AGRARIAN SCIENCES

Somatic embryogenesis from leaf tissues of macaw palm [*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.]

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Abstract: A somatic embryogenesis protocol was developed from the immature leaves of adult plants of the macaw palm. Leaf explants from different regions of the palm heart were used for callus initiation in a modified Y3 medium, supplemented with 2,4-D or Picloram at 450 µM. Calli were separated from the leaf explants at 6-, 9- and 12-month periods and transferred to a fresh culture medium of the same composition. They were multiplied for up to 120 days. Reduced concentrations of 2,4-D and Picloram were used to differentiate somatic embryos. They were then germinated in a medium without plant growth regulators. Morphological and anatomical analyses were conducted at different stages of the embryogenic process. The best results for callus induction were achieved by Picloram, when explants were maintained for up to 9 months on culture medium (64.9%). The farthest portions of the apical meristem were those that provided the biggest calli formation. The formation of the somatic embryos was observed from the calli multiplication phase. Reduction in concentrations of growth regulators failed to promote the formation of complete plants. Picloram at 450 µM promotes high callogenesis in leaf tissues of macaw palm, with a potential for somatic embryo formation.

Key words: Arecaceae, somatic embryogenesis, leaf, callus culture, woody plants.

INTRODUCTION

The macaw palm [*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.], a species of the Arecaceae family, is widely distributed in the states of São Paulo and Rio de Janeiro, throughout Minas Gerais and the Mid-West, Northeast and North regions of Brazil (Wandeck & Justo 1988, Henderson et al. 1995). Macaw palm has one of the highest productivity rates in vegetable oil among oil plants, with a potential yield of 1,500 to 6,200 kg oil ha^{-1} (Wandeck & Justo 1988, Dias 2011).

Since asexual conventional propagation of macaw palm is practically unfeasible, *in vitro* regeneration offers a useful tool for mass propagation in the palm species analyzed (Moura et al. 2009, Nguyen et al. 2015, Luis & Scherwinski-Pereira 2014). Among the *in vitro* culture techniques, somatic embryogenesis may ensure the propagation of specimens in large scale and in a homogeneous way, either for the production of elite plantlets or for accelerating genetic improvement programs (Moura et al. 2009, Soh et al. 2011, Luis & Scherwinski-Pereira 2014). By this technique and under favorable experimental conditions, differentiated or undifferentiated somatic cells are determined by following specific morphogenic routes that culminate in the development of somatic embryos at the end of the process, without gamete fusion (Williams & Maheswaran 1986, Emons 1994).

Studies on somatic embryogenesis of the macaw palm are scanty, although the technique using zygotic embryos as an explant source has already been described (Teixeira et al. 1986, Moura et al. 2009, Luis & Scherwinski-Pereira 2014, Granja et al. 2018). Indeed, studies on the somatic embryogenesis in macaw palm seem to be still in the infance phase, when results are taken into account. This is especially true when dealing with the use of propagules originating from somatic tissues, such as leaves and inflorescences, as starting material for cultivation. Such explants have the additional advantages of allowing the cloning of the original genotype and providing a significant amount of material by collection.

In general, the development of protocols for palm trees by somatic embryogenesis, including *Acrocomia aculeata*, involves the addition of growth regulators, especially 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-tri chloropicolinic acid (Picloram) (Moura et al. 2009, Luis & Scherwinski-Pereira 2014, Granja et al. 2018), usually employed at high concentrations in the induction phase. In fact, growth regulators play a central role in mediating signal transduction cascades leading to gene reprogramming (Dudits et al. 1991) and subsequent somatic embryogenesis.

Due to the scarcity of reports on somatic embryogenesis using somatic tissues of adult plants as explants for *in vitro* culture, current study aims at inducing somatic embryogenesis in the macaw palm from the leaf tissues of adult plants. To date, no report exists on the somatic embryogenesis of *A. aculeata* employing adult plants' somatic tissues.

MATERIALS AND METHODS

Selection of explant

Immature and unexpanded leaves (palm heart) of *Acrocomia aculeata*, derived from adult plants grown near the municipality of Sobradinho DF Brazil, were used as explant source. Plant material was sent to the laboratory where the outermost leaves were removed and the size of the palm heart reduced to approximately 30 cm in length (measured from the meristem base to the leaf apex of the central cylinder). The palm heart, consisting exclusively of achlorophyllous leaves, was disinfested with 70% (v/v) alcohol in a laminar flow for three minutes, followed by sodium hypochlorite (1.5% active chlorine) for 20 minutes. The explants were then rinsed thrice with distilled and autoclaved water. After disinfestation, the leaves were excised into 1.0 cm^2, with three leaf blades per explant.

The experiments were divided into four phases: callus induction, callus multiplication, somatic embryo differentiation and plant regeneration. Table I shows components of the culture media used in each different stage.

Callus induction and multiplication

The induction medium consisted of modified Y3 (Eeuwens 1976). Fe-EDTA and vitamin source were maintained according to original MS concentration (Murashige & Skoog 1962), supplemented with 30 g/L sucrose (Dinâmica, Indaiatuba, Brazil), 0.5 g/L glutamine (Sigma, St. Louis MO, USA), 2.5 g/L activated charcoal (Sigma, St. Louis, MO, USA), 2.5 g/L Phytagel (Sigma, St. Louis, MO, USA), and either 450 µM 4-amino-3,5,6-trichloropicolinic acid - Picloram (Sigma, St. Louis, MO, USA) or 2,4-dichlorophenoxyacetic acid - 2,4-D (Sigma) (Table I). At this stage, two experiments were performed:

Effect of auxins and 2-isopentenyladenine - 2iP on callus induction: Callus induction was

Table I. Composition of culture media used in somatic embryogenesis studies from leaf explants of the macaw palm (*Acrocomia aculeata*).

Components	Callus induction (6, 9 and 12 months)	Callus multiplication (4 months)	Somatic embryo differentiation (1 month)	Plant regeneration (>1 month)
Basal medium (Inorganic salts)	Y3	Y3	Y3	Y3
Vitamins and Fe-EDTA	MS	MS	MS	MS
Glutamine (mg/L)	500	500	500	500
Picloram (µM)	450	450	0, 10, 20, 40, 80 and 120	-
2,4 – D (µM)	450	-	0, 5, 10, 20, 40 and 80	-
2iP (µM)	0 and 20	-	-	-
Sucrose (%)	3.0	3.0	3.0	3.0
Phytagel (g/L)	2.5	2.5	2.5	2.5
Activated charcoal (g/L)	2.5	2.5	2.5	2.5

MS Murashige and Skoog medium (1962), Y3 Eeuwens (1976), *Picloram* 4-amino-3,5,6-trichloropicolinicacid, 2iP 2-isopentenyladenine, 2,4-D 2,4-dichlorophenoxyacetic acid.

performed by evaluating auxins associated with 2iP. Leaf explants were inoculated in a basic medium consisting of salts of Y3 culture medium and vitamins of MS medium, supplemented with 2.5 g/L activated charcoal and 2.5 g/L Phytagel. Auxins 2,4-D and Picloram were added to the medium at a concentration of 450 µM, with or without 2ip (20 µM) (Table I). Effect of the palm heart regions on callus induction: Three regions of the palm heart, namely, proximal (close to the meristem), median (intermediate area) and distal (close to the leaf apex), about 10 cm each long, were used as explants (Fig 1a, b). In current experiment, the basic culture medium comprised

salts of Y3 culture medium and vitamins of MS medium, supplemented with 2.5 g/L activated charcoal, 2.5 g/L Phytagel and 450 µM Picloram (according to previous results) (Table I).

