induction, auxins exert greater effect than the culture media, suggesting that this phase is that which governs the initial responses due to strong pressure in the cells for cell reprogramming and acquisition of embryogenic competence.

After 150 days, the calli formed at the distal region started the change from the primary phase to the embryogenic. These calli exhibited an increase in yellow pigmentation and then progressed to the formation of embryogenic calli (EC) (Fig. 1g). At this stage we also observed nodular structure formation, similar to SEs on calli formed from the mesocotyl (Fig. 1h). These structures were more evident in treatments containing picloram.

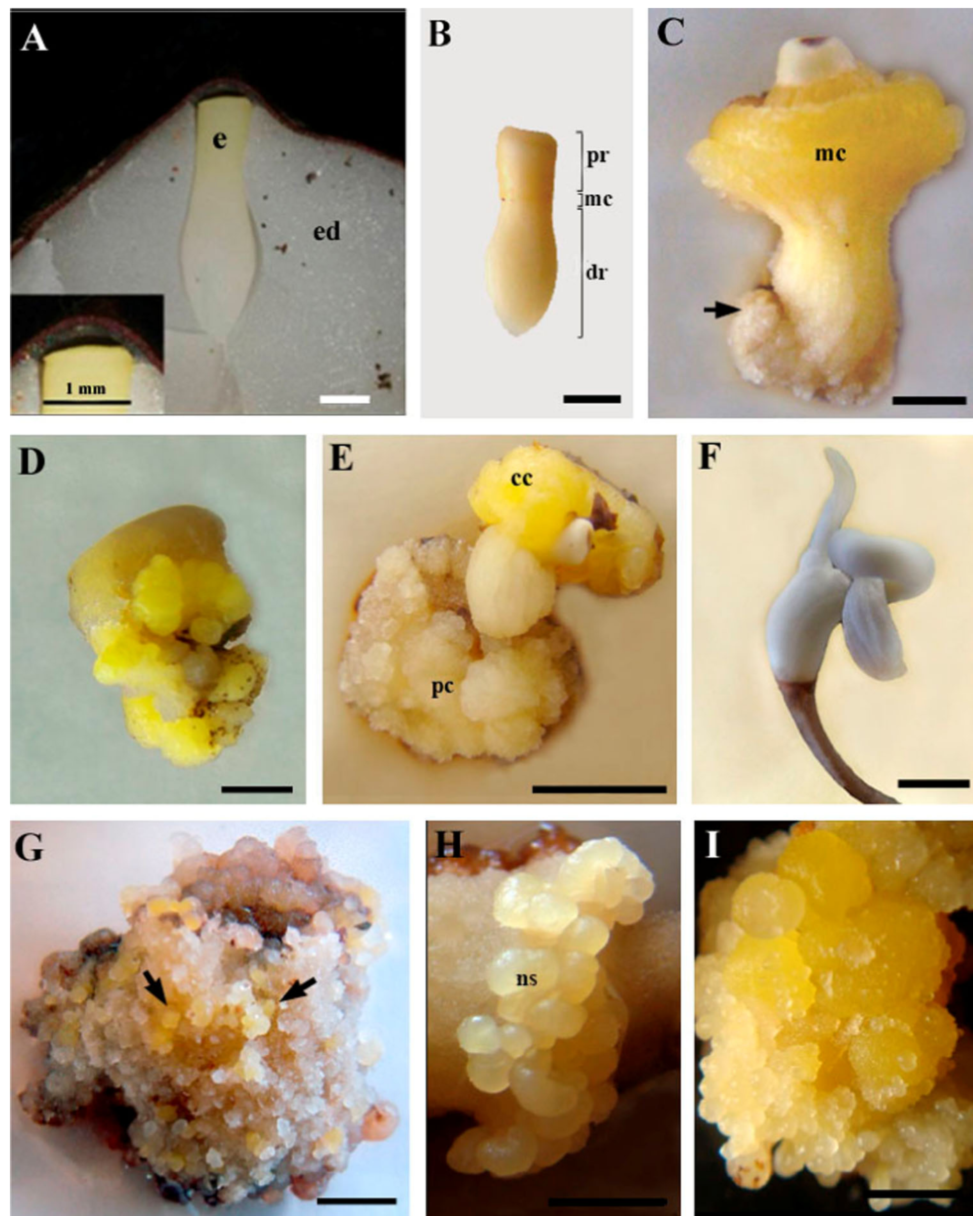

Fig. 1 Morphological stages of calli induction during somatic embryogenesis of macaw palm (*A. aculeata*). **a** Macaw palm seed section showing the zygotic embryo inserted in the endosperm. **b** Detail of the proximal and distal regions and mesocotyl of the zygotic embryo. **c** Swollen zygotic embryo, presenting early primary callus formation in the distal region (*arrow*) and compact callus in the mesocotyl region after 30 days in medium containing picloram (3.0 mg L^{-1}). **d** Zygotic embryo formation with yellowish compact callus after 30 days in medium with 2,4-D (1.5 mg L^{-1}). **e** Zygotic embryo showing primary callus in the distal region and compact callus in the mesocotyl after 30 days in medium containing dicamba (1.5 mg L^{-1}). **f** Detail of the zygotic embryo germination in medium devoid of auxin. **g** Primary callus presenting initiation of embryogenic callus formation (*arrows*). **h** Mesocotyl with nodular structures at 150 days of culture. **i** Embryogenic callus, yellowish and nodular. *cc* compact callus, *dr* distal region, *e* zygotic embryo, *ed* endosperm, *mc* mesocotyl, *ns* nodular structures, *pc* primary callus, *pr* proximal region. *Bars* **a**, **b** = 1 mm; **c**, **d** = 3 mm; **e**–**i** = 1 cm

