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ORIGINAL PAPER

Somatic embryogenesis and plant regeneration in açaí palm (*Euterpe oleracea*)

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Abstract An efficient and simple system for inducing somatic embryogenesis and regenerating plantlets from immature zygotic embryos of açaí palm (*Euterpe oleracea*) has been developed. Embryogenic calli (ECs) were induced from immature zygotic embryos of açaí palm on Murashige and Skoog (MS) modified medium with 2,4-dichlorophenoxyacetic acid and picloram. Embryogenic frequency was dependent on auxin type and concentration. The optimal concentration of picloram for the high-frequency induction of embryogenic calli (72%) was 225 μM. ECs were then subcultured on a differentiation and maturation medium composed of MS modified medium with 2-isopentenyladenine and naphthaleneacetic acid with subcultures at 4-week intervals. SEs were converted to plants on MS modified medium with half-strength macro- and micronutrients, 20 g l^{-1} sucrose, and 2.5 g l^{-1} activated charcoal and gelled with 2.5 g l^{-1} Phytagel. Detailed morpho anatomical changes during the different stages of somatic embryogenesis were characterized. The development of SEs was asynchronous, and ontogenic studies confirmed that the initial cell divisions occur in the epidermal and subepidermal regions of the zygotic embryos. Broad base attachment of SEs to the epidermis indicates the presence of a suspensor.

Keywords Arecaceae · Acai berry palm · Euterp palm · Morphogenesis · Embryo origin · Ontogenesis · Somatic embryos

Abbreviations

EC	Embryogenic calli
MS	Murashige and Skoog medium (1962)
2iP	2-Isopentenyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
NAA	Naphthaleneacetic acid
SEs	Somatic embryos

Introduction

The açaí palm tree (*Euterpe oleracea*) is native to tropical Central and South America and grows mainly in floodplains and swamps. Although its soft interior stem can be used as a source for heart of palm, açaí is traditionally consumed as juice extracted from the fruit pulp and skin. At present, great effort has been devoted to improving the agronomic aspects of açaí palm, especially regarding the development of high-yielding hybrids through breeding programs (Oliveira et al. 2010).

However, breeding programs are complex and take too long due to the extended cycle of this species and the absence of conventional methods of vegetative

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propagation; vegetative propagation using offshoots is limited because only a few plantlets are produced, which are difficult to separate and have very low survival rates in the field. Traditionally, the açaí palm has been propagated by seed (Farias Neto et al. 2005). This makes the improvement, selection, and propagation of elite individuals from the natural population difficult. Therefore, any time-saving methodology will be beneficial for improvement programs in this species.

Somatic embryogenesis can be described as the process through which haploid or diploid somatic cells develop into different plants through characteristic embryological stages without gamete fusion (Namasivayam 2007; Ramakrishna et al. 2011). Somatic embryogenesis of açaí palm has been obtained from mature zygotic embryos (Ledo et al. 2002). Although immature embryos have been used as explant sources for other monocotyledons (Teixeira et al. 1993; Schaik et al. 1996; Eudes et al. 2003; Anami et al. 2010), in açaí palm, the morphogenetic responses of immature zygotic embryos are restricted to a germination process with the development of abnormal seedlings and of granular structures on the cotyledon node without the progression of embryogenic cultures (Ledo et al. 2002). The use of immature zygotic embryos as starting explants is justifiable in the sense that they have different physiological status, which could lead to different results in culture compared to mature zygotic embryos (Dodeman et al. 1997; Eudes et al. 2003). Moreover, mature açaí fruits are difficult to transport and store until use because they are recalcitrant, soft, and easily injured in few days; meanwhile, immature fruits have hard skin and a soft bud endosperm that facilitate easy zygotic embryo extraction.

In the literature, there is a consensus regarding the role of growth regulators added to cultivation media in inducing embryogenic competence; auxins and cytokinins are the 2 most involved in the activation and regulation of cellular division and differentiation (Geldner et al. 2000; Fehér et al. 2003; Chen et al. 2010). Considering these two categories of growth regulators, the exogenous application of auxins considered to be strong, including 2,4-dichlorophenoxyacetic (2,4-D), picloram, and dicamba, is well documented to induce the transition of somatic cells into embryogenic cells in many vegetal systems (Prakash and Gurumurthi 2010; Don Palmer and Keller 2011). In the specific case of the açaí palm, only 2,4-D has been used to induce somatic embryogenesis (Ledo et al. 2002). Alternatively, some studies provide evidence of the superiority of picloram in inducing somatic embryos (SEs) in several species of palms including peach, arecanut palm, and oil palm (Valverde et al. 1987; Steinmacher et al. 2007; Scherwinski-Pereira et al. 2010).