Culture media were dispensed into 15 x 90 mm Petri dishes and cultures were sealed with transparent plastic film. During callus induction, the explants were incubated in the dark, at 25±2°C, and sub-cultured after every eight weeks on fresh media with the same composition. Explants were maintained in the induction medium until primary callus was obtained. In all culture media, pH was adjusted at 5.8±0.1 prior

to sterilization, by autoclaving at 120°C, under 1 atm pressure, for 20 minutes.

During the callus induction phase, calli were separated from the source explants and immediately transferred to a new culture medium of the same composition as the induction one, called multiplication medium (Table I). Leaf explants that originated the primary calli remained in the induction medium after each calli collection. Three calli collections were carried out at 6, 9 and 12 months. At each period, the calogenic formations were evaluated, individualized and inoculated in multiplication medium. Isolated calli were evaluated for multiplication (grams) and final increase rates in fresh mass, obtained by weight averages retrieved every 30 days during four successive subcultures.

Differentiation of somatic embryos

Two experiments were carried out to differentiate somatic embryos. Experiment 1 tested different concentrations of Picloram (0, 10, 20, 40, 80 and 120 μM), whilst different concentrations of 2,4-D - (0, 5, 10, 20, 40 and 80 μM) were used in Experiment 2. In the two experiments, Y3 culture medium, supplemented with Fe-EDTA and vitamins of MS medium, had the addition of 0.5 g/L glutamine, 2.5 g/L activated charcoal and 2.5 g/L Phytagel. During this phase, the calli were evaluated every 30 days to verify the differentiation of somatic embryos.

Regeneration of somatic embryos

For plant regeneration, the somatic embryos were transferred to Y3 culture medium supplemented with Fe-EDTA and vitamins of MS, without growth regulators and supplemented with 0.5 g/L glutamine, 2.5 g/L Phytagel and 2.5 g/L activated charcoal (Table I). The somatic embryos were incubated in a culture room at 25±2°C, in the presence of light, with an intensity of 100 μmol.m^{-2}.s^{-1}, until they showed enough growth to be individualized.
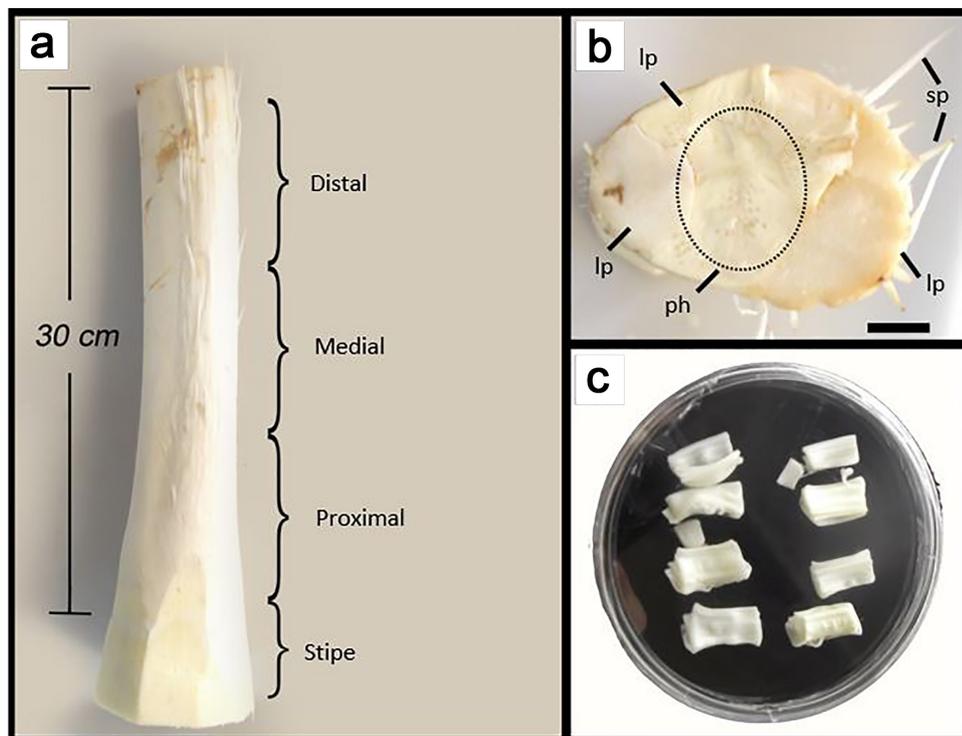
Figure 1. Morphological aspects of the palm heart when explants are excised for somatic embryogenesis induction in macaw palm (*Acrocomia aculeata*) (a-b); Leaf explants on culture medium (c). Abbreviations: ph: palm heart; lp: leaf petiole: sp: immature aculeus. Bars: a: 30 cm; b: 1 cm.

Morphological characterization and anatomical analysis

The morphological and histological characteristics of calli and explants' oxidation rate were evaluated in all experiments. The morphological analysis was performed visually and calli´s characteristics, observed during induction and multiplication stages of the different experiments, were described and photographed. In the case of histological analysis, samples of the material were retrieved *in vitro* at different stages of development. Samples of *in vitro* plant material consisted of immature leaves and leaves with callus. Samples of the different callus types obtained during the multiplication phase were also collected for characterization, coupled to somatic embryos for differentiation and development (Meira et al. 2019).

Fixation, dehydration and embedding stages of collected plant materials were carried out, following Luis & Scherwinski-Pereira (2014). Subsequently, longitudinal and transversal cuts (7 μm), obtained by manual rotary microtome (Leica®, RM212RT), were stretched and adhered to microscopic slides on a plate heated to 40°C. Sections were stained with toluidine blue (0.5%) (O'Brien et al. 1964). Results were recorded under a microscope locked to a computer with image capture software (LAS EZ 2.0).

Statistical analysis

All experiments were conducted in a completely randomized design (CRD). In the case of the experiment with auxins, associated or non-associated with 2iP, a factorial scheme 2 x 2 (auxins x presence or absent of cytokinins), totaling 4 treatments comprising ten replicates with eight explants per plot, was employed. In the experiment with different regions of the palm heart, associated with different harvest collection times, a factorial scheme 3 x 3 (regions of the palm heart x callus collection time), totaling 9 treatments comprising ten replicates with eight explants per plot, was employed. Experiment involving multiplication and differentiation of somatic embryos comprised three replicates with five calli per treatment. Data were submitted to variance analysis (ANOVA) and averages compared by Tukey's test, at 5% probability, calculated by Sisvar software (Ferreira 2011).

