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Somatic embryogenesis from immature and mature zygotic embryos of the açaí palm (*Euterpe oleracea*): Induction of embryogenic cultures, morphoanatomy and its morphological characteristics

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ABSTRACT

A somatic embryogenesis protocol for the açaí palm (*Euterpe oleracea* Mart.) was developed, based on mature and immature zygotic embryos, to define morphoanatomically the process's different stages and to analyze the homogeneity of nuclear DNA content by flow cytometry from calli, somatic embryos and regenerated plants. Auxin picloram (4-amino-3,5,6-trichloropicolinic acid) was tested to induce embryogenic calli at 225 and 450 μM concentrations, coupled to the physiological maturing stages of zygotic embryos (mature and immature). Murashige and Skoog (MS) medium with 30 g l^{-1} sucrose, 2.5 g l^{-1} Phytagel, 2.5 g l^{-1} activated charcoal and 0.5 g l^{-1} L-glutamine was employed for callus induction. Embryogenic calli with somatic embryos in initial differentiation were transferred to a culture medium with 12.3 μM of 2iP and 0.6 μM of NAA and 300 mg l^{-1} of activated charcoal for differentiating and maturing somatic embryos. Plant regeneration occurred in a medium with 1.0 μM BAP (N^6-benzylaminopurine) and 0.5 μM GA_3 (gibberellic acid). The formation of embryogenic calli in all treatments was observed in the induction medium, regardless of the stage of development of the zygotic embryo. Picloram at 450 μM concentration provided the best results in forming embryogenic calli (84.7%). In the differentiating and maturing stage, 100% of the explants that had an embryogenic callus formation resulted in somatic embryos. The largest rate of plant regeneration (58.7%) was noted in treatment with an induction medium of 450 μM of picloram and somatic embryos obtained from immature zygotic embryos. Morphoanatomical analyses evidenced that induction of somatic embryogenesis reflected stages characteristic of the indirect kind. Regenerated plants showed normal development, with growth of roots and aerial part. Calli, somatic embryos and plants, analyzed by flow cytometry, revealed no significant differences in the estimated rates of DNA content.

1. Introduction

The açaí palm (*Euterpe oleracea* Mart.) is a typical tropical palm tree commonly found in the estuary of the Amazon River (Shanley et al., 2010). The population of the Amazon region prepare the "açaí" beverage from its fruit, featuring very high energetic and nutritional value (Ribeiro et al., 2007; Menezes et al., 2008, 2011). Açaí pulp is rich in phenolic compounds, among which the outstanding compounds are flavonoids, chiefly anthocyanins (Santos et al., 2008; Gordon et al., 2012). In the last decades, the market for açaí sales has expanded in Brazil and overseas owing to the worldwide trend in consuming healthier foods. Consequently, an increasing interest by Brazilian producers has emerged to grow this species on a commercial scale, thus expanding even more the area dedicated to this fruit-bearing palm tree in the Amazon region (Pompeu et al., 2009; Pacheco-Palencia et al., 2009; Menezes et al., 2011; Gordon et al., 2012).

The açaí palm is endowed with strategies for sexual (seeds) and asexual propagation (tillers). However, the production of seedlings on a commercial scale takes place by seeds only, since vegetative propagation by tillers is highly limited due to low efficiency and plant survival rates during the process. Hence, the key propaga-

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tion mechanism employed has been with seeds, a relatively uneven mechanism that often gives rise to the formation of heterogeneous plants (Farias Neto et al., 2005). Furthermore, açaí palm seeds are recalcitrant, short-lived and easily dehydrated. These factors impair preservation, genetic improvement programs for the species and propagation of genotypes of interest (Martins et al., 2004).

Under the circumstances, somatic embryogenesis arises as an alternative for multiplying genotypes of interest, either to produce elite plants or to accelerate genetic improvement programs (Litz and Gray, 1995). In fact, somatic embryogenesis is possible due to the notable plasticity of vegetable cells (Fehér, 2008). However, so that cells result in somatic embryos, care should be taken with key factors that influence somatic embryogenesis, such as growth regulators, types of explants, formulations of culture media and genotype (Badawy et al., 2005; Asemota et al., 2007; Namasivayam, 2007; Steinmacher et al., 2007; Gueye et al., 2009; Perera et al., 2009).

As a rule, explants with differentiated or undifferentiated cells become embryogenic when exposed to stress conditions usually caused artificially by adding growth regulators to the culture medium (Thompson, 2008; Viñas and Jiménez, 2011). Henceforth, the cells will undergo a series of morphological, biochemical and genetic changes, and will be able to revert their state of differentiation under specific conditions, and enter again into the mitotic cycle, following morphogenic routes which result in the development of somatic embryos (Williams and Maheswaran, 1986; Fehér et al., 2003; von Arnold, 2008; Gueye et al., 2009).

