Somatic embryogenesis in Euterpe edulis Martius is improved by wounding, explant orientation, and suspension culture

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Abstract

Illegal extraction of the heart of palm is threatening *Euterpe edulis* Martius with extinction. Here, we investigated the induction of somatic embryogenesis in segments of *E. edulis* seedlings as a means of propagating this palm species. Immature seeds were harvested from the wild and germinated *in vitro*. After six months, the seedlings were excised in the middle of the caulicle and cut either transversely into two explants, or longitudinally with the wounded surface face down, up or sideways on the medium. Friable calli formed from upward facing explants were transferred to a suspension culture with different concentrations of picloram (15, 25, 35, and 45 μM) and then matured in the presence of abscisic acid (1, 5, 10, and 20 μM). Explants derived from upward facing segments were placed in culture medium containing L-glutamine or hydrolyzed casein (0.0, 0.5, and 1.0 g L^{-1}). Induction in medium with 150 μM picloram was strongest for stems with longitudinal wounds positioned upward and/or sideways; while medium with 15 μM picloram enabled strong growth of friable calli. The highest average number of proembryos (16.33) was obtained with 1.0 g L^{-1} hydrolyzed casein and differentiation of somatic embryos was greatest with 1 μM abscisic acid. Therefore, somatic embryogenesis of *E. edulis* is best achieved by placing segments from longitudinally wounded stems face up on medium containing 150 μM picloram, followed by suspension cultivation with 15 μM picloram and maturation with 1 μM abscisic acid.

1 INTRODUCTION

Euterpe edulis Martius is a palm tree native to the Atlantic Forest, whose sweet heart of palm is an economically valuable delicacy. However, the heart of palm includes the apical meristem, and its harvest leads to the death of the plant. As a result, *E. edulis* figures now on the endangered species list (Wendt et al. 2011; Schulz et al. 2016). A more sustainable exploitation of this tree involves its fruits, which have sensory characteristics comparable to those of açaí (*Euterpe oleracea* Martius), but with superior antioxidant, anti-inflammatory, and cardioprotective properties (Schulz et al. 2016; Cardoso et al. 2018).

As it does not tiller, seeds are the only means by which *E. edulis* propagates naturally. However, the seeds are recalcitrant, lose their vigor quickly after harvest, and present slow and uneven germination (Cursi and Cicero 2014; Schulz et al. 2016). An alternative propagation route is represented by somatic embryogenesis, which could help preserve the species but also allow the selection of superior progeny for commercial plantations.

Induction of complex structures, such as somatic embryos, in explants after an injury initiates a genetically determined developmental program for wound healing, cell dedifferentiation, cell proliferation, and formation of new organs (Lup et al. 2016). When cauline segments are cut in half, auxin transported from the cauline apex accumulates at the upper end of the wound site and is depleted at the lower end, inducing gene expression and cell proliferation to heal the gap at the wound site (Ikeuchi et al. 2013).

The genes of the *WOUND-INDUCED DEDIFERENTIATION* family (WIND1, WIND2, WIND3, and WIND4) are key regulators of this response, as injury promotes changes in genome methylation in the wound region (Ikeuchi et al. 2013; IWASE et al. 2017; FARIA et al. 2019). Sequential activation of the WIND1 gene and the embryonic regulator *LEAFY COTYLEDON2* (LEC2) induces somatic embryogenesis at both excised and non-excised sites (Iwase et al. 2015).

Injury and specific orientation of the explant in culture medium can increase responsiveness, which is necessary for developmental plasticity and morphogenesis of new organs (Faria et al. 2019). These new organs can be used to establish highly productive embryogenic suspension cultures.

Medium consistency and supplementation with growth regulators directly influence the response of plant tissue. Consistency changes with the concentration of gelling agent, which may affect the availability of nutrients, oxygen, water, and osmotic potential of the medium (Costa et al. 2012). At the same time, growth in liquid medium can be affected by oxygen deprivation and overhydration. To meet the respiratory needs of submerged cells and tissues, the concentration of oxygen can be increased through constant agitation of the medium or by allowing the samples to be in direct contact with air (George et al. 2008).

One of the most used inducers of embryogenesis is picloram (4-amino-3,5,6-trichloropicolinic acid), a synthetic organic chemical that belongs to the group of pyridinecarboxylic acid herbicides (Marco-Brown et al. 2014), also known as auxin mimetics. Picloram benefits from efficient absorption and mobilization, as well as rapid metabolism in specific parts of the plant (Karun et al., 2004). It provides good results in palm trees, such as *Bactris gasipaes* (Valverde et al. 1987; Steinmacher et al. 2007), *Calamus merrillii* Becc., *Calamus subinermis* Wendl. ex Becc. (Goh et al. 1999, 2001), E. *oleracea* (Scherwinski-Pereira et al. 2012), *Euterpe precatoria* (Ferreira et al. 2022)d *edulis* (Oliveira et al. 2022; Mello et al. 2023).

In the present study, we analyzed the embryogenic response in segments of *E. edulis* seedlings subjected to different lesions, orientation of the explant on culture medium, cultivation mode, and amino acid supplementation.