For açaí palm, an efficient protocol for improving calli induction, synchronous embryo proliferation, maturation, and efficient germination is proving difficult. Furthermore, the current information on these events is still not adequate. Moreover, the basic understanding of the nature of SEs that behave like zygotic embryos and studies involving cultural conditions that facilitate quality in vitro embryo production are not enough to exploit embryogeny practically. To our knowledge, this study is the first to report the regeneration of açaí palm from immature zygotic embryos via somatic embryogenesis. The ontogeny of SEs was also studied in order to characterize the ontogenetic routes followed by these embryos using histological approaches.

Materials and methods

Plant material, media, and culture conditions

Immature zygotic açaí palm embryos were used as an explant source. They were obtained from adult açaí palm plants of the Germplasm Bank of Embrapa Acre, Rio Branco, Acre, Brazil and were still dark green, approximately 1.2 cm in diameter, and 100–110 days post-anthesis. At this age, the zygotic embryos were apparent and hardening, facilitating handling (Fig. 3a, b). First, the fruits were rinsed in tap water for 5 min followed by surface sterilization through immersion in 70% ethanol for 15 s and then in sodium hypochlorite solution consisting of 50% commercial bleach (2.0–2.5% active chlorine) plus 3 drops of Tween-20®/100 ml. Thereafter, under aseptic conditions, the materials were rinsed 3 times in sterile distilled water and then cultured on Murashige and Skoog (MS) (1962) medium with 3.0% sucrose and 1.5 g l^{-1} activated charcoal, and gelled with 6 g l^{-1} Bacto-Agar (Sigma, St. Louis, MO). Picloram and 2,4-dichlorophenoxyacetic (2,4-D) were added at various concentrations (0, 225, or 450 μM) to evaluate their effects on the production of embryogenic calli (ECs). Each treatment consisted of 70 embryos (7 flasks, 250 ml capacity with 40 ml medium; 10 embryos per flask). ECs were evaluated 150 days after inoculation. The percentages of responsive explants and EC induction were evaluated in all explants. No subculturing was applied until ECs were obtained.

The ECs were maintained in a differentiation and maturation medium composed of basal media, 0.537 μM naphthaleneacetic acid (NAA), and 12.30 μM 2-isopentenyladenine (2iP) with subcultures at 4-week intervals for 12 weeks until the polyembryos differentiated. To convert SEs into plants, SE clumps were transferred to modified medium with half-strength macro- and micronutrients and 2.5 g l^{-1} activated charcoal and were gelled with 6 g l^{-1} Bacto-Agar without growth regulators. The compositions of the culture media in the different phases of somatic embryogenesis induction in açaí palm can be seen in Table 1.

Table 1 Components of the culture media used in açai palm for somatic embryogenesis and plant recovery

Components	Stage I Calli induction (20 weeks)	Stage II Differentiation/maturation (up to 12 weeks)	Stage III Plant recovery (12–18 weeks)
Culture medium	MS	MS	½ MS
Myo-inositol (mg l^{-1})	2.0	2.0	2.0
Picloram (μM)	0; 225 and 450	–	–
2,4-D (μM)	0; 225 and 450	–	–
Activated charcoal (g l^{-1})	1.5	–	2.5
NAA (μM)	–	0.537	–
2iP (μM)	–	12.30	–
Bacto-Agar (g l^{-1})	6.0	6.0	6.0

MS Murashige and Skoog medium (1962); *NAA* naphthaleneacetic acid; *2iP*, 2-isopentenyladenine; *2,4-D* 2,4-dichlorophenoxyacetic acid

All culture media were adjusted to pH 5.8 prior to adding the gelling agent and were autoclaved for 15 min at 1.3 kgf cm^{-2}. During the induction and maturation of SEs, the cultures were kept in the dark at 25 ± 2°C. For somatic conversion and plant growth, the cultures were kept in a growth chamber at 25 ± 2°C under a 16-h light photoperiod with an intensity of 35–40 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps until the plants were 6–8 cm tall; the plants were then acclimatized in plastic dibble tubes (115 cm^3) with Plantmax® as substrate.