RESULTS AND DISCUSSION

Effect of auxins and 2iP on callus initiation

Different responses in callus formation were detected with regard to the effect of auxins associated or non-associated with 2iP, at different evaluation times (Table II). Picloram provided higher callus formation when compared to 2,4-D, at all collection times. The highest production rate (64.9%) of callus occurred with Picloram after a 9-month induction. Further, results in Table II showed that the addition of 2iP did not improve calli induction. It rather caused a decrease in the variable when associated with Picloram after an induction of 9 months. Nevertheless, several authors reported the use of 2iP in the callus induction phase in *Phoenix dactylifera* (Veramendi & Navarro 1996, Badawy et al. 2005, Eshraghi et al. 2005). Veramendi & Navarro (1996), using leaves of adult plants of *P. dactylifera*, reported positive effects of the association of 15 μM of 2iP with 453 μM of 2,4-D on calli induction. Contrastingly to current study, Badawy et al. (2005) reported that shoot apices had higher callus formation in the induction phase when the effect of 100 mg L^{-1} of 2,4-D associated with 15 μM of 2iP was tested. According to Abohatem et al. (2011), the use of cytokinins, such as 6-benzylaminopurine (BAP), during the callus induction phase in palm trees may increase the accumulation of phenolic

compounds and negatively affect callus induction. Consequently, the development of somatic embryos is impaired.

Effect of the palm heart region explants on callus induction

Significant results concerning callus formation were observed with regard to the different regions of the palm heart used as explant source, associated with different collection times (Table III). The maintenance of explants for 9 months in induction medium enhanced a higher average rate of callus formation (58.8%), regardless of palm heart region. In general, the farthest regions from the meristem (distal and median) were the most responsive in callus production considering the three periods of callus collection. Gueye et al. (2009a) observed that the region which was most distal to the meristem in the leaves of young date palm plants provided a better response to callus formation when compared to that at the more basal region. As noted by Gueye et al. (2009a), above results are a contrast to the general hypothesis that, in somatic embryogenesis studies on adult plants, explants from less differentiated or younger tissues are the most responsive to dedifferentiate and acquire embryogenic competence. As reported by Stasolla & Yeung (2003), different types of tissues in the same plant or the same tissue

at various stages of development may present different responses under *in vitro* conditions. In addition, the level of endogenous auxin and the orientation of explants (Almeida et al. 2012), especially the type and characteristic of tissue vascularization, are factors that probably influence *in vitro* responses (Gomes et al. 2017).

Callus multiplication

Table IV shows callus multiplication from different portions of leaves at several periods. Calli maintenance from the induction phase in multiplication medium provided continuous and significant increases in the calli fresh mass after 120 days of cultivation. The multiplication phase provided an increment of 6.22, 5.81, and 5.27 times in the initial mass of calli originating from the basal region and collected at 6, 9 and 12 months of induction, respectively.

For multiplication, the calli obtained during the induction phase were maintained in the medium with the same composition as that used during the induction phase. Konan et al. (2010) used the same procedure with calli of *Elaeis guineensis* and did not report any decline in mass calli proliferation. The above is a contrast to conclusions by Balzon et al. (2013) who observed that reduction in auxin concentration was important to establish repetitive cycles of cell division and inhibit differentiation processes,

Table II. Effect of Picloram and 2,4-D, associated (+) or not (-) with 2iP, on callus induction in young leaves obtained from the collected palm heart of macaw palm (*Acrocomia aculeata*) at different inoculation times.

Plant growth regulators (PGRs)	Callus induction (%)					
	6 months		9 months		12 months	
	(-)2iP	(+)2iP	(-)2iP	(+)2iP	(-)2iP	(+)2iP
2,4-D	9.5 bA	6.1 bA	25.9 bA	11.9 bA	4.6 bA	3.4 bA
Picloram	35.7 aA	30.1 aA	64.9 aA	34.2 aB	30.0 aA	32.6 aA

Upper case letters represent significant differences between presence or absence of 2iP at each period of calli induction, and lower case letters represent differences between PGRs at each condition of 2iP, according to Tukey's test ($p \leq 0.05$).

Table III. Effect of different leaf positions on percent callus induction in macaw palm (*Acrocomia aculeata*) at different callus induction periods (using the same leaf explant), associated with Picloram.

Leaf position in the palm heart	Calli induction (%)		
	6 months	9 months	12 months
Distal	62.3 aA	76.0 aA	20.6 bB
Median	27.5 bB	69.7 aA	56.0 aAB
Proximal	7.9 bA	30.8 bA	3.9 bA

Upper case letters represent significant differences among periods of calli induction, and lower case letters represent differences among leaf positions in the palm heart at each period of calli induction, according to Tukey's test ($p \leq 0.05$).

allowing calli of *E. guineensis* to multiply. According to Guerra & Handro (1998), successive subcultures in culture media with high auxin concentrations during the multiplication phase may inhibit the development of embryos during subsequent phases.

The influence of collection times and different regions of palm heart on callus multiplication in palm trees is still not fully documented. However, with regard to the influence of the region of the palm heart on the multiplication of primary calli, Luis (2013) insisted that there was no difference between regions when auxin concentrations were reduced at this stage.

Differentiation and *in vitro* germination of somatic embryos

Differently to what was expected, the differentiation of somatic embryos was observed during the callus multiplication stage. However, the rate of somatic embryos formation could not be evaluated due to the asynchrony process. For germination, the somatic embryos obtained were inoculated in culture medium devoid of growth regulators (Table I). In fact, differentiation of somatic embryos in palm trees is usually reported in the literature as an asynchronous appearance, as has been observed in the açaí

palm (Scherwinski-Pereira et al. 2012), peach palm (Steinmacher et al. 2007), oil palm (Silva et al. 2012) and macaw palm (Luis & Scherwinski-Pereira 2014). On the other hand, Padilha et al. (2015) observed that moving primary calli of *A. aculeata* from the induction medium directly to a culture medium, devoid of regulators and activated charcoal, caused the oxidation and death of the calli. Moreover, the early removal of auxin from the medium inhibited the formation of somatic embryos. Current experiment revealed that reduced concentrations of auxins failed to promote the formation of somatic embryos from the calli obtained. In current study, somatic embryos were missing during the differentiation experiments of the calli in liquid medium (data not shown) and the calli remained in the multiplication stage. Contrastingly, Steinmacher et al. (2011) observed that calli of *Bactris gasipaes* exhibited high embryogenic capacity and high frequency of somatic embryos in liquid medium. However, improvements in the maturation stages of somatic embryos and germination in a semi-solid medium were reported.

Further, non-germinating somatic embryos collapsed and grew abnormally. However, germinating somatic embryos went into senescence and died after a few weeks of

Table IV. Effect of different leaf positions in the palm heart, periods of callus induction (using the same leaf explant), and multiplication time on the increase of fresh mass of calli in macaw palm (*Acrocomia aculeata*).