At 180 days of culture in induction medium, we observed the formation of EC in all treatments containing auxin. These calli had yellowish coloration with two distinct regions: a central portion with compact appearance and a nodular peripheral that detached easily from the explant (Fig. 1i). In MS medium, significant results were achieved when the explants were cultured with 1.5 or 3.0 mg L^{-1} of dicamba and 1.5 mg L^{-1} 2,4-D, with 75, 50 and 55 % of explants with EC, respectively (Table 2).

In the Y3 medium all auxins tested provided results to obtain EC, except for the treatment containing 3.0 mg L^{-1} dicamba, in which we obtained a significantly lower value

Table 2 Effect of the MS or Y3 culture media and different concentrations of dicamba, picloram or 2,4-D on the percentage of explants with primary calli on the 90th day of cultivation and embryogenic on the 180th day of cultivation in macaw palm zygotic embryos (*A. aculeata*)

Medium	Concentration of PGRs (mg L^{-1})			Primary callus (%)	Mean[a]	Embryogenic callus (%)	Mean[a]
	Dicamba	Picloram	2,4-D				
MS	0.0	–	–	0.0 ± 0.0 c	41.7 ± 8.5 a	0.0 ± 0.0 b	39.2 ± 10.0 b
	1.5	–	–	75.0 ± 10.0 a		75.0 ± 15.0 a	
	3.0	–	–	25.0 ± 12.0 b		50.0 ± 18.0 a	
	–	0.0	–	0.0 ± 0.0 c		0.0 ± 0.0 b	
	–	1.5	–	30.0 ± 13.0 b		25.0 ± 2.0 b	
	–	3.0	–	25.0 ± 9.0 b		20.0 ± 14.0 b	
	–	–	0.0	0.0 ± 0.0 c		0.0 ± 0.0 b	
	–	–	1.5	60.0 ± 8.0 a		55.0 ± 18.0 a	
	–	–	3.0	35.0 ± 5.0 a		10.0 ± 7.0 b	
Y3	0.0	–	–	0.0 ± 0.0 c	51.7 ± 5.3 a	0.0 ± 0.0 b	60.8 ± 5.7 a
	1.5	–	–	60.0 ± 8.0 a		65.0 ± 6.3 a	
	3.0	–	–	30.0 ± 4.0 b		35.0 ± 8.5 a	
	–	0.0	–	0.0 ± 0.0 c		0.0 ± 0.0 b	
	–	1.5	–	45.0 ± 5.0 a		60.0 ± 15.0a	
	–	3.0	–	50.0 ± 7.0 a		70.0 ± 12.0 a	
	–	–	0.0	0.0 ± 0.0 c		0.0 ± 0.0 b	
	–	–	1.5	60.0 ± 9.0 a		60.0 ± 6.3 a	
	–	–	3.0	65.0 ± 6.4 a		75.0 ± 11.0 a	