Histological analyses

Samples of the responsive explants, ECs, and SEs were prepared for histological analysis to determine the ontogenic route followed by the SEs. The samples were prepared for optical microscopy according to the method reported by James et al. (1994) with some modifications. The samples were fixed in 2.5% glutaraldehyde solution in 0.1 M (pH 7.2) phosphate buffer for 24 h at room temperature. After fixing, the samples were washed three times in phosphate buffer and dehydrated in increasing ethanol series (20–100%). After dehydration, the samples were embedded in acrylic resin, LR White medium grade (London, UK) for 7 days in a refrigerator for resin infiltration. The samples were then embedded in transparent gelatin capsules containing resin and heated to 55°C for 18 h for polymerization. From this material, 1-μm-thick sections were obtained using an ultramicrotome (Leica), stained with 1% toluidine blue solution, and examined under an optical microscope (Zeiss). Histological analyses were performed using Image Pro Plus® 4.5 software.

Statistical analysis

Statistical analysis was carried out using Sanest (Zonta and Machado 1984) software. The effects of 3 picloram and 2,4-D concentrations (0, 225, and 450 μM) on EC production were evaluated. The experiment was a factorial completely randomized with 7 replicates. The effects of treatment were tested by analyses of variance (one-way ANOVA) with a 5% level of significance; means were compared with Tukey's multiple range test at 95% significance. Prior to analysis, percentage data were arcsine-square-root transformed.

Results and discussion

In general, all explants in the culture medium were viable. Therefore, different morphogenic responses were observed with the use of immature zygotic embryos cultivated in MS medium supplemented with 0, 225, or 450 μM picloram or 2,4-dichlorophenoxyacetic (2,4-D) in the presence of activated charcoal after 20 weeks of cultivation. In culture media without the presence of growth regulators (control), the most common result observed was the germination of immature zygotic embryos (Fig. 2c). However, in the other tested concentrations of auxins with the exception of 225 μM 2,4-D, the explants differentiated into either non-embryogenic primary friable calli or EC after 20 weeks of cultivation (Table 2).

Primary callus formation started after approximately 30 days of cultivation in induction culture medium. In this period, the response of the explants that formed calli was an initial swelling of the zygotic embryos in culture with the formation of calli initiating in the mid-region of the explants and typical formation of a cell mass with a white–light yellow appearance (Fig. 2d), progressing to the formation of compact ECs with a yellowish color throughout their length (Fig. 2e).

In general, the best treatments for both primary and EC induction were obtained with the use of picloram. In media with 2,4-D, the zygotic embryos in cultivation only formed non-embryogenic primary calli even at the highest concentration used (450 μM). In the media with picloram, with the exception of the control treatment, all concentrations led to the formation of primary calli or ECs (Table 2). Therefore, the differences between responses were due to the auxin concentration. Thus, the picloram concentration

Table 2 Effect of different concentrations of 2,4-D and Picloram on percentage of zygotic embryos with response, percentage of primary callus formation and percentage of embryogenic callus formed after 20 weeks, and percentage of callus with somatic embryos after 12 weeks in maturation and differentiation medium during somatic embryogenesis in açaí palm from immature zygotic embryos

Stage I—Callus induction media (20 weeks)		
Auxins	(μM)	% Responsive explants
Picloram	0	100 a
	225	100 a
	450	100 a
2,4-D	0	100 a
	225	100 a
	450	100 a
Auxins	(μM)	% Primary callus formation
Picloram	0	0.0 c
	225	25.2 b
	450	44.8 a
2,4-D	0	0.0 c
	225	0.0 c
	450	21.2 b
Auxins	(μM)	% Embryogenic callus formation
Picloram	0	0.0 c
	225	72.0 a
	450	46.7 b
2,4-D	0	0.0 c
	225	0.0 c
	450	0.0 c
Stage II—Differentiation/maturation (12 weeks)		
Auxins	(μM)	% Embryogenic callus with somatic embryos
Picloram	0	0.0 c
	225	72.0 a
	450	46.7 b
2,4-D	0	0.0 c
	225	0.0 c
	450	0.0 c