Leaf position in the palm heart	Callus harvest (months)	Callus multiplication (days of culture)					Fresh mass increase (x)
		Initial weight (gr)	30	60	90	120	
Distal	6	2.04±0.7	4.30±0.5	5.89±1.9	7.84±1.3	8.23±0.1	4.04
	9	3.53±0.1	4.80±0.7	7.65±1.4	9.62±0.9	11.96±0.5	3.38
	12	1.85±0.4	2.76±0.8	4.65±0.6	5.55±0.4	5.52±0.4	2.98
Median	6	0.60±0.01	1.36±0.3	1.96±0.5	2.04±0.4	2.09±0.2	3.48
	9	5.25±1.2	8.11±3.2	10.25±3.6	15.94±5.3	23.23±6.7	4.42
	12	4.47±2.2	6.87±5.1	11.85±6.5	14.94±8.8	16.10±9.6	3.60
Proximal	6	0.36±0.09	0.93±0.2	1.56±0.3	1.93±0.2	2.23±0.1	6.22
	9	0.40±0.005	0.62±0.03	1.27±0.1	1.73±0.2	2.33±0.3	5.81
	12	0.22±0.08	0.44±0.1	0.71±0.1	1.0±0.1	1.19±0.1	5.27

Each value represents the mean of three replicates ± standard error.

germination. Senescence in micropropagated plants may be associated with several causes. It is more common when they are exposed for prolonged periods to growth regulators, which affect endogenous levels of auxin, cytokinin and, primarily, ethylene (Jones 2001). Results corroborate those by Moura et al. (2009) who, although observing the germination of somatic embryos of macaw palm from zygotic embryos, failed to obtain the formation of complete plants, since there was no differentiation of the aerial part. Results were similar to those in current study, since there was no success in the regeneration of plants. Current study provides important information on *in vitro* multiplication via somatic embryogenesis starting from macaw palm leaves. However, further tests are necessary during differentiation, maturation and germination stages of somatic embryos to obtain complete macaw palm plants *in vitro*.

Morphological characterization

During the callus induction phase, the explants' first response occurred during the first three days of culture, whereby the swelling of the inoculated leaves, characterized as an increase in the volume of the explant, was registered (Fig. 1c, 2a). Three months after inoculation, the first callus formation at the edges of the explants was detected in the treatment with Picloram alone (Fig. 2b). The oxidation of leaf material started during this period.

These responses on callus formation are associated with *in vitro* culture, with high concentrations of synthetic auxins. No explant responses were extant in the absence of synthetic auxins. Similar results were obtained in researches with high concentrations of synthetic auxins (Othmani et al. 2009). Picloram is notable among these auxins, with responses detected in other palm trees, such as the date palm (Gueye et al. 2009b), the oil palm (Scherwinski-Pereira et al. 2010, Silva et al. 2012, Padua et al. 2013) and
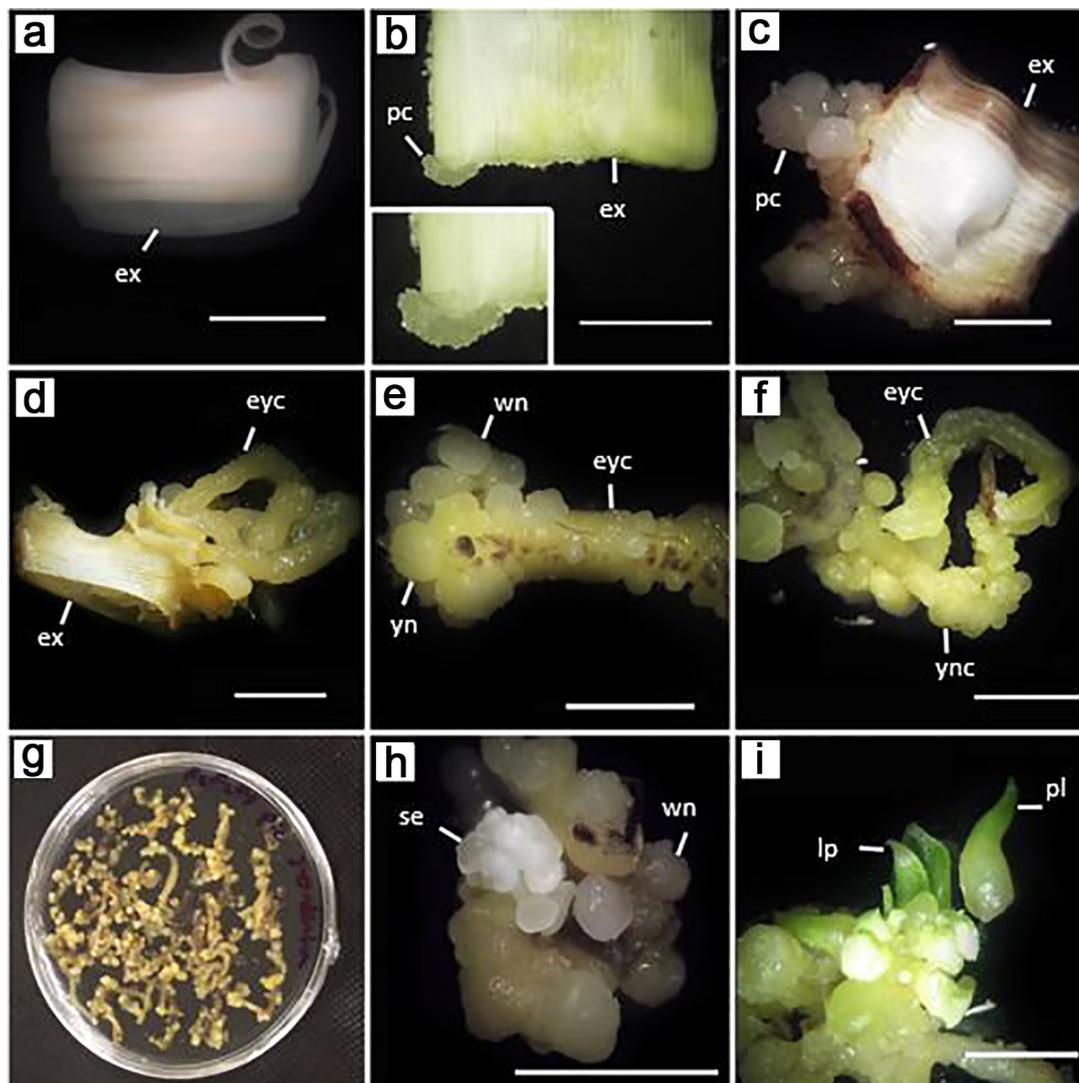
Figure 2. Morphological aspect of somatic embryogenesis in macaw palm (*Acrocomia aculeata*) from leaf tissues. a: Explant. b: Explant after 3 months of cultivation, with inception of callus formation. c: Explant at 4 months, with development of primary calli at the edges. d: Explants at 6 months with elongated yellowish calli. e: Elongated yellowish callus at 9 months of cultivation, showing calli formation with white and yellow nodular appearance. f: Elongated yellowish callus at 9 months of cultivation, showing calli with a yellow nodular appearance. g: Petri dish at 9 months of in vitro culture, showing amount of calli formed after multiplication stage. h: Somatic embryos observed after 12 months of *in vitro* culture. i: Germination of somatic embryos with leaf primordia. Legend: pc: primary callus, ex: explant, eyc: elongated yellowish callus; WN: whitish nodular callus; YW: yellowish nodular callus; se: somatic embryos; lp: leaf primordia; pl: young plantlets. Bars: 5 mm.

the açaí palm (Scherwinski-Pereira et al. 2012), besides macaw palm (Padilha et al. 2015, Luis & Scherwinski-Pereira 2014).