Somatic embryogenesis may occur directly or indirectly. Whereas in the former case somatic embryos originate directly from explant tissues, in the indirect manner embryos originate from explant's pre-formed cellular masses, based on consecutive differentiation cycles, until they arrive at the embryogenic callus stage, or rather, the last and decisive step prior to differentiation of the somatic embryos themselves (Williams and Maheswaran, 1986; Fehér et al., 2003). Previous studies with *E. oleraceae* demonstrated that somatic embryogenesis in the species occurs on the formation of calli (Scherwinski-Pereira et al., 2012). This form is recognizably more susceptible to the occurrence of a buildup of cytogenetic abnormalities in the material under cultivation, owing to the intense cellular division and reprogramming activity by the calli in their different morphogenic stages. Consequently, changes resulting from *in vitro* culture reflect features of self-imposed mutagenesis due to the normal cellular cycle's absence of control (Larkin and Scoweroft, 1981; Karp et al., 1987; Bajaj, 1990; Phillips et al., 1994; Thiem and Sliwinska, 2003; Lema-Ruminska, 2011; Silva and Carvalho, 2014). Thus, any perturbation affects the synchrony of cell events duplication, often associated with euploidy and/or aneuploidy (Lee and Phillips, 1988; Orzechowska et al., 2013).

Although such variation often occurs during the formation of calli, it is not safe to assume when the change begins. Thus, it is necessary to avoid any kind of cell variation, particularly in new lines derived from calli during somatic embryogenesis, once the cells are multiplied originating new cells that, in the last case, will originate great quantities of *in vitro* plants (Zhao et al., 2012; Gong et al., 2013; Gomes et al., 2016). Thus, monitoring steps of somatic embryogenesis, especially during the callus phase, and some advanced formations, may prevent the proliferation of malformed cell lines (somaclones), and consequently malformed plants.

Flow cytometry has recently been suggested as an auxiliary tool to assess, define and correlate morphogenic profiles, as well as to allow advance detection of likely behavior, including cytogenetic abnormalities by propagules or *in vitro* plants (Rival et al., 1997; Dolezel et al., 2007a; Prado et al., 2010; Naing et al., 2013; Medrano et al., 2014). Cytometry is currently being employed to assess nuclear DNA contents, ploidy levels, as well as cell cycle studies and, in some cases, as a tool to detect genomic instability (for example, gains or losses in genetic material) of micropropagated plants, as in *Quercus robur* (Endemann et al., 2001), *Eucalyptus globulus* (Pinto et al., 2004), *Scutellaria baicalensis* (Alan et al., 2007), *Juniperus phoeniceae* (Loureiro et al., 2007), *Vitis vinifera* (Prado et al., 2010), *Passiflora cincinnata* (Silva and Carvalho, 2014), *Saccharum* spp. (Nogueira et al., 2015) and others (Dolezel et al., 2007b; Tulin and Cross, 2014).

Therefore, the availability of a somatic embryogenesis protocol for *E. oleraceae* which defines and minimizes or eliminates cytogenetic abnormalities of the multiplied material is essential to increase efficiency of the species' asexual propagation. To date, only two studies have described açai palm somatic embryogenesis from zygotic embryos (Ledo et al., 2002; Scherwinski-Pereira et al., 2012). However, results demonstrated lack of in-depth knowledge on the development phases of the somatic embryogenesis, especially on the manner morpho-anatomical events progress during the embryogenic process and improve the understanding of the technique. Current study improves the somatic embryogenesis protocol based on the use of zygotic embryos originating from mature and immature fruits. It also characterizes morphoanatomically and by flow cytometry the different stages involved in the process.

2. Material and methods

2.1. Plant material and cultivation conditions

Mature fruit zygotic embryos (160–180 days, post-anthesis) and immature fruit (80–100 days, post-anthesis) of *Euterpe oleracea* were used. They originated from adult plants at the Embrapa's açaí palm Gene Active Bank in the Eastern Amazon region, Belém PA Brazil.

The fruits were washed for 5 min running water and commercial detergent. They were then immersed in ethanol 70% for 3 min, immersed in sodium hypochlorite (2.5% active chlorine) for 30 min, and subsequently rinsed three times in sterile distilled water.

The basic medium comprised MS medium (Murashige and Skoog, 1962), salts and vitamins, plus 30 g l^{-1} sucrose and 2.5 g l^{-1} Phytagel (Sigma, St. Louis, MO, USA). Other organic and inorganic compounds and growth regulators were added to the culture medium, specifically for each cultivation stage (Table 1). The culture medium's pH was always adjusted to 5.8 ± 0.1 with 0.1 N of HCl or 0.1N of NaOH prior to sterilizing for 20 min at 121 °C and pressure at 1.05 kg/cm^2.

During the somatic embryogenesis induction stages and differentiation and during the maturing of somatic embryos, the cultures were kept in darkness, while during the regeneration stage the cultures were placed in the light, at 52 μm m^{-2} s^{-1} for 16/8 h (light/dark) photoperiod. The growth room's temperature was 25 ± 2 °C during the entire experiment.

2.2. Induction of somatic embryogenesis

Excised zygotic embryos were cultivated in Petri dishes (15 × 90 mm), sealed with parafilm, containing 20 ml of induction culture medium, plus 2.5 g l^{-1} of activated charcoal, 0.5 g l^{-1} of L-glutamine and supplemented with picloram at concentrations 225 μM and 450 μM. Explants were kept in this culture medium up to 24 weeks after which the percentage formation of embryogenic calli was assessed. Sub-cultures were performed after every 30 days interval.

2.3. Differentiation and maturation

Basic medium was with 12.3 μM of 2-isopentenyl-adenine (2iP), 0.6 μM of naphthalenoacetic acid (NAA), and 300 mg l^{-1} of activated charcoal to mature differentiated somatic embryos in an