Means followed by different small letters in columns are statically different ($p = 0.05$) (Tukey's test)

that provided the highest levels of non-embryogenic primary calli was 450 μM. For the formation of ECs, the optimal picloram concentration was 225 μM, with 72% of the explants in cultivation presenting EC formation compared with 46.7% at 450 μM. In *E. oleracea*, with the use of 2,4-D, Ledo et al. (2002) also observed that at concentrations close to 225 μM, the root and cotyledonary coleoptile emerged, giving rise to normal healthy seedlings through the germination of the zygotic embryos. These authors only observed the formation of granular structures characterized by calli at higher concentrations of 2,4-D, similar to the findings of our study. In addition, they report that it is necessary to use even higher concentrations for the formation of ECs; however, the observed frequency of ECs even at concentrations above 500 μM was only 38%, which is much lower than 72% observed in our work with 225 μM picloram (Table 2).

After 20 weeks in induction culture medium, the ECs (Fig. 2e) were transferred to new culture media supplemented with 0.537 μM naphthaleneacetic acid (NAA) and 12.3 μM 2-isopentenyladenine (2iP) to induce the differentiation and maturation of the SEs. The conversion of the ECs to the differentiation/maturation phase occurred; SEs started to differentiate after approximately 30 days of cultivation. At 90 days of cultivation, asynchronous differentiation was observed with SEs in the globular and torpedo stages and in different stages of maturation (Fig. 2f). Besides the asynchronous development, some cultures presented the formation of SEs from pre-existing formations, indicating a high-frequency long-duration secondary or repetitive embryogenesis model. This same standard was also observed by Ledo et al. (2002) and Guerra and Handro (1998) in their works on somatic embryogenesis from zygotic embryos in *E. oleracea* and *E. edulis*, respectively. However, in our study, an indirect pattern of somatic embryogenesis was observed contrary to the findings of Ledo et al. (2002) and Guerra and Handro (1998) who observed a direct model for somatic embryogenesis with the use of 2,4-D.

In the differentiation and maturation phase, 100% of the explants that exhibited EC formation in the induction phase differentiated into SEs (Table 2). The auxin 2,4-D did not produce significant results for the induction of ECs, and consequently, did not induce SEs. In fact, the differentiation of SEs from ECs can be seen in various cultures that had initially been treated in the induction phase with high concentrations of auxins followed by reduced concentrations or transfer to other culture media. This confirms that in general, the process of somatic embryogenesis can be divided into two phases: induction and expression. During the induction phase, somatic cells are generally undifferentiated; they acquire embryogenic competency and proliferate as embryogenic formations. In the expression phase, the embryogenic cells exhibit their acquired embryogenic potential in the previous phase, differentiating to form SEs (Jiménez and Bangerth 2001; Fehér et al. 2003; Rai et al. 2011).

In general, the conversion of SEs in plants began after 40 weeks of cultivation and 4 weeks after transfer of the

calli in differentiated SEs to the regeneration medium (Fig. 3g). Although the present results indicate a high-frequency long-duration repetitive model of somatic embryogenesis, after 16 weeks in regeneration medium, the number of regenerated plants was evaluated. Significant statistical differences were found between 225 and 450 μM picloram used in the induction phases with respect to the number of embryos regenerated per callus (Fig. 1). Thus, the best results with respect to the number of SEs regenerated were observed at the lowest concentration of picloram used, with an average of 20.2 SEs regenerated per callus. This number is significantly higher than that at 450 μM, at which the number of embryos regenerated reached an average of 4.2; at this point, the aerial parts had grown to approximately 0.5–1.0 cm and could be isolated from the other developing embryos (Fig. 3h, i). The results obtained with picloram are similar to those obtained with 2,4-D in a study by Ledo et al. (2002) in which 14.2 SEs were regenerated per EC; however, this is approximately 42% lower than that obtained with picloram in the present study.

The plants regenerated from the SE clumps were gradually individualized according to their differentiation and were transferred individually to fresh medium without growth regulators for complete development (Fig. 3h, i). It should also be noted that isolating germinated SEs only after their differentiation is important because it allows the continuous proliferation and differentiation of plants from the formed clusters of SEs, suggesting that this could be applied for large-scale cultivation of this species.