Calogenic masses were developed between four and six months of induction (Fig. 2c, d). They exhibited morphological characteristics, predominantly elongated and yellowish, mainly appearing at the edges of the sectioned explants in a medium containing Picloram (Table V). The callus observed in the medium supplemented with 2,4-D had a mucilaginous consistency and no definite shape. After 9

months of callus proliferation, the emergence of a callus with nodular appearance and coloration ranging between yellow and a whitish hue was detected, especially in explants under the effect of Picloram (Fig. 2e, f). During this period, an intensification of calli multiplication was also reported (Fig. 2g).

During the multiplication phase, proembryos emerged from white or yellowish nodular calli. After 12 months in culture medium, the proembryos progressed to somatic embryos in the globular stage (Fig. 2h, i).

There was a significant increase in the explant oxidation from the sixth month in callus-induction medium, especially those under the effect of 2,4-D. Longer periods of cultivation (9 and 12 months) under this auxin provided a higher oxidation rate, causing the partial necrosis of the materials. Thus, oxidation seems to be a limiting factor for the calli proliferation for long periods. After transfer to *in vitro* culture, plant tissue in some species may be oxidized due to the release of phenolic compounds. Accumulation of polyphenols and oxidation products around the excised surface usually modifies the composition of the culture medium and, consequently, absorption. In fact, toxic substances normally inhibit the growth of explants, not infrequently causing their death (Van Winkle et al. 2003).

When certain species are cultured *in vitro* with high auxin concentrations, they may exhibit the oxidation of explants, a commonly observed fact in palms (Moura et al. 2009, Scherwinski-Pereira et al. 2010). The issue may be solved when activated charcoal is added to the medium, which adsorbs the inhibiting substances released by the tissues (Van Winkle et al. 2003). In current assay, the combination of activated charcoal and Picloram maintained tolerable levels of oxidation, unlike that observed for 2,4-D. Further, Padua et al. (2013), working on young

leaves of oil palm associated with Picloram, detected four types of calli which were classified according to color and shape: translucent-elongated, translucent-aqueous, beige-globular and white-globular. According to the anatomical and ultrastructural characteristics, the beige-globular and white-globular calli had a greater embryogenic potential. Moreover, Sané et al. (2012) reported, in date palm leaf explants, highly callogenic activity, characterized by the proliferation of compact globular-type calli, using 2,4-D in two of the four cultivars tested.

The aptitude for primary callogenesis appears to be strongly dependent on the type of explant, genotype and growth regulators used (Sané et al. 2012). Other authors working with palm species found similar results on its morphology when they employed Picloram as a regulator for callus induction in *in vitro* culture, such as in *Areca catechu* (Karun et al. 2004), peach palm (Steinmacher et al. 2011), oil palm (Scherwinski-Pereira et al. 2010, Silva et al. 2012) and açaí palm (Scherwinski-Pereira et al. 2012).

Histological analysis

The histological analysis of leaf explants after 60 days revealed the inception of callus formation from the vascular bundle (Meira et al. 2019). This was evidenced by cells with typical meristematic characteristics (smaller diameter, isodiametric form, cells with large nuclei and dense cytoplasm) in process of cell division (Fig. 3a). After 90 days of culture, continuity of callus formation was perceived in the vascular bundle and observed at an advanced stage of development. At this stage, cell proliferation inside the callus was reported, resulting in the removal of epidermal surfaces, whereby the calli were exposed on the leaf surface (Fig. 3b). Repeated mitotic divisions, at different directions, formed isolated cell clusters that subsequently gave rise to inception of primary calli. Rose et al. (2006) suggested that

Table V. Effect of different leaf positions in the palm heart, periods of callus induction or non-associated with 2iP, and the leaf position in the palm heart on callus responses and morphology and degree of oxidation in macaw palm (*Acrocomia aculeata*).

Callus harvest	Auxin	Leaf position in the palm heart	Cytokinin		Degree of callus oxidation[a]	
			2iP(-)	2iP(+)	2iP(-)	2iP(+)
6 months	Picloram	1	Yellowish elongated	Yellowish elongated, Yellowish nodular	+	+
		2	Yellowish elongated,	Yellowish elongated	+	+
		3	Yellowish elongated,	Yellowish elongated	+ +	+ +
	2,4-D	1	No callus formed	No callus formed	+	+ +
		2	Whitish mucilaginous	No callus formed	+	+
		3	Whitish mucilaginous	Whitish mucilaginous	+ +	+ +
9 months	Picloram	1	Yellowish elongated, Yellowish nodular	Yellowish elongated, Yellowish nodular	+	+ +
		2	Yellowish nodular	Yellowish elongated, Yellowish nodular	+	+ +
		3	Yellowish elongated, Yellowish nodular	Yellowish elongated, Yellowish nodular	+ +	+ +
	2,4-D	1	No callus formed	No callus formed	+ +	+ + +
		2	Whitish nodular	Whitish nodular	+ + +	+ + +
		3	No callus formed	Whitish nodular	+ +	+ +
12 months	Picloram	1	Yellowish elongated, Yellowish nodular, Whitish nodular	Yellowish elongated, Yellowish nodular, Whitish nodular	+ +	+ +
		2	Yellowish elongated, Yellowish nodular	Yellowish elongated, Yellowish nodular, Whitish nodular	+ +	+ +
		3	Yellowish nodular, Whitish nodular	yellowish nodular, Whitish nodular	+ +	+ +
	2,4-D	1	No callus formed	Whitish nodular	+ + +	+ +
		2	No callus formed	No callus formed	+ + +	+ + +
		3	No callus formed	No callus formed	+ +	+ +