MS Murashige and Skoog medium (1962), *Y3* Eeuwens (1976), *Dicamba* 3,6-dichloro-2-methoxybenzoic acid, *Picloram* 4-amino-3,5,6-trichloropicolinic acid, *2,4-D* 2,4-dichlorophenoxyacetic acid

Mean values ± standard error followed by the same letter belong to the same group at 5 % probability by the Scott-Knott test. Data transformed according to $(x + 1)^{0.5}$. Data were the mean from 25 explants per treatment

[a] Mean excluding the control treatment.

than the other treatments, with 35 % of explants with EC induction.

When analyzing the influence of the culture medium, there were significant influences on the EC induction, since explants cultured on Y3 showed, on average, 60.8 % calli formation significantly higher than the value observed in MS, with 39.2 % of the explants with EC (Table 2).

Differentiation of somatic embryos

Embryogenic calli formed in induction medium were transferred to media containing low concentrations of auxins seeking the conversion of the embryogenic calli into SEs. In *Cocos nucifera* the transfer to medium with low concentrations of auxin and cytokinin addition, increased the number of differentiated SE (Fernando and Gamage 2000). Other studies have also reported this behavior, after reducing the concentration of auxin used in calli induction (Dias et al. 1994; Huong et al. 1999; Silva et al. 2012).

At 30 days in differentiation medium, we observed the development of the embryogenic mass (Fig. 2a). At that stage, it was possible to visualize the globular and translucent structures, characterizing SEs at a stage similar to the globular. After 60 days in differentiation medium, there were several embryos at the globular stage and others at a more advanced stage (Fig. 2b). At this stage, the SE formed were morphologically normal. In most cases, at the base of the globular embryo, we observed the suspensor making the connection from the somatic embryo to the explant of origin (Fig. 2b detail). In the following the globular stage, the suspensor was no longer observed, suggesting degeneration.

At 90 days in differentiation medium, the assessment of the number of SEs formed per explant was conducted (Table 3). In general, the responses related to the number of embryos formed were significantly higher in Y3 (7.0) compared with the MS (2.5). These results suggest that the composition of the Y3 medium was more efficient for differentiation of SEs. The Y3 medium was described by Eeuwens (1976), who revealed it to be the most suitable for the in vitro culture of *C. nucifera*, compared with MS medium (Murashige and Skoog 1962), possibly because this species requires high levels of iodine and potassium, and less nitrogen.

In Y3 medium containing dicamba, we observed better results in the differentiation of SEs when the EC, coming