2 MATERIAL AND METHODS

2.1 Plant material

The fruits of *E. edulis* were harvested from a mother plant of 6.7 m in height and diameter at breast height (1.30 m) of 13.4 cm, located in the district of Pedra Menina, municipality of Dores do Rio Preto, ES, Brazil. The plant was growing near Caparaó National Park, at coordinates 20°32'45.4" S, 41°49'35.7" W, and an altitude of 951 m. The fruits were harvested approximately 168 days after anthesis and were sent to the Laboratory of Forest Seeds and Laboratory of Plant Tissue Culture at the Center for Agricultural Sciences and Engineering, Federal University of Espírito Santo.

After cleaning the fruits, the tegument was removed and immersed in 2% ascorbic acid to prevent phenolic oxidation. Using a laminar flow chamber, *E. edulis* seeds were immersed in 70% ethyl alcohol for 1 min, 2.5% sodium hypochlorite (Candura) for 10 min, and 3 g L^{-1} amoxicillin solution (Germed) for 10 min, with triple washes in distilled and autoclaved water after each step.

The seeds were arranged in test tubes (25 × 150 mm) containing 10 mL MS culture medium (MURASHIGE and SKOOG 1962) (Sigma), supplemented with 1 g L^{-1} polyvinylpyrrolidone (PVP) (Synth), 30 g L^{-1} sucrose (Dinâmica), 0.1 g L^{-1} myo-inositol (Sigma), and 6 g L^{-1} agar (Kasvi). The pH of the medium was adjusted to 5.7 ± 0.1 with 1.0 N KOH (Alphatec) and/or HCl (Vetec) before autoclaving. The explants were maintained in a growth room at a temperature of 27 ± 2°C, photoperiod of 8/16 h light/dark, and light intensity of 30 µmol m^{-2} s^{-1} until seedlings were obtained (six months).

2.2 Induction

Using a laminar flow chamber, a 20-mm median portion of the seedlings was excised (Fig. 1). The excised segment was sectioned either transversely into two 10-mm explants (TW), or longitudinally with the wounded surface facing downward (LWD), upward (LWU) or sideways (LWS) on the culture medium (Experiment I) (Fig. 1h–o).

The explants were placed in Petri dishes (90 × 15 mm), with basal MS medium (Sigma) supplemented with 1 g L^{-1} PVP, 30 g L^{-1} sucrose, and 0.1 g L^{-1} myo-inositol. The pH of the medium was adjusted to 5.7 ± 0.1 with 1.0 N KOH and/or HCl, and finally solidified with 6 g L^{-1} agar. After autoclaving, the medium was supplemented with 150 µM picloram (Sigma) under laminar flow. The dishes were maintained in a growth room at 27 ± 2°C in the dark for 60 days.

After 60 days, the explants were transferred to basal MS medium, where they remained for 30 days. Following this period, the friable calli (0.2 g) formed from LWU explants were transferred to Erlenmeyer flasks (250 mL) containing 50 mL liquid basal MS supplemented with filtered picloram (15, 25, 35, and 45 µM). The flasks were maintained in an incubator with orbital agitation (Solab) at 100 rpm and 27°C in the dark (Fig. 1p, q) for 12 weeks (Experiment II). Mass (g) was recorded every 14 days. The growth index was calculated as (final mass - initial mass) / initial mass, and the growth rate (%) was calculated as growth index × 100.

In Experiment III (Fig. 1r, s), LWU explants were placed in Petri dishes (90 × 15 mm) with basal MS medium solidified with 6 g L^{-1} autoclaved agar and supplemented with 150 µM filter-sterile picloram. L-glutamine or hydrolyzed casein were added at 0.0, 0.5 or 1.0 g L^{-1} as sources of amino acids. The dishes were maintained in a growth room at 27 ± 2°C in the dark for 60 days.

2.3 Genetic diversity assessment

2.3.1 Genomic DNA extraction

DNA was extracted from the roots of each seedling used for embryogenic induction. The roots were macerated in liquid nitrogen as described by Doyle and Doyle (1990). Next, 50 mg macerated plant tissue was transferred to microtubes and mixed with 700 µL 200 mM Tris-HCl extraction buffer (pH 7.5) containing 288 mM NaCl, 25 mM cetyltrimethylammonium bromide, 0.5% sodium dodecyl sulfate, 0.2% beta-mercaptoethanol, and 1% PVP.

The samples were placed in a dry bath at 65°C for 30 min and homogenized every 10 min. Next, 650 µL of a chloroform:isoamyl alcohol (24:1 v/v) mixture was added, the samples were homogenized for 5 min, and centrifuged at 12,000 rpm for 10 min. Transfer of the supernatant and addition of the chloroform:isoamyl alcohol mixture was repeated twice.

DNA was precipitated with ice-cold isopropanol and 230 µL ammonium acetate, centrifuged at 12,000 rpm for 10 min, washed with 250 µL 70% alcohol, and centrifuged again at 12,000 rpm for 3 min. DNA precipitation was repeated twice. The samples were dried in a dry bath, resuspended in 40 µL TE buffer with RNase (40 µg mL^{-1}), and left in a dry bath at 37°C for 30 min.