[a]**Degree of callus formation: + low; + + moderate; + + + high. Leaf position: 1- Proximal; 2- Median; 3- Distal.**
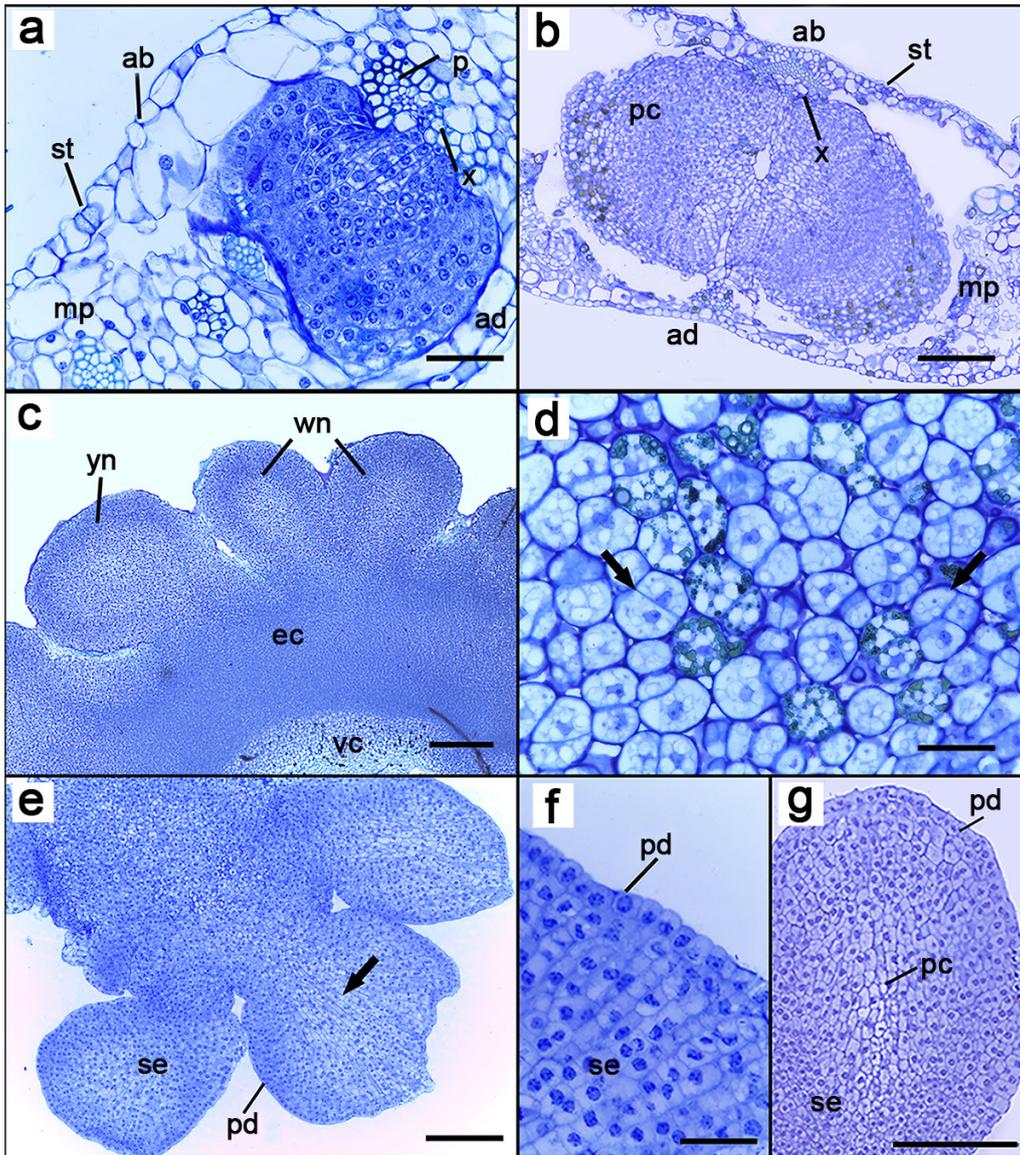
Figure 3. Histological events of macaw palm (*Acrocomia aculeata*) during somatic embryogenesis. a: Leaf after 60 days, showing inception of primary callus in the vascular bundle region. b: Callus formed in the vascular bundle region after 90 days of cultivation, showing epidermal face separation. c: Anatomical cut of the yellow elongated callus showing formation of white and yellow nodular calli on its surface. d: Proembryos delimited by thickening of the cell wall. e: Cluster of somatic embryos delimited by the protoderm, with elongated cells in the central region (arrow). f: Detail of the protoderm in the peripheral region of the embryo. g: Detail of the procambium in the central region of the embryo. Abbreviations: (ab) abaxial epidermis; (ad) adaxial epidermis; (st) stomata; (p) phloem; (x) xylem; (mp) mesophyll; (pc) primary callus; yn: yellowish nodular callus; wn: whitish nodular callus; (ec) elongated callus; (vc) vacuolated cells; (se) somatic embryo; (pd) protoderm; (pc) procambium. Bars: a-c: 0.1 mm; d-f: 0.05 mm; g: 0.2 mm.

the procambium cells, that would normally be differentiated in vascular tissues constituting the rib of the leaf after being stimulated by the auxin in the culture medium, dedifferentiated and reprogramed themselves, enhancing cell proliferation and callus formation.

After being transferred to a new medium for maintenance and proliferation, the elongated

calli obtained in the induction phase started to multiply. New calli strains were formed from the elongated calli in the first phase, exhibiting a yellow and whitish nodular appearance. The strain of yellow nodular calli exhibited anatomical characteristics similar to the whitish nodules, consisting predominantly of meristematic cells (Fig. 3c). Classifying oil palm callus strains, Padua et al. (2013) suggested that beige-globular and white-globular calli revealed a higher embryogenic potential based on the anatomical aspects studied. Similarly, Luis & Scherwinski-Pereira (2014), working with zygotic embryos, observed that the calli of nodular strains in macaw palm showed greater embryogenic potential. Differentiation of somatic embryos from these calli was recorded.

Further, the formation of proembryos was registered during the callus proliferation phase (Fig. 3d). As underscored by Silva et al. (2013) and Balzon et al. (2013), the formation of proembryos could be detected from the development of the mass of embryogenic calli, characterized by the isolation of a group of cells through the apparent thickening of the wall. Verdeil et al. (2001) and Sané (2006) studied the acquisition of competence of embryogenic calli respectively in *Cocos nucifera* and in *P. dactylifera*, and mentioned the isolation of proembryos due to the thickening of the cell wall, characterized by closure of callose deposition in plasmodesmata. The above suggested that these events led towards the isolation required for cell reprogramming and inception of embryogenic events.

Somatic embryos, formed in the proliferation medium, were seen in a globular stage with whitish coloration. They consisted of meristem cells of different shapes and sizes. The outermost layer of cells of the embryos is the protoderm, or rather, the first tissue that may be identified histologically in the process of embryogenesis (West & Harada 1993), as well as a prerequisite for the development of later embryogenic stages (De Jong et al. 1992). Several cells in the central region of the embryo exhibit an elongated shape, with the larger axis parallel to the larger axis of the somatic embryo, characterizing procambium cells (Fig. 3e-g).

CONCLUSION

The current study describes, for the first time, the different phases of the somatic embryogenesis in macaw palm from leaf tissues of adult plants. The protocol confirms the importance of auxins at high concentrations, particularly at 450 µM using Picloram, in callus induction and, later, formation of somatic embryos. It is also worth noting the greater responsiveness of the explants from heart regions farthest from the plant meristem, which reduces the chances of irreversible damage to the donor plant. Calli multiplication, conventionally called "calli harvest" or "calli collection", is efficient for the production of a satisfactory volume of calli. It is easy to perform and it allows the formation of somatic embryos. Finally, somatic embryos could be obtained from the somatic tissues of adult macaw palm plants. However, this phase still requires further studies, mainly to synchronize somatic embryos differentiation and to improve plant regeneration rates.

REFERENCES

ABERLENC-BERTOSSI F, NOIROT M & DUVAL YBA. 1999. BA enhances the germination of oil palm somatic embryos derived from embryogenic suspension cultures. Plant Cell Tiss Organ Cult 56(1): 53-57.