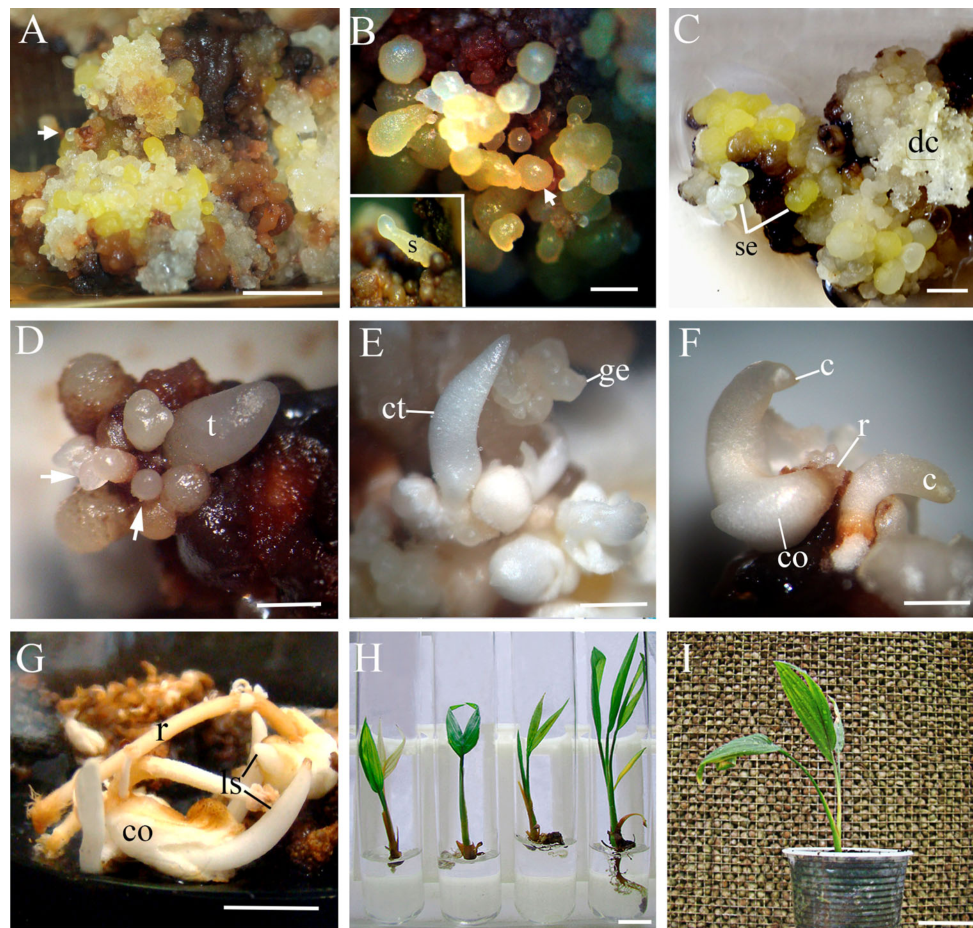

Fig. 2 Morphological aspects of plant regeneration from zygotic embryo culture of macaw palm (*A. aculeata*) through somatic embryogenesis. **a** Embryogenic callus containing SEs (*arrow*) after 30 days in differentiation medium. **b** SEs at the globular stage (*arrow*), detail of the globular embryo with suspensor. **c** SEs and region of the explant with dehydrated aspect after 30 days in synchronization and maturation medium. **d** SEs in different phases, globular (*arrow*) and torpedo after 60 days in medium synchronization and maturation embryos. **e** Embryo in cotyledon stage after 60 days in synchronization and maturation medium. **f** Somatic embryo with beginning of radicle protrusion containing cotyledon and coleoptile after 45 days in plant regeneration medium. **g** Germination of SEs in regeneration medium containing radicle, coleoptile and leaf sheath. **h** Individualized clones presenting roots and shoots. **i** Acclimatized plant. *c* cotyledon, *ct* embryo in cotyledon stage, *dc* dehydrated callus, *ge* embryo globular stage, *Co* coleoptile, *ls* leaf sheath, *r* radicle, *s* suspensor, *se* somatic embryo, *t* torpedo stage embryo. *Bars* **a**, **b**, **g**, **h** = 1 cm; **c**–**f** = 0.4 cm; **i** = 5 cm

from both induction concentrations were transferred to 0.5 mg L^{-1}. In treatments using picloram, the auxin concentration did not affect the differentiation of the SE. In 2,4-D, the EC induced in 1.5 mg L^{-1} showed higher percentage of conversion into SEs when transferred to 1.0 mg L^{-1}, while those induced in 3.0 mg L^{-1} differentiated more in 0.5 mg L^{-1}, with an average of 12.5 and 6.7 embryos per explant, respectively.

In MS media, only treatment with dicamba in 1.0 mg L^{-1} from induction medium with concentration of 1.5 mg L^{-1} showed significant results. However, the development and germination of these embryos was not observed, demonstrating that this treatment is not suitable for macaw palm plant regeneration.