A Nanodrop 2000 spectrophotometer (Thermo Scientific) was used to assess DNA quality and quantity. DNA integrity was analyzed using a Bio-Rad Gel DocTM XR photo documenter after running a 1.2% agarose gel.

2.3.2 DNA amplification and electrophoresis

After extracting genomic DNA, the following inter simple sequence repeat primers were used to analyze the genetic variability of seedlings (annealing temperatures are indicated in parenthesis): UBC 826 (56°C), UBC 841 (50°C), UBC 852 (50°C), UBC 853 (50°C), UBC 868 (50°C), UBC 873 (50°C), UBC 880 (50°C), UBC 812 (50°C), UBC 818 (50°C), UBC 808 (56°C), and UBC 811 (58°C or 56°C).

Amplification reactions included the following reagents: 1× buffer; 1 U Taq DNA polymerase in 0.2 µL, 0.8 µM primer, 0.8 mM dNTPs, 2 mM $MgCl_2$, 7 ng $µL^{-1}$ genomic DNA, and 7.8 µL H_2O for a final volume of 15 µL. A Veriti 96-well thermal cycler (Applied Biosystems) was used for amplification with the following parameters: denaturation at 94°C for 4 min, followed by 40 cycles at 94°C for 1 min, annealing at the primer-specific temperature for 1 min, extension at 72°C for 1 min, plus a final extension at 72°C for 5 min.

The amplification products were separated on a 1.2% agarose gel at 80 V for 2 h and the bands were stained with GelRed® dye prior to visualization. Using RStudio, we performed cluster analysis employing the Ward.D2 method.

2.4 Histological and structural analyses

For anatomical studies, samples were collected and fixed in Karnovsky's (Karnovsky 1965) solution containing 2.5% glutaraldehyde, 4.0% paraformaldehyde, 5 mM $CaCl_2$ and 0.1 M cacodylate buffer. Further, samples were dehydrated in an ethanol series and soaked in methacrylate (Historesin®; Leica).

Transversal and longitudinal sections (thickness of 5 µm) were obtained with an automatic rotating microtome (RM2155; Leica) equipped with a disposable glass knife, and stained with toluidine blue at pH 4.0 (O'BRIEN and McCULLY 1981) for 15 min. The slides were mounted in a synthetic resin (Permount). Analysis and photo documentation were performed using a light microscope (Olympus-AX 70) coupled with a photomicrography system (Olympus U-Photo) at the Laboratory of Plant Anatomy, Department of Plant Biology, Federal University of Viçosa.

2.5 Transmission electron microscopy (TEM)

Samples were collected and fixed in Karnovsky's solution containing 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1 M cacodylate buffer. Following, the tissues were post-fixed in osmium tetroxide, dehydrated in graded ethanolic series (30%, 50%, 70%, 90%, and 100%), and embedded in epoxy resin (Embed- 812, Eletron Microscopy Sciences). Ultrathin sections (60–80 nm thick) were accomplished using an ultramicrotome (UCT, Leica Microsystems) before staining with uranyl acetate and lead citrate. Representative electron micrographs were captured using a transmission electron microscope (JEOL JEM-1400, Japan) at 120 kV and 12000 x magnification.

2.6 Atomic force microscopy (AFM)

Samples of approximately 0.5 cm^2 obtained from the nodular callus following induction were dehydrated in an ethylic series to absolute alcohol, subjected to critical point drying with CO_2 (Autosamdri 815; Tousimis), and arranged on glass slides using enamel as a bonding medium.

AFM images were collected using an alpha300 R confocal microscope (WITec) operated in non-contact mode and, in selected regions, using a light microscope. The confocal microscope was operated with a Si_3N_4 cantilever, a nominal constant of 42 N m^{-1}, resonant frequency of ~ 285 kHz, sweep rates of 0.3–1.0 Hz, and scan range of 2500–10000 nm.

Phase and light microscopy images were collected along with topographical images. Phase images were used to estimate the physical properties of the calli, such as hardness, adhesion, and viscoelasticity.

Surface asymmetry (SSK) was obtained from Eq. 1. Peak-to-peak height, given by the difference between the highest and lowest peak heights, was used to assess surface roughness.

$$SSK = \frac{1}{RQ^3}\sum_{i}^{n} Z_i^3 \qquad \text{Equation 1}$$

Where *Zi* is the height at position *i*, *RQ* is the mean square of the height deviation, and *n* is the number of points in the image grid.

In general, SSK = 0 suggests a symmetrical or even data distribution around the median plane; whereas SSK ≠ 0 suggests an asymmetric distribution, which points to a flat surface with small peaks (SSK > 0) or small valleys (SSK < 0).

Kurtosis (SKU) was determined by Eq. 2 using WITec data evaluation software. Kurtosis indicates whether the data are arranged horizontally or perpendicularly around the mean.

$$SKU = \frac{1}{MNSQ^4} \sum_{j=1}^{N} \sum_{i=1}^{M} \in_{i,j} [z(x_i, y_j) - \bar{z}]^4 \quad \text{Equation 2}$$

Where *M* and *N* are the numbers of data points in *X* and *Y*, respectively; *SQ* is the root mean square deviation; and *Z* is the height of the surface relative to the mean plane. SKU > 3.00 indicates the presence of excessively high peaks or deep valleys; whereas SKU < 3.00 indicates surfaces with a protuberant texture. If the surface heights are normally distributed, SKU = 3.00.