ABOHATEM M, ZOUINE J & EL HADRAMI I. 2011. Low concentration of BAP and high rate of subcultures improve the establishment and multiplication of somatic embryos in date palm suspension cultures by limiting oxidative browning associated with high levels of total phenols and peroxidase activities. Sci Horticult 130: 344-348.

ALMEIDA M, ALMEIDA CV, GRANER EM, BRONDANI GE & ABREU-TARAZI MF. 2012. Pre-procambial cells are niches for pluripotent and totipotent stem-like cells for organogenesis and somatic embryogenesis in the peach palm: a histological study. Plant Cell Rep 31: 1495-1515.

BADAWY EM, HABIB, AMA, EL-BANA A & YOSRY GM. 2005. Propagation of date palm (*Phoenix dactylifera*) plants by using tissue culture technique. Arab J Biotechnol 8: 343-354.

BALZON TA, LUIS ZG & SCHERWINSKI-PEREIRA JE. 2013. New approaches to improve the efficiency of somatic embryogenesis in oil palm (*Elaeis guineensis* Jacq.) from mature zygotic embryos. *In Vitro* Cell Dev Biol Plant 49: 41-50.

BEULÉ T, CAMPS C, DEBIESSE S, TRANCHANT C, DUSSERT S, SABAU X, JALIGOT E, ALWEE SSRS & TREGEAR JW. 2011. Transcriptome analysis reveals differentially expressed genes associated with the mantled homeotic flowering abnormality in oil palm (*Elaeis guineensis*). Tree Genet Genomes 7: 169-182.

DE JONG AJ, CORDEWENER J, LO SCHIAVO F, TERZI M, VANDEKERCKHOVE J, VAN KAMMEN A & DE VRIES SC. 1992. A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4: 425-433

DIAS LADS. 2011. Biofuel plant species and the contribution of genetic improvement. Crop Breed Appl Biotechnol. 11: 16-26.

DUDITS D, BÖGRE L & GYÖRGYEY J. 1991. Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. J Cell Sci 99: 475-484.

EEUWENS CJ. 1976. Mineral requirements for growth and callus initiation of tissue explants Excised from mature coconut palms (*Cocos nucifera*) and cultured *in vitro*. Physiol Plant 36: 23-28.

EKE CR, AKOMEAH P & ASEMOTA O. 2005. Somatic embryogenesis in date palm (*Phoenix dactylifera* L.) from apical meristem tissues from 'Zebia' and 'Loko' landraces. Afr J Biotechnol 4: 244-246.

EMONS AMC. 1994. Somatic embryogenesis: cell biological aspects. Acta Bot Neerl 43: 1-14.

ESHRAGHI P, ZARGHAMI R & MIRABDULBAGHI M. 2005. Somatic embryogenesis in two Iranian date palm cultivars. Afr J Biotechnol 4: 1309-1312.

FERNÁNDEZ-GUIJARRO B, CELESTINO C & TORIBIO M. 1995. Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber*. Plant Cell Tiss Organ Cult 41: 99-106.

FERREIRA DF. 2011. Sisvar: a computer statistical analysis system. Ciênc agrotec 35: 1039-1042.

GOMES HT, BARTOS PMC & SCHERWINSKI-PEREIRA JE. 2017. Dynamics of morphological and anatomical changes in leaf tissues of an interspecific hybrid of oil palm during acquisition and development of somatic embryogenesis. Plant Cell Tiss Organ Cult 131: 269-282.

GRANJA MMC, MOTOIKE SY, ANDRADE APS, CORREA TR, PICOLI EAT & KUKI KN. 2018. Explant origin and culture media factors drive the somatic embryogenesis response in *Acrocomia aculeata* (Jacq.) Lodd. ex Mart., an emerging oil crop in the tropics. Ind Crops Prod 117: 1-12.

GUERRA MP & HANDRO W. 1998. Somatic embryogenesis and plant regeneration in different organs of *Euterpe edulis* Mart. (Palmae): control and structural features. J Plant Res 111: 65-71.

GUEYE B ET AL. 2009a. Acquisition of callogenic capacity in date palm leaf tissues in response to 2,4-D treatment. Plant Cell Tiss Organ Cult. 99: 35-45.

GUEYE B, SAID-AHMED H, MORCILLO F, BORGEL A, SANÉ D, HILBERT JL, VERDEIL JL & BLERVACQ AS. 2009b. Callogenesis and rhizogenesis in date palm leaf segments: are there similarities between the two auxin-induced pathways? Plant Cell Tiss Organ Cult 98: 47-58.

HENDERSON A, GALEANO G & BERNAL R. 1995. Field guide to the palms of the Americas. Princeton (NJ): Princeton University Press, 1st ed., 352 p.

HIANE PA, RAMOS FILHO MM, RAMOS MIL & MACEDO MLR. 2005. Bocaiúva, *Acrocomia aculeata* (Jacq.) Lodd., Pulp and kernel oils: characterization and fatty acid composition. Braz J Food Technol 8: 256-259

JONES AM. 2001. Programmed cell death in development and defense. Plant Physiol 125: 94-97.

KARUN A, SIRIL E, RADHA E & PARTHASARATHY VA. 2004. Somatic embryogenesis and plantlet regeneration from leaf and inflorescence explants of arecanut (*Areca catechu* L.) Curr Sci 86: 1623-1628.

KOMATSUDA T, LEE W & OKA S. 1992. Maturation and germination of somatic embryos as affected by sucrose and plant growth regulators in soybeans *Glycine gracilis*

Skvortz and *Glycine max* (L.) Merr. Plant Cell Tiss Organ Cult 18: 103-113.

KONAN KE, GASSELIN TD, KOUADIO YJ, FLORI A, RIVAL A, DUVAL Y & PANNETIER C. 2010. *In vitro* conservation of oil palm somatic embryos for 20 years on a hormone-free culture medium: characteristics of the embryogenic cultures, derived plantlets and adult palms. Plant Cell Rep 29: 1-13.

LLERAS E & CORADIN L. 1985. Palmeras nativas como oleaginosas: situación actual y perspectivas para América Latina. In: Forero LE (Ed). Informe del seminário taller sobre oleaginosas promisorias. Asociación Interciencia/Asociación Colombiana para el Avance de la Ciencia: Bogotá, 1st ed., 92-143 p.

LUIS ZG. 2013. Estratégias para a embriogênese somática e conservação *ex situ* de germoplasma de macaúba [*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.]. Tese de doutorado. Universidade de Brasília.

LUIS ZG & SCHERWINSKI-PEREIRA JE. 2014. An improved protocol for somatic embryogenesis and plant regeneration in macaw palm (*Acrocomia aculeata*) from mature zygotic embryos. Plant Cell Tiss Organ Cult 118: 1-12.

MEIRA FS, LUIS ZG, SILVA-CARDOSO IMA & SCHERWINSKI-PEREIRA JE. 2019. Developmental pathway of somatic embryogenesis from leaf tissues of macaw palm (*Acrocomia aculeata*) revealed by histological events. Flora 250: 59-67.