Synchronization and maturation of somatic embryos

In differentiation medium, calli and SE in different stages of development were observed in a same explant, characterizing an asynchronized process. In search of synchronization and maturation of SE, the explants were transferred to new culture medium devoid of growth regulators and sucrose containing a higher concentration (45 g L^{-1}), to improve the synchronization and maturation. According to Ikeda-Iwai et al. (2003), stress periods, such as dehydration during in vitro culture, can promote increased induction, maturation and germination rates of SEs. The type and concentration of carbohydrates in the culture medium have been suggested as factors that

Table 3 Influence of the differentiation and maturation medium on the average number of SEs formed per explant and regeneration frequency of macaw palm plants (*A. aculeata*) after 90 days in regeneration medium

Medium	Induction (mg L^{-1})	Differentiation/maturation medium (mg L^{-1})			No. of somatic embryos/callus	Mean medium	Regeneration frequency (%)[a]	Mean medium
		Dicamba	Picloram	2.4-D				
MS	1.5	0.5	–	–	0.0 ± 0.0 b	2.5 ± 0.9 b	0.0 ± 0.0 c	0.0b
		1.0	–	–	5.0 ± 0.5 a		0.0 ± 0.0 c	
	3.0	0.5	–	–	1.5 ± 2.5 b		0.0 ± 0.0 c	
		1.0	–	–	0.0 ± 0.0 b		0.0 ± 0.0 c	
	1.5	–	0.5	–	0.0 ± 0.0 b		0.0 ± 0.0 c	
		–	1.0	–	0.0 ± 0.0 b		0.0 ± 0.0 c	
	3.0	–	0.5	–	0.0 ± 0.0 b		0.0 ± 0.0 c	
		–	1.0	–	1.0 ± 1.0 b		0.0 ± 0.0 c	
	1.5	–	–	0.5	0.0 ± 0.0 b		0.0 ± 0.0 c	
		–	–	1.0	0.0 ± 0.0 b		0.0 ± 0.0 c	
	3.0	–	–	0.5	0.0 ± 0.0 b		0.0 ± 0.0 c	
		–	–	1.0	0.0 ± 0.0 b		0.0 ± 0.0 c	
Y3	1.5	0.5	–	–	6.7 ± 2.7a	7.0 ± 1.2 a	13.0 ± 5.0 b	14.0 ± 4.0 a
		1.0	–	–	0.0 ± 0.0 b		0.0 ± 0.0 c	
	3.0	0.5	–	–	11.5 ± 3.5 a		9.1 ± 2.0 b	
		1.0	–	–	0.0 ± 0.0 b		0.0 ± 0.0 c	
	1.5	–	0.5	–	4.7 ± 1.7 a		0.0 ± 0.0 c	
		–	1.0	–	6.3 ± 2.7 a		0.0 ± 0.0 c	
	3.0	–	0.5	–	10.3 ± 5.0 a		3.3 ± 1.2 c	
		–	1.0	–	6.7 ± 3.2 a		0.0 ± 0.0 c	
	1.5	–	–	0.5	1.5 ± 0.5 b		0.0 ± 0.0 c	
		–	–	1.0	12.5 ± 2.2 a		31.9 ± 4.6 a	
	3.0	–	–	0.5	6.7 ± 1.7 a		12.5 ± 5.5 b	
		–	–	1.0	2.2 ± 0.7 b		0.0 ± 0.0 c	

MS Murashige and Skoog medium (1962), *Y3* Eeuwens medium (1976), *Dicamba* 3,6-dichloro-2-methoxybenzoic acid, *Picloram* 4-amino-3,5,6-trichloropicolinic acid, *2,4-D* 2,4-dichlorophenoxyacetic acid

Mean values ± standard error followed by the same letter belong to the same group at 5 % probability by the Scott–Knott test. Data transformed according to $(x + 1)^{0.5}$. Data were the mean from 25 explants (embryogenic calli) per treatment

[a] Mean excluding the control treatment.

strongly affect maturation and conversion of SEs (Troch et al. 2009). These factors are able to enhance the maturation and germination because they can simulate the developmental environment of ZE (Te-chato and Hilae 2007). Shi et al. (2009) in studies with *Cinnamomum camphora* L. found that the best results for SE maturation were obtained with concentrations varying from 20 to 50 g L^{-1} of sucrose, while higher concentrations, 70–90 g L^{-1} of sucrose, resulted in a decrease in the mean number of SE.