The analyses were performed at the Laboratory for Research and Development of Methodologies for Oil Analysis, Department of Chemistry, Federal University of Espírito Santo, Vitória.

2.7 Experimental design

The design was completely randomized. Experiment I included four treatments (TW, LWU, LWD, and LWS) and 10 replications with four explants. Experiment II included four treatments (15, 25, 35, and 45 µM picloram) and four replications. Experiment III included two amino acid sources (hydrolyzed casein or L-glutamine) supplemented at three concentrations (0.0, 0.5, and 1.0 g L^{-1}) and 10 replications with four explants. Data were subjected to analysis of variance, regression, and Tukey's test ($p < 0.05$) (R CORE TEAM, 2023).

2.8 Maturation

Friable calli (0.2 g) from the best experimental treatment were transferred to Erlenmeyer flasks (250 mL) containing 50 mL liquid basal maturation medium supplemented with filter-sterile abscisic acid (ABA) at 1, 5, 10, and 20 µM (Sigma). The flasks were maintained in an incubator with orbital agitation at 100 rpm, 27°C, and in the dark for 8 weeks. Subculture and mass analysis were performed every two weeks. The final mass (g), index, and growth rate (%) were determined.

2.9 Conversion of emblings and acclimatization

After maturation, somatic embryos were transferred to MS culture medium, supplemented with 0.1 g L^{-1} myo-inositol, 1 g L^{-1} PVP, and 30 g L^{-1} sucrose. The pH of the medium was adjusted to 5.7 ± 0.1 prior to the addition of agar (5.5 g L^{-1}).

After that period (30 days), they were placed in an *ex-vitro* environment consisting of plastic cups (590 mL) containing autoclaved substrate (TerraNutri®) moistened with distilled and autoclaved water. Each cup was covered with another cup (300 mL) to maintain the humidity during the first 7 days. Following that period, two holes (Θ = 0.5 cm) were made in the upper cup every 2 days, and the cultures were incubated for another 10 days (Mello et al. 2023).

3 RESULTS

3.1 Embryogenic induction: growth of wounded *E. edulis* seedlings in solid medium containing picloram

Seven days after transfer to induction medium containing 150 µM picloram, seedling segments began to grow longitudinally and, 60 days later, somatic proembryos emerged directly from non-oxidized stem segments. In explants with a transverse injury, proembryos appeared only at the growing end (Fig. 2a); whereas in explants with a longitudinal injury, proembryos appeared throughout the length of the segments (Fig. 2b–d). After 30 days in basal medium, the explants harboring proembryos grew and multiplied (Fig. 2e, f).

After 60 days in embryogenic induction medium, the maximum average number of proembryos (14) and induction rate (48% and 44%) were obtained with LWU and LWS explants, respectively (Fig. 3a, b). These samples displayed also the lowest oxidation rates (52% and 56%) (Fig. 3c). The advantageous characteristics of LWU and LWS explants were independent of their mass (Fig. 3d).

Using the ward.D method, a dendrogram was constructed, revealing two distinct groups (Fig. 4). Group 1 comprised the parent plant, seedlings that were responsive to embryogenic induction (R2 and R3), and non-responsive seedlings (NR2). Group 2 included seedlings responsive to embryogenic induction (R1 and R4) and the non-responsive ones (NR1, NR3, and NR4).

3.2 Embryogenic induction: cultivation of LWU-derived friable calli in picloram-containing liquid medium under agitation

All calli transferred to suspension culture displayed embryogenic characteristics, including embryogenic cells, proembryogenic cells in the meristematic zones, and parenchymal cells in the peripheral regions (Fig. 5). Embryogenic cells presented stronger staining than proembryogenic cells. They also formed an embryogenic zone with smaller cells characterized by voluminous nuclei with evident heterochromatin, near absence of vacuoles, dense cytoplasm, and absence of intercellular spaces (Fig. 5b). The meristematic zones, composed of proembryogenic cells, exhibited intense cell division, with clearly visible nuclei and numerous nucleoli, a greater number of fragmented vacuoles, thicker cell walls, and evident intercellular spaces (Fig. 5b). The same pattern was observed irrespective of callus type (Fig. 5c–l).

The friable embryogenic calli had irregular surfaces (Fig. 5m–p), as evidenced by AFM ultrastructural analysis. The peak-to-peak distance was 2192.27 nm, with a predominance of small peaks (SSK > 0) and asymmetrical peaks (SKU > 3).

The friable calli in the suspension culture began to grow in the second week. After the fourth week, their multiplication was evident, with the appearance of new pink-colored globular structures (Fig. 6a) characteristic of anthocyanin peppers. After 12 weeks, the globular structures incubated with the highest

concentration of picloram (45 μM) darkened and oxidized (Fig. 6b). The highest average mass (0.6716 g), growth index (2.358), and growth rate (235.8%) were observed in calli incubated with 15 μM picloram (Fig. 6c–e).