MOURA EF, MOTOIKE SY, VENTRELLA MC, SÁ JÚNIOR AQ & CARVALHO M. 2009. Somatic embryogenesis in macaw palm (*Acrocomia aculeata*) from zygotic embryos. Sci Hort 119: 447-454.

MURASHIGE T & SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

NGUYEN QT, BANDUPRIY AHDD, LOPEZ-VILLALOBOS A, SISUNANDAR S, FOALE M & ADKINS SW. 2015. Tissue culture and associated biotechnological interventions for the improvement of coconut (*Cocos nucifera* L.): a review. Planta 242: 1059-1076.

O' BRIEN, TP, FEDER N & MCCULLY ME. 1965. Polychromatic staining of plant cell walls by toluidine blue. Protoplasma 59: 368-373.

OTHMANI A, BAYOUDH C, DRIRA N, MARRAKCHI M & TRIFI M. 2009. Somatic embryogenesis and plant regeneration in date palm *Phœnix dactylifera* L., cv. Boufeggous is significantly improved by fine chopping and partial desiccation of embryogenic callus. Plant Cell Tiss Organ Cult 97: 71-79.

PADILHA JHD, RIBAS LLF, AMANO E & QUOIRIN M. 2015. Somatic embryogenesis in *Acrocomia aculeata* Jacq. (Lodd.) ex Mart using the thin cell layer technique. Acta Bot Bras 29: 516-523.

PADUA MS, PAIVA L, LABORY CRG, ALVES E & STEIN VC. 2013. Induction and characterization of oil palm (*Elaeis guineensis* Jacq.) pro-embryogenic masses. An Acad Bras Cienc 85: 1545-1556.

PAN MJ & VAN STADEN J. 1998. The use of charcoal in *in vitro* culture - a review. Plant Growth Regul 26: 155-163.

POETSCH J, LEWANDOWSKI DHI, OBERLÄNDER D & HILGER T. 2012. *Acrocomia aculeata*—a sustainable oil crop. Rural 21: 41-44.

ROBERTS DR, FLINN BS, WEBB DT, WEBSTER FB & SUTTON BCS. 1989. Characterization of immature embryos of interior spruce by SDS–PAGE and microscopy in relation to their competence for somatic embryogenesis. Plant Cell Rep 8: 285-288.

ROSE RJ, WANG XD, NOLAN KE & ROLFE BG. 2006. Root meristems in *Medicago truncatula* tissue culture arise from vascular derived procambial-like cells in a process regulated by ethylene. J Exp Bot 57: 2227-2235.

SANÉ D, ABERLENC-BERTOSSI F, DIATTA L, GUEYE B, DAHER A, SAGNA M, DUVAL Y & BORGEL A. 2012. Influence of growth regulators on callogenesis and somatic embryo development in date palm (*Phoenix dactylifera* L.) Sahelian cultivars. Sci World J 2012: 1-8.

SANÉ D, ABERLENC-BERTOSSI F, GASSAMA-DIA YK, SAGNA M, TROUSLOT MF, DUVAL Y & BORGEL A. 2006. Histocytological analysis of callogenesis and somatic embryogenesis from cell suspensions of date palm (*Phoenix dactylifera*). Ann Bot 98: 301-308.

SCARIOT AO, LLERAS E & HAY JD. 1991. Reproductive biology of the palm *Acrocomia aculeata* in central Brazil. Biotropica 23: 12-22.

SCHERWINSKI-PEREIRA JE, GUEDES RS, FERMINO PCP, SILVA TL & COSTA FHS. 2010. Somatic embryogenesis and plant regeneration in oil palm using the thin cell layer technique. *In Vitro* Cell Dev Biol -Plant 46: 378-385.

SCHERWINSKI-PEREIRA JE, GUEDES RS, SILVA RA, FERMINO JR, LUIS ZG & FREITAS EO. 2012. Somatic embryogenesis and plant regeneration in açaí palm (*Euterpe oleracea*). Plant Cell Tiss Organ Cult 109: 501-508

SILVA JA. 2013. Avaliação do Programa Nacional de Produção e Uso do Biodiesel no Brasil – PNPB. Rev Pol Agr 22: 18-31.

SILVA RC, LUIS ZL & SCHERWINSKI-PEREIRA JE. 2012. Differential responses to somatic embryogenesis of different genotypes of Brazilian oil palm (*Elaeis guineensis* Jacq.). Plant Cell Tiss Organ Cult 111: 59-67.

SOH AC, WONG G, TANG CC, CHEW PS, CHONG SP, HO YW, WONG CK, CHO CN, NOR AZURA H & KUMAR H. 2011. Commercial-scale propagation and planting of elite oil palm clones: Research and development towards realization. J Oil Palm Res 23: 935-952.

STEINMACHER DA, CLEMENT CR & GUERRA MP. 2007. Somatic embryogenesis from immature peach palm inflorescence explants: towards development of an efficient protocol. Plant Cell Tiss Organ Cult 89: 15-22.

STEINMACHER DA, GUERRA MP, SAARE-SURMINSKI K & LIEBEREI R. 2011. A temporary immersion system improves *in vitro* regeneration of peach palm through secondary somatic embryogenesis. Ann Bot 108: 1463-1475.

STASOLLA C & YEUNG EC. 2003. Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tiss Organ Cult 74: 15-35.

TEIXEIRA JB, SÖNDAHL MR & KIRBY EG. 1986. Asexual embryogenesis in mature embryo callus of palms (*Acrocomia aculeata* and *Elaeis oleifera*). VI International Congress of Plant Tissue and Cell Culture, August 3-8, University of Minnesota, Minneapolis, Minnesota, USA, 6th ed., 191 p.

TEIXEIRA LC. 2005. Potencialidades de oleaginosas para produção de biodiesel. Inf Agrop 26: 18-27.

TOMLINSON PB & HUGGETT BA. 2012. Cell longevity and sustained primary growth in palm stems. Am J Bot 99: 1891-1902.

VAN WINKLE S, JOHNSON S & PULLMAN GS. 2003. The impact of gelrite and activated carbon on the elemental composition of plant tissue culture media. Plant Cell Rep 21: 1175-1182.

VERAMENDI J & NAVARRO L. 1996. Influence of physical conditions of nutrient medium and sucrose on somatic embryogenesis of date palm. Plant Cell Tiss Organ Cult 45: 159-164.

VERDEIL JL, HOCHER V, HUET C, GROSDEMANGE F, ESCOUTE J, FERRIERE N & NICOLE M. 2001. Ultrastructural Changes in Coconut Calli Associated with the Acquisition of Embryogenic Competence. Ann Bot 88: 9-18.

WANDECK FA & JUSTO PG. 1988. A macaúba, fonte energética e insumo industrial: sua significação econômica no Brasil. Simpósio sobre o Cerrado, Savana, 6th ed., Brasília, p 541-577.

WEST MAL & HARADA JJ. 1993. Embryogenesis in higher plants: an overview. Plant Cell 5: 1361-1369.

WILLIAMS ES & MAHESWARAN B. 1986. Somatic embryogenesis: Factors influencing coordinated behaviour of cells as an embryogenic group. Ann Bot 57: 443-462.

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