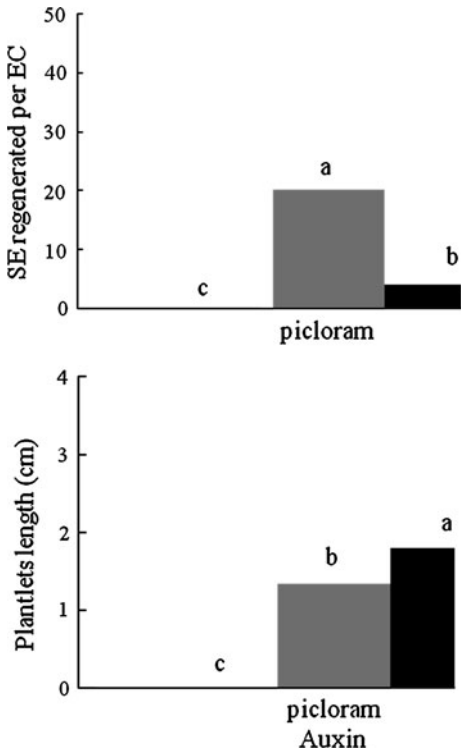

Fig. 1 Effect of different concentrations of picloram (0, 225 and 450 μM) on total number of somatic embryos (SE) regenerated per embryogenic calli (EC) and plantlets length during somatic embryogenesis in açaí palm from immature zygotic embryos. Means followed by *same letters* were not significantly different ($p = 0.05$) (Tukey's test)

Figure 2 illustrates the ontogenic stages of the somatic embryogenesis of *E. oleracea*. The ontogenic analysis shows that the initial cell divisions occur in the epidermal and subepidermal regions of the explants. In this region of the zygotic embryo, small cells are observed with a prominent nucleus, dense cytoplasm, and thin cell walls dividing on various levels (Fig. 2b–d), characterizing cells with embryogenic competency. Rodriguez and Wetzstein (1998) also observed that the start of cell division in immature *Carya illinoinensis* embryos induced by NAA and 2,4-D occurs in the subepidermal layer of the cotyledons.

Within the zygotic embryo and in the region where the frequency of cell division was high, cells were large and contained starch grains but had lower cytoplasm density (Fig. 2a). In general, the starch grains accumulated only in cells that did not exhibit embryogenic characteristics and were located close to the regions where cell division was occurring. Kanchanapoom and Domyoas (1999) also detected the accumulation of starch in calli and bipolar embryoids of *Elaeis guineensis*, suggesting that starch increases with the formation of SEs in this species.

The successive cell divisions initially form a protuberance containing 1 or 2 meristematic cells (Fig. 2b), progressing to a numerous multicellular formation that evolves to nodular calli, leading to the formation of somatic proembryos (Fig. 2d). According to Zimmerman (1993) and Fehér et al. (2003), the process of the induction of somatic embryogenesis, which includes the formation of the callus, characterizes an indirect regenerative route.

From the development of the proembryogenic mass, the formation of SEs is observed with numerous spherically arranged cells characterizing SEs in the globular stage (Fig. 2e, f) of multicellular origin. At the base of the formed globular structure, cells similar to the suspensor, which connects the SEs to the tissues of the zygotic embryo, were observed. Embryos of multicellular origin are fused to their tissue of origin (Williams and Maheswaran 1986), share a common protoderm, and give rise to nodular calli consisting of embryogenic masses, leading to the formation of globular embryos. Saénz et al. (2006) also observed that SEs of *Cocos nucifera* exhibit multicellular origin and are formed from the nodular calli. In the present study, besides the embryos of multicellular origin, SEs of unicellular origin, which are characterized by their independence from the explant of origin, also