3.3 Embryogenic induction: cultivation of LWU explants in solid medium with amino acids

The effect of amino acid source and content on embryogenic induction (Fig. 7) revealed no statistical difference with respect to the highest average number of proembryos between explants cultivated in the absence of amino acids (14.92), with 1.0 g L^{-1} hydrolyzed casein (16.25), and with 0.5 g L^{-1} L-glutamine plus 1.0 g L^{-1} hydrolyzed casein (16.33) (Fig. 7a).

The lack of any statistical difference between the absence of amino acids and addition of 0.5 g L^{-1} L-glutamine with respect to the number of proembryos was confirmed by similarity at the morphological and cellular level between the two treatments, but it contrasted with the 1.0 g L^{-1} L-glutamine case (Figs. 8 and 9). In both the absence and presence of 0.5 g L^{-1} L-glutamine, globular somatic embryos were formed directly from the explant tissue via direct secondary embryogenesis (Fig. 8a–h). In comparison, 1.0 g L^{-1} L-glutamine elicited callus formation and the appearance of globular embryos even in non-embryogenic regions (Fig. 8i–l).

The explants induced with 0.5 g L^{-1} L-glutamine presented cells with characteristics similar to those of cells incubated in the absence of the amino acid (Fig. 9a–f, g–l), including a dense cytoplasm, evident nuclei, large and electron-dense nucleoli, irregularly shaped nuclei, large vacuoles, lower heterochromatin to euchromatin content (Fig. 9a, b, g, h), numerous mitochondria, a developed endoplasmic reticulum, a prominent Golgi complex, amyloplasts with visible starch granules (Fig. 9c–f, i–k), cell components closely attached to the cell wall, thick cell walls, and visible intercellular spaces (Fig. 9e, f). In contrast, at 1.0 g L^{-1} L-glutamine, the cells displayed the typical characteristics of an embryogenic callus, including the fragmentation of vacuoles (Fig. 9m, n).

3.4 Maturation

The somatic embryos in the suspension culture began to grow as early as the second week and, after the fourth week, embryos induced with 1 μM ABA began to multiply, as denoted by the emergence of new globular structures (Fig. 10a). The highest average final mass (0.35 g), growth index (0.73), and growth rate (73.11%) were obtained at eight weeks with 1 μM ABA, decreasing linearly as the concentration of ABA increased. Globular somatic embryos induced with 10 and 20 μM ABA began to show oxidation signs from the seventh week onwards, likely explaining the lower final mass, growth index, and growth rate of the corresponding embryos (Fig. 10b–d).

3.5 Conversion of emblings and acclimatization

After maturation, somatic embryos still showed asynchronous development and growth. The coleoptillar embryos transferred to the basal culture medium with white color (Fig. 11a), after 15 days, began to form

the primary root characterized with a reddish-pink color (Fig. 11b). After 30 days, complete emblings had been removed, with primary roots and shoots colored pink (Fig. 11c).

The emblings showed normal growth and basal medium (Fig. 11d), and when transferred to the *ex-vitro* environment, they remained green, without wilting characteristics or the presence of fungi (Fig. 11e).

4 DISCUSSION

Caulicles of *E. edulis* cut lengthwise and arranged upward and/or sideways in MS medium supplemented with 150 µM picloram were the most suitable for inducing somatic embryogenesis, with the explant arranged upward being more practical to deal with. In *Medicago truncatula* Gaertn., injury-induced stress response and defense reaction during the first week of callus growth involves increased superoxide dismutase and catalase activities, followed by that of ascorbate peroxidase (Orlowska and Kępczyńska 2020). In *Arabidopsis*, the AP2/ERF transcription factor WIND1 and its close homologs WIND2–4 are rapidly induced at the wound site, promoting dedifferentiation and subsequent cell proliferation to form a mass of pluripotent cells termed calli (Iwase et al. 2011a, b). According to these authors, ectopic overexpression of WIND1 is sufficient to establish and maintain the undifferentiated status of somatic cells, without addition of exogenous auxin and cytokinin. These two plant hormones are normally required for cell dedifferentiation. According to Iwase et al. (2015), sequential activation of WIND1 and the embryonic regulator LEC2 induces somatic embryogenesis at both cut and uncut sites; whereas activation of LEC2 alone allows embryogenesis only at cut sites.

Stem segments of *E. edulis* seedlings sectioned longitudinally (≅ 0.2 mm in thickness) with the wound facing upward and/or sideways yielded better results than the explant sectioned only transversely (Figs. 2 and 3). This finding suggests that the *in vitro* response depends on the size of the explant. A thin cell layer consisting of small explants (1 × 0.5 or 10 mm) may include only one type of tissue, such as a monolayer of epidermal cells, or several (3–6) layers of cells from different tissues (Van and Gendy 1996). A thin cell layer proved efficient in explants of *B. gasipaes*, whereby low callus induction was observed with 1-cm-thick explants, but induction increased to 83–97% with primary calli (Steinmacher et al. 2007).

Larger explants maintain normal tissue interactions, which can inhibit cell division by retaining symplast domains. However, in smaller explants, there is greater superficial contact with the medium and a higher level of stress, which promotes cellular metabolism. In addition, the synthesis of new cell wall components such as oligosaccharides acts as a signal for the cell to enter the mitotic cycle (Steinmacher et al. 2007).