In this study, after 30 days in synchronization/maturation medium, the SE were more developed and whitish, while the regions with calli exhibited a dehydrated appearance (Fig. 2c), progressing to intense oxidation and necrosis. After 60 days, the SE were visualized at different stages of development (Fig. 2d). SE were visualized featuring white coloration and differentiated cotyledon (haustorium), which characterized the cotyledon stage (Fig. 2e). According to Arnold et al. (2002), only morphologically normal and mature SE were able to accumulate sufficient reserve substances, and thus tolerate dehydration periods and be converted into normal plants.

Plant regeneration

The germination of the SE occurred, on average, after 45 days in regeneration medium devoid of auxin, and was initially confirmed by the expansion of the region near the embryo where the embryonic axis is located, followed by radicle protrusion (Fig. 2f). After growth of at least 1.0 cm of the radicle, emission of the aerial part occurred by the coleoptile (Fig. 2g). In general, SEs formed were isolated and those that remained bound together and to the explants were separated and placed for development. The use of

culture medium devoid of growth regulators has been cited in several studies with palm trees. Scherwinski-Pereira et al. (2012) and Silva et al. (2012) when using culture medium without added growth regulators and containing activated charcoal, obtained successful germination of SE in studies performed with the palms *Euterpe oleracea* and *E. guineensis*, respectively.

The treatments used during induction, differentiation and synchronization/maturation of SEs were instrumental in the success of macaw palm germination and plant regeneration. The maximum plant regeneration frequency (31.9 %) was obtained when the SE were induced in Y3 medium with 1.5 mg L^{-1} 2,4-D and transferred to 1.0 mg L^{-1} (Table 3). These results corroborate those obtained by Thuzar et al. (2011) in studies with *E. guineensis*, in which the authors also observed the superiority of results with the use of 2,4-D compared to treatments with dicamba and picloram, since germination was obtained only for SE induced in 2,4-D.

Several studies reporting the success of somatic embryogenesis in palm trees from different explants were successfully developed principally using the auxin 2,4-D (Teixeira et al. 1993; Eke et al. 2005; Othmani et al. 2009). However, picloram has also been used frequently in somatic embryogenesis protocols for the palm. In studies carried out by Moura et al. (2009), the most suitable SE germination were achieved using picloram, however, these authors reported that in the regeneration medium few completed germination.

In the regeneration medium, besides the germination of SE, we observed different morphological responses of EC, such as the formation of adventitious roots and cases of SE abnormality. Some SE germinated with normal root development, however, the apical meristem appeared to be absent or poorly developed. The lack of SE shoot development was also observed during germination of the control treatment ZE.

Most of the regenerated plants showed normal development, with root and shoot growth (Fig. 2h). Once regenerated, the plants were acclimatized after the shoot development and emission of at least two roots (Fig. 2i). It was observed that the plants that did not show at least two roots did not survive acclimatization.

The process of somatic embryogenesis in macaw palm, from induction to the complete regeneration of plants was carried out in 15 months.

Histological analysis

Histological analysis showed differences between the callus formed in the distal region of the embryo (cotyledon) and that of the mesocotyl. The callus formed from the distal region of the zygotic embryo (Fig. 3a) has a different cellular make up: vacuolated cells with low nucleus/cytoplasm ratio in greater quantity and meristematic cells with large nuclei and dense cytoplasm in smaller amounts (Fig. 3b).

The compact callus formed in the mesocotyl region presents different cellular make up than the callus formed in the distal region of the ZE. Meristematic cells predominate in these calli (Fig. 3c). The difference observed between the mesocotyl callus and that the distal region, is probably related to the cellular constituents of these regions of the explant (zygotic embryo). The mesocotyl is located near the embryonic axis which contains higher amounts of meristematic cells, while the distal region is mostly formed by parenchyma cells (Ribeiro et al. 2012). Therefore, the type of callus formed is influenced by the region of the explant used for induction.