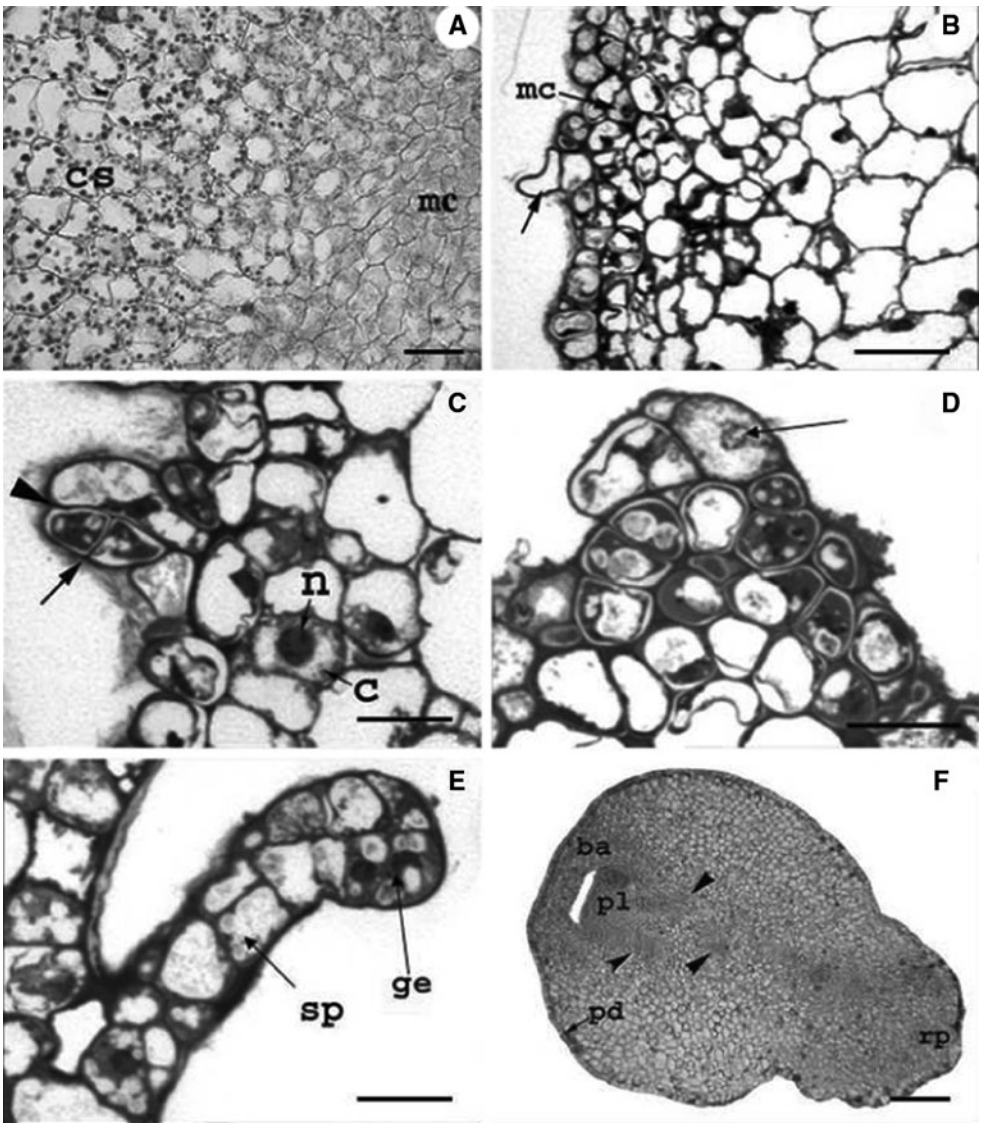

Fig. 2 Ontogenesis of somatic embryos of the açaí palm (*Euterpe oleraceae*). **a** Embryogenic callus containing cells with starch grains situated close to regions with intensely-dividing meristematic cells; **b** protoderm with intensely-dividing meristematic cells (*arrows*); **c** anticlinal (*arrow point*) and periclinal cell divisions (*arrow*); **d** proembryos containing cells with prominent nuclei and dense cytoplasm; **e** globular somatic embryo with suspensor cell; **f** somatic embryo presenting a sheath around the apical or plumular meristem, procambial strands (*head point*), protoderm, and root pole with little differentiation; *cs* cells with starch grains; *mc* meristematic cells; *n* nucleus; *c* dense cytoplasm; *ge* globular embryo; *sp* suspensor cell; *ba* sheath; *pl* plumule; *pd* protoderm; *rp* root pole. *Scale bars* **a** = 20 μm; **b**, **f** = 50 μm; **c**–**d**, **e** = 10 μm

formed. In zygotic embryos of *E. guineensis* Jacq., the process of somatic embryogenesis begins at the subepidermal cells; according to the authors, the embryos exhibit a unicellular origin (Kanchanapoom and Domyoas 1999) (Fig. 3).