Besides external factors, genetic factors are also important during embryogenic induction. In the present study, the genotype of the male parent (pollen donor) may have been essential for the embryogenic response, as dissimilarity analysis of responsive and non-responsive daughter seedlings in relation to the female parent revealed two groups, each of them including both types of seedlings.

Histological analysis and AFM confirmed the embryogenic potential of calli grown in suspension medium (liquid MS), identifying the embryogenic and proembryogenic zones inside the callus, as well as qualitative and quantitative differences in topography, surface profile, roughness, and symmetry.

After multiplication in suspension medium, supplementation with 15 µM picloram yielded better results than higher concentrations of this synthetic auxin. Agitation of the medium allowed for faster multiplication of calli obtained from solid medium. This enabled secondary somatic embryogenesis, as manifested by the emergence of small isolated embryogenic nodules and the development of proembryos.

Cell suspension is considered superior to callus culture as it provides greater contact between medium and tissue, rapid and uniform transfer of nutrients, easier access to growth regulators, and a reduced effect of the toxins commonly released by tissues. Thus, constant exchange of liquid medium promotes rapid tissue multiplication (Kong et al. 2020).

Cell suspension cultures have been used with several species of palm trees for propagation via somatic embryogenesis and subsequent regeneration of seedlings. Such efforts have focused mainly on *Elaeis guineensis* Jacq, *Phoenix dactylifera* L., *Cocos nucifera* L., and *B. gasipaes* Kunth (Kong et al. 2020), making this the first study whereby suspension cultivation has been applied to members of the *Euterpe* genus.

L-glutamine and hydrolyzed casein have also been used as supplements in culture media for many species of the Arecaceae family (Ledo et al. 2002; Steinmacher et al. 2007; Zouine and El Hadrami 2007; Moura et al. 2009; Konan et al. 2010; Saldanha and Martins-Corder 2012; Scherwinski-Pereira et al. 2012; Freitas et al. 2016; Ferreira et al. 2022). They serve as alternative organic nitrogen sources to increase cell metabolism, the synthesis of reserve proteins and sugars, differentiation, and cell growth (Zouine and El Hadrami 2007; Parast et al. 2011). The marginal benefit of adding L-glutamine observed in the present study (Fig. 8), does not justify its supplementation or that of hydrolyzed casein during embryogenic induction of stem segments from *E. edulis* seedlings.

In *P. dactylifera* L., addition of ABA to a suspension culture increased the production and maturity of somatic embryos, along with the accumulation of proteins, lipids, and starch (Alwael et al., 2017). ABA suppresses the formation of abnormal embryonic structures and prevents the maturation of embryos during early germination (Zouine et al. 2005; Rai et al. 2011). Directly influencing the later stage, the conversion of somatic embryos into emblings, and their acclimatization.

5 CONCLUSIONS

Caulicles with longitudinal wounds facing upward when grown in MS medium supplemented with 150 µM picloram achieved the highest rates of embryogenic induction.

The cultivation of friable *E. edulis* calli in liquid medium under agitation was achieved for the first time in this species, with the strongest growth occurring in MS medium supplemented with 15 μM picloram.

The highest average number of proembryos was obtained in the presence of 1.0 g L^{-1} hydrolyzed casein (16.33), although this value was not statistically different from that obtained in the absence of amino acid supplementation.

During maturation, addition of 1 μM ABA led to the greatest differentiation of somatic embryos.

DECLARATIONS

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CRediT authorship contribution statement

Tamyris de Mello: Investigation, Methodology, Writing – review and editing. **Tatiane Dulcineia Silva**, **TadeuÉriton Caliman Zanardo**, **Francine Alves Nogueira de Almeida**, **Luciano Bestete Oliveira**, and **Clovis Eduardo Nunes Hegedus**: Investigation, Methodology. **Breno Benvindo dos Anjos**, **Edilson Romais Schmildt**, and **Adésio Ferreira**: Formal analysis. **Márcia Flores da Silva Ferreira**, **José Carlos Lopes**, **Glória Maria de Farias Viégas Aquije**, and **Wagner Campos Otoni**: Supervision, Writing – original draft, Writing – review and editing. **Rodrigo Sobreira Alexandre**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – original draft, Writing – review and editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Declaration of data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