Despite the constitutive differences, both regions have developed embryogenic calli with a nodular aspect (Fig. 3d), characterized by embryogenic meristematic cells. In the peripheral region of the embryogenic callus (EC), bicellular proembryos were visualized presenting one apical cell and the other basal, responsible for the formation of embryonic tissue and the suspensor, respectively (Fig. 3e). Subsequently, we observed the globular proembryos with multicellular suspensor (Fig. 3f). The presence of the suspensor confirmed the unicellular origin of these embryos, since such a structure indicates that the suspensor and the embryo are formed from the asymmetric division of a single cell (Heidstra 2007). Additionally, De Almeida et al. (2012) reported that embryos of unicellular origin form from external tissue cells and easily isolate themselves from the explant, while the multicellular are formed internally from a group of meristematic cells and thus, isolation from the explant is hindered.

Globular structures apparently isolated and consisting of meristematic cells were also seen among the meristematic cells in the central region of the nodular callus (Fig. 3g). This type of structure characterizes the formation of embryos of multicellular origin. These results corroborate those reported by Moura et al. (2008), who reported that embryos of multicellular origin formed from nodular calli. Globular embryos of multicellular origin were also observed by Balzon et al. (2013) from embryogenic calli of *E. guineensis*.

In differentiation medium, SEs were seen at different developmental stages, including those in post-globular stage connected to the explant stadium (Fig. 3h, i). Clusters of embryos delimited by protoderm and fused to the callus were observed with frequency (Fig. 3j). SEs at later stages like torpedo or cotyledonary, characterized by containing procambial bundles, differentiated vessel elements and protoderm with a single layer of juxtaposed rectangular cells with cell divisions in the anticlinal direction, were visualized after transfer to regeneration medium (Fig. 3k).