In conclusion, we described a procedure for establishing a protocol for obtaining a high frequency of EC and SE regeneration from immature zygotic embryos of açaí palm. The auxin picloram combined with activated charcoal significantly improves the process of EC induction. Morphoanatomical analyses revealed the origin and changes that occur during the different stages of somatic embryogenesis. These findings may offer an invaluable contribution for future studies on açaí palm since the detailed descriptions of the protocol and morphological routes involved in the different stages of the process will help other researchers to more easily clone this species from immature zygotic embryos.

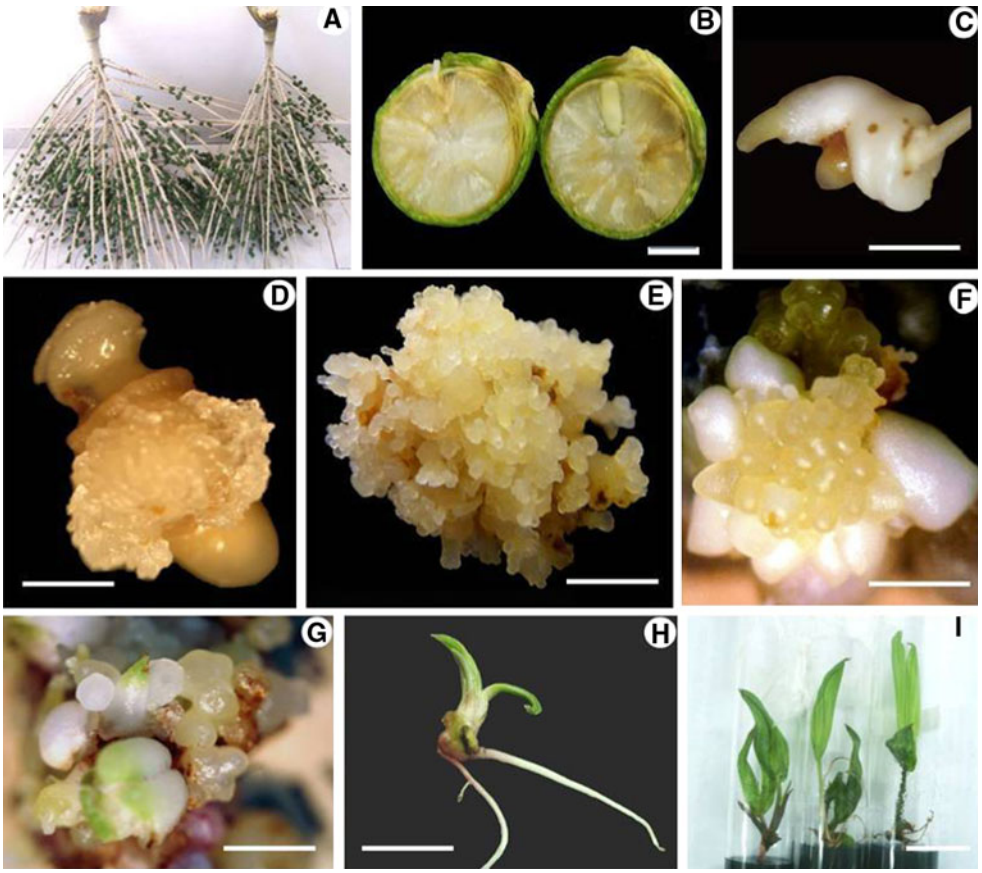

Fig. 3 Somatic embryogenesis from immature zygotic embryo in açaí palm (*Euterpe oleracea* Mart.) **a** Racemes with immature fruits of açai palm. **b** Immature fruits of açaí palm, 100–110 days post-anthesis showing the zygotic embryo used to induce somatic embryogenesis. **c** Germination of immature zygotic embryo in culture medium without the presence of growth regulators (control). **d** Formation of primary callus after around 30 days of culture in induction culture medium. **e** Progression of embryogenic callus from primary callus after 20 weeks on callus induction medium. **f** Cluster of somatic embryos from embryogenic callus on differentiation and maturation culture medium. **g**, **h** Conversion of somatic embryos on regeneration culture medium. **i** Açaí palm plants regenerated from somatic embryogenesis. *Scale bars* **b**, **c**, **e**–**g** = 2 mm; **d** = 1 mm; **h** = 5 mm; **i** = 20 mm

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