REFERENCES

1. Alwael HA, Naik PM, Al-khayri JM (2017) Synchronization of somatic embryogenesis in date palm suspension culture using abscisic acid. In: Al-Khayri, J.; Jain, S.; Johnson, D. (eds) Date palm biotechnology protocols. Humana Press: New York. https://doi.org/10.1007/978-1-4939-7156-5_18
2. Cardoso A, Liz S, Rieger D, Farah A, Vieira FK, Assis MA, Di Pietro P (2018) An update on the biological activities of *Euterpe edulis* (juçara). Planta Med 84:487-499. https://doi.org/10.1055/s-0044-101624
3. Costa AS, Silva JHS, Torres MF, Arrigoni-Blank MF, Blank AF (2012) Composição do meio de cultura na redução da hiperidricidade *in vitro* em híbrido de manjericão. Hort Bras30:S168-S172.
4. Cursi PR, Cicero SM (2014) Fruit processing and the physiological quality of *Euterpe dulis* Martius seeds. J Seed Sci 36:134-142. https://doi.org/10.1590/2317-1545v32n2847
5. Faria DV, Correia LNF, Matos EM, Souza MVC, Batista DS, Costa MGC, Paiva Neto VB, Xavier A, Rogalski M, Otoni WC (2019) Wounding and medium formulation affect de novo shoot organogenic responses in hypocotyl-derived explants of annatto (*Bixa orellana* L.). In Vitro Cell Dev Biol – Plant 55:277-289. https://doi.org/10.1007/s11627-019-09978-2
6. Ferreira JCB, Silva-Cardoso IMA, Meira RO, Scherwinski-Pereira JE (2022) Somatic embryogenesis and plant regeneration from zygotic embryos of the palm tree *Euterpe precatoria* Mart. Plant Cell, Tiss Organ Cult148:667-686. https://doi.org/10.1007/s11240-022-02227-2
7. Freitas EO, Monteiro TR, Nogueira GF, Scherwinski-Pereira JE (2016) Somatic embryogenesis from immature and mature zygotic embryos of the açai palm (*Euterpe oleracea*): Induction of embryogenic cultures, morphoanatomy and its morphological characteristics. Sci Hortic 212:126-135. http://dx.doi.org/10.1016/j.scienta.2016.09.044
8. George EF, Hall MA, de Klerk GJ (2008) Plant Propagation by Tissue Culture. 3 ed., v. 1, Dordrecht: Springer, 503p. https://doi.org/ 10.1007/978-1-4020-5005-3
9. Goh DKS, Michaux-Ferrière N, Monteuuis O, Bon MC (1999) Evidence of somatic embryogenesis from root tip explants of the rattan *Calamus manan*. In Vitro Cell Dev Biol – Plant 35:424-427. https://doi.org/10.1007/s11627-999-0060-7
10. Goh DKS, Bon MC, Aliotti F, Escoute J, Ferrière N, Monteuuis O (2001) *In vitro* somatic embryogenesis in two major rattan species, *Calamus merrillii* and *Calamus subinermis*. In Vitro Cell Dev Biol – Plant 37:375-381. https://doi.org/10.1007/s11627-001-0066-2
11. Ikeuchi M, Sugimoto K, Iwase A (2013) Plant callus: mechanisms of induction and repression. The Plant Cell 25:3159-3173. https://doi.org/10.1105/tpc.113.116053
12. Iwase A, Harashima H, Ikeuchi M, Rymen B, Ohnuma M, Komaki S, Morohashi K, Kurata T, Nakata M, Ohme-Takagi M, Grotewold E, Grotewold E, Sugimoto K (2017) WIND1 promotes shoot regeneration

through transcriptional activation of *ENHANCER OF SHOOT REGENERATION1* in Arabidopsis. The Plant Cell 29:54-69. https://doi.org/10.1105/tpc.16.00623

13. Iwase A, Mitsuda N, Koyama T, Hiratsu K, Kojima M, Arai T, Inoue, Y, Seki M, Sakakibara H, Sugimoto K, Ohme-Takagi M (2011a) The AP2/ERF transcription factor WIND1 controls cell dedifferentiation in Arabidopsis. Curr Biol 21:508-514. https://doi.org/10.1016/j.cub.2011.02.020
14. Iwase A, Ohme-Takagi M, Sugimoto K (2011b) WIND1: A key molecular switch for plant cell dedifferentiation. Plant Signal. Behav 6:1943-1945. https://doi.org/10.4161/psb.6.12.18266
15. Iwase A, Mita K, Nonaka S, Ikeuchi M, Koizuka C, Ohnuma M, Ezura H, Imamura J, Sugimoto K (2015) WIND1-based acquisition of regeneration competency in Arabidopsis and rapeseed. J Plant Res 128:389-397. https://doi.org/10.1007/s10265-015-0714-y
16. Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 27:137-138. http://www.jstor.org/stable/1604673
17. Karun A, Siril EA, 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. https://doi.org/10.1590/0102-33062015abb0109
18. Konan KE, Durand-Gasselin T, Kouadio YJ, Flori A, Rival A, Duval Y, Pannetier C (2010) *In vitro* conservation of oil palm somatic embryo 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. https://doi.org/10.1007/s00299-009-0787-y
19. Kong EYY, Biddle J, Foale M, ADKINS, S. W. (2020) Cell suspension culture: A potential *in vitro* culture method for clonal propagation of coconut plantlets via somatic embryogenesis. Ind Crops Prod147:112125. https://doi.org/10.1016/j.indcrop.2020.112125
20. Ledo AS, Lameira OA, Benbadis AK, Menezes IC, Oliveira MSP, Medeiros Filho S (2002) Somatic embryogenesis from zygotic embryos of *Euterpe oleracea* Mart. Rev Bras Frutic 24:601-603. https://doi.org/10.1590/S0100-29452002000300004
21. Lup SD, Tian X, Xu J, Pérez-Pérez JM (2016) Wound signalling of regenerative cell reprogramming. Plant Sci 250:178-187. https://doi.org/10.1016/j.plantsci.2016.06.012
22. Marco-Browna JL, Arecoa MM, Sánchez RMT (2014) Adsorption of picloram herbicide on montmorillonite: Kinetic and equilibrium studies. Colloids Surf A: Physicochem Eng Asp 449:121-128. https://doi.org/10.1016/j.colsurfa.2014.02.038
23. Mello T, Correia LNF, Hegedus CEN, Schmildt ER, Ferreira A, Lopes JC, Otoni WC, Alexandre RS (2023) Cell reprogramming via direct somatic embryogenesis in an Atlantic Forest species vulnerable to extinction: *Euterpe edulis* stem segments induced with picloram. Plant Cell, Tiss Organ Cult 154, 131–140. https://doi.org/10.1007/s11240-023-02521-7
24. Moura EF, Motoike SY, Vendrella MC, Sá AQ, Carvalho M (2009) Somatic embryogenesis in macaw palm (*Acrocomia aculeata*) from zygotic embryos. Sci Hortic 119:447-454. https://doi.org/10.1016/j.scienta.2008.08.033