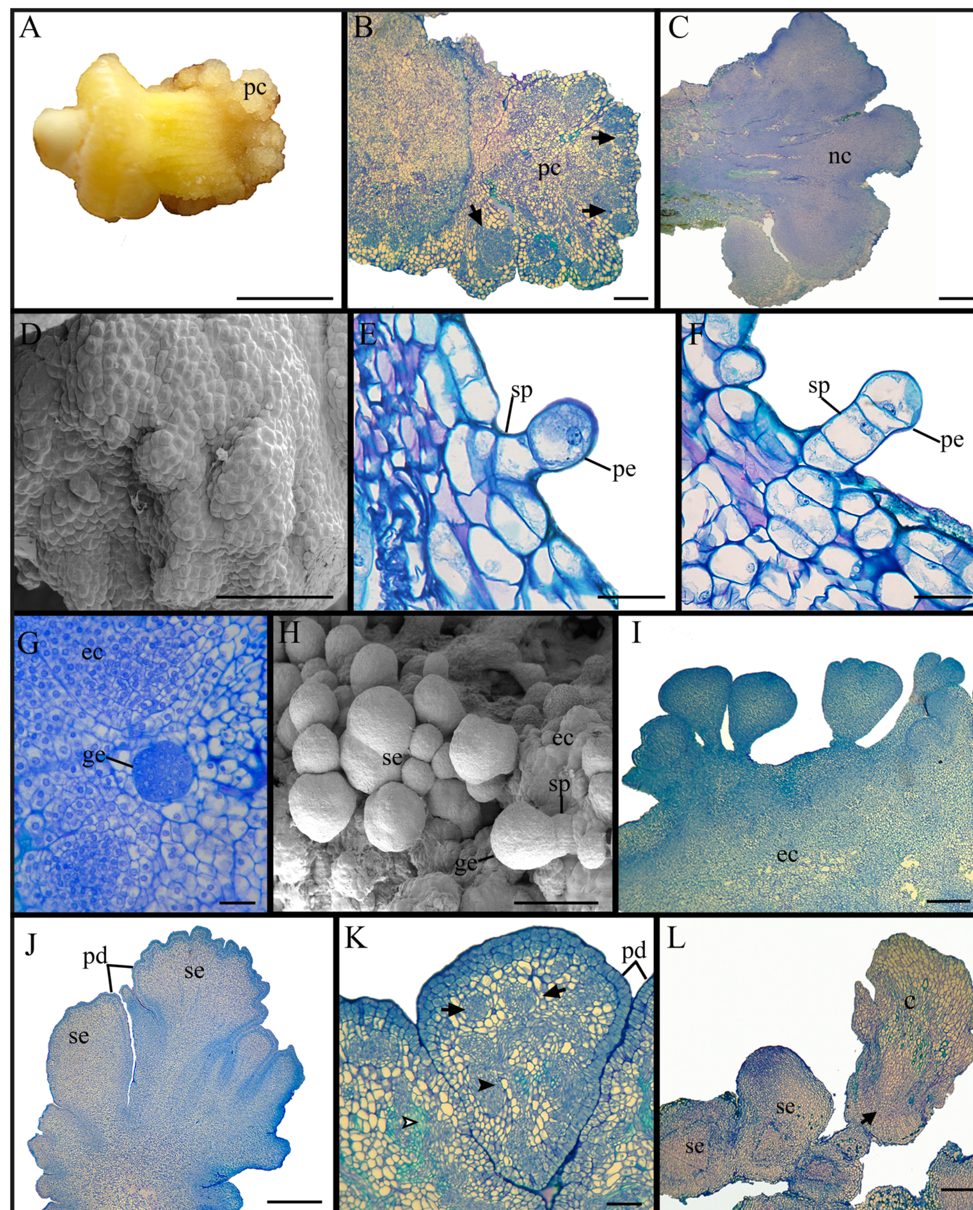

Fig. 3 Histological characterization of somatic embryogenesis in macaw palm (*A. aculeata*). **a** morphological appearance of zygotic embryo containing primary callus on the edge of the distal region. **b** Primary callus containing meristematic clusters on the periphery (*arrows*) (callus distal region). **c** Nodular embryogenic callus composed of meristematic cells (callus mesocotyl region). **d** Scanning electron micrograph showing the appearance of embryogenic callus. **e** Bicellular proembryo embryo with suspensor. **f** Proembryo showing multicellular suspensor. **g** Globular embryo isolated among cells in the central region of embryogenic callus. **h** Scanning electron micrograph showing globular SEs formed from embryogenic calli. **i** SEs formed at the end of the callus and connected by the suspensor. **j** Fused SEs delimited by protoderm. **k** SEs at an advanced stage presenting protoderm, procambial bundles (*arrows*) and vessel elements in early differentiation (*arrowhead*). **l** Somatic embryo in torpedo stage with the proximal (*arrow*) and distal region represented by cotyledon. *c* cotyledon, *ec* embryogenic callus, *ge* globular embryo, *nc* nodular embryogenic callus, *pc* primary callus, *pd* protoderm, *se* somatic embryo, *sp* suspensor. *Bars* **a** = 1 cm; **b**, **m** = 150 µm; **c**, **d**, **h–j** = 200 µm; **e**, **f** = 15 µm; **g** = 50 um; **l** = 100 µm

The polarity of the SE was established in the torpedo stage, showing the distinction of a distal and proximal pole (Fig. 3l). On the distal pole the cotyledon of the somatic embryo containing procambial bundles, parenchyma cells and protoderm was observed. The proximal pole, so named due to its proximity to the suspensor, contains the future embryonic axis. In somatic embryogenesis of *C. nucifera* and *E. guineensis*, Fernando et al. (2003) and Silva et al. (2013) also characterized SEs showing bipolarity and differentiated cotyledon, respectively.

In conclusion, our experiments have led to significant improvement of the overall efficiency of somatic

embryogenesis of macaw palm. This protocol is comprehensive, covering all steps from explants to plant. Our results contribute for future studies by confirming the effects of the Y3 culture medium and the 2,4-D, picloram or dicamba on enhancing the embryogenic response. We also conclude that somatic embryogenesis and plantlets regeneration could be achieved through continuous monitoring of cultures and by reducing the auxin concentrations during the different process phases. Studies are now in progress in order to establish competent and synchronous embryogenic cell lines to improve the regenerative protocol, as well as to determine the embryogenic competence from different natural population, including immature leaves from adult plants as initial explant.

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