25. Murashige T, SKOOG F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x
26. Oliveira LB, Mello T, Araujo CP, Oliveira JPB, Ferreira A, Zanardo TEC, Vieira LM, Otoni WC, Alexandre RS, Carvalho VS (2022) Morphoanotomical aspects of auxin herbicides-induced somatic embryogenesis in *Euterpe edulis* Martius, a symbol and threatened species of the Atlantic Forest. Sci Hortic 299:111051. https://doi.org/10.1016/j.scienta.2022.111051
27. Orłowska A, Kępczyńska E (2020) Oxidative status in *Medicago truncatula* Gaertn. non-embryogenic and embryogenic tissues with particular reference to somatic embryogenesis. Plant Cell, Tiss Organ Cult140:35-48. https://doi.org/10.1007/s11240-019-01709-0
28. Parast BM, Chetri SK, Sharma K, Agrawal V (2011) *In vitro* isolation, elicitation of psoralen in callus cultures of *Psoralea corylifolia* and cloning of psoralen synthase gene. Plant Physiol Biochem 49:1138-1146. http://dx.doi.org/10.1016/j.plaphy.2011.03.017
29. Rai MK, Shekhawat NS, Harish, Gupta AK, Phulwaria M, Ram K, Jaiswal U (2011) The role of abscisic acid in plant tissue culture: a review of recent progress. Plant Cell, Tiss Organ Cult 106:179-190. https://doi.org/10.1007/s11240-011-9923-9
30. R Core Team (2023) R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Disponível em: <https://www.rproject.org/>. Acesso em: 10 fev. 2023.
31. Saldanha CW, Martins-Corder MP (2012) *In vitro* germination and embryogenic competence acquisition of *Euterpe edulis* Martius immature zygotic embryos. Crop Breed Appl Biotechnol 12:171-178. https://doi.org/10.1590/S1984-70332012000300002
32. 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. https://doi.org/10.1007/s11240-012-0115-z
33. Schulz M, Borges GDSC, Gonzaga LV, Costa ACO, Fett R (2016) Juçara fruit (*Euterpe edulis* Mart.): Sustainable exploitation of a source of bioactive compounds. Food Res Int 89:14-26. https://doi.org/10.1016/j.foodres.2016.07.027
34. Steinmacher DA, Krohn NG, Dantas AC, Stefenon VM, Clement CR, Guerra MP (2007) Somatic embryogenesis in peach palm using the thin cell layer technique: induction, morpho-histological aspects and AFLP analysis of somaclonal variation. Ann Bot 100:699-709. https://doi.org/10.1093/aob/mcm153
35. Valverde R, Arias O, Thorpe TA (1987) Picloram-induced somatic embryogenesis in pejibaye palm (*Bactris gasipaes* HBK). Plant Cell, Tiss Organ Cult 10:149-156. https://doi.org/10.1007/BF00035913
36. Van KTT, Gendy C (1996) Thin cell layer (TCL) method to programme morphogenetic patterns. In: Lindsey, K. (eds), Plant tissue culture manual. Dordrecht: Springer, 1065-1089. https://doi.org/10.1007/978-94-009-0181-0_5
37. Wendt T, Cruz DD, Demuner VG, Guilherme FAG, Boudet-Fernandes H (2011) An evaluation of the species boundaries of two putative taxonomic entities of *Euterpe* (Arecaceae) based on reproductive

and morphological features. Flora 206:144-150. http://dx.doi.org/10.1016/j.flora.2010.03.002

38. Zouine J, Bellaj ME, Meddich A, Verdeil Jl, El Hadrami I (2005) Proliferation and germination of somatic embryos from embryogenic suspension cultures in Phœnix dactylifera. Plant Cell, Tiss Organ Cult 82:83-92. https://doi.org/10.1007/s11240-004-6914-0
39. Zouine J, El Hadrami I (2007) Effect of 2,4-D, glutamine and BAP on embryogenic suspension culture of date palm (*Phoenix dactylifera* L.). Sci Hortic112:221-226. https://doi.org/10.1016/j.scienta.2006.12.041