



Explant age and genotype drive the somatic embryogenesis from leaf explants of *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. (Arecaceae), an alternative palm crop for oil production

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Abstract

Key message A complete system of regeneration, via somatic embryogenesis, from the in vitro culture of leaflets explants of young *A. aculeata* donor-plants has been reported.

Abstract In the present study, a complete regeneration protocol of *Acrocomia aculeata* (Jacq.) Lodd. ex Mart., via somatic embryogenesis is reported, and the influence of the genotype and its age on the induction of the embryogenic process determined. Leaflets explants of 4 genotypes, aged 2 and 5 years, were inoculated in the induction medium consisting of salts and vitamins Y3 supplemented with different concentrations of picloram (9.0, 18.0 and 36.0 μM). In the control, no plant growth regulators were added. Picloram concentrations of 18.0 and 36.0 μM induced greater formation of embryogenic calluses in all genotypes studied. However, 2-year-old genotypes had higher percentages of embryogenic calluses. In addition, at the highest concentration of picloram (36.0 μM), 5-year-old genotypes had the highest oxidation rates. Differentiation of somatic embryos was observed in medium supplemented with 9.0 and 18.0 μM picloram and 1 mM putrescine. However, at a concentration of 9.0 μM , the somatic embryos showed a high degree of fusion. Embryogenic lines were only obtained in medium supplemented with 18.0 μM picloram and 1 mM putrescine. Histochemical analysis showed the presence of pectins in embryogenic cultures and starch grains in peripheral regions of embryogenic calluses, which were not directly involved in regeneration. Somatic embryos were converted into plantlets after 90 days in germination medium containing 0.54 μM NAA, 1 mM putrescine and 3.0 g L^{-1} activated charcoal, highlighting the potential of the propagation system proposed here for clonal propagation of *A. aculeata*.

Keywords Macaw palm · Oil palm · Palm micropropagation · Picloram · Somatic embryo

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Introduction

The demand of the industrial market for vegetable oils and the expansion of renewable energy sources have accelerated the development of the agro-industrial chain of oilseeds in the world (Yang et al. 2018; Oliveira et al. 2021). Soybean, palm oil and rapeseed dominate the plant-based oil production, maintaining their participation in the different industries of derived products (Rodionova et al. 2017). However, with the approval of laws that prohibit the import of products obtained from deforestation, as done by the European Union, prospecting alternatives for this agricultural commodity is paramount. The tropical region because of its great plant biodiversity, adequate climatic conditions and unoccupied

arable areas represents a potential source for new crops and for planting oilseeds.

Acrocomia aculeata (Jacq.) Lodd. ex Mart. (Arecaceae), popularly known as macaw palm, is a palm tree with fruits that have a high oil content, a characteristic that makes it a potential species for use in food, cosmetic, and feed industries (César et al. 2015; Cardoso et al. 2017), as well as for biofuel production (Motoike and Kuki 2009; César et al. 2015; Granja et al. 2018). The species is also known for its rusticity towards prolonged drought periods, fire and herbivory (Motoike et al. 2013). Despite all of that, this palm is still poorly explored, mainly by extractive manner in native populations (Abreu et al. 2011). Information related to the domestication and propagation of *A. aculeata* is scarce, which hinders the selection and multiplication of genotypes of interest, as well as the obtaining of seedlings with genetic and phytosanitary quality (Motoike et al. 2013).

Acrocomia aculeata is traditionally propagated by seeds because, unlike many palm trees, this species does not generate clumps (Moura et al. 2009; Motoike et al. 2013). However, there is high genetic variability, as the species is a predominantly allogamous (Lanes et al. 2016). The irregular production of fruits throughout the year and the long juvenile period of this species (Manfio et al. 2012) have stimulated the development of alternative systems for cloning superior individuals of *A. aculeata*, such as somatic embryogenesis.

The regeneration of *A. aculeata* plants, via somatic embryogenesis, was initially proposed in the early 1990s (Tabai 1992), although the maturation of somatic embryos and their conversion into plantlets have failed due to problems related to phenolic oxidation. The induction of embryogenic calluses of *A. aculeata* has also been reported by Moura et al. (2009) and Luis and Scherwinski Pereira (2014). However, the regeneration systems reported by these authors were initiated from zygotic embryos, which limits the use of the technique for cloning selected mother plants. In addition, factors such as genotype and explant age seem to have a significant effect on the embryogenic process of *A. aculeata*, so that some materials are recalcitrant and not responsive to the technique, showing genotype-dependent behavior (Granja et al. 2018).

Recently, embryogenic lines of *A. aculeata* have been obtained from the in vitro culture of immature leaflets segments, which is an important step in establishing a clonal multiplication system for the species (Meira et al. 2020), but callus production occurred after a long period of in vitro culturing (270 days). In addition, difficulties in the synchronization and maturation of the somatic embryos have been reported (Meira et al. 2020), highlighting that the routine use of the technique is far from being a commercial reality and that further studies are necessary to optimize the embryogenic process of *A. aculeata*.

In the present study, a complete in vitro regeneration system of *A. aculeata* plantlets was achieved. We also reported the influence of the genotype and its age on the efficiency of somatic embryogenesis induction and described the structural changes involved in the differentiation of pro-embryogenic structures.

Materials and methods

Plant material

The plant material used as a source of explants was obtained from four genotypes of *A. aculeata*, established in the Macaw Palm Germplasm Bank (BGP—Macaw), located in the municipality of Araponga, Minas Gerais, Brazil, consisting of two young plants with two years of age (BGP49 and BGP21) and two adult plants with five years of age (BGP40 and BGP27) (Fig. 1). These genotypes come from fruits collected from plants located in the municipalities of Bocaiúva (BGP49), Belo Horizonte (BGP21), Pitangui (BGP40) and Abaeté (BGP27), in Minas Gerais, Brazil (Fig. 1).

To obtain explants in young plants (BGP49 and BGP21), a careful dissection was performed using a stainless blade until reaching the immature leaves being separated in layers of approximately 20 cm. Next, the leaflets of each leaf were detached from the rachis, with 1/3 of the leaflet apex being eliminated. In adult plants (BGP40 and BGP27), the shoot containing the young leaves (heart of palm) was extracted using chainsaw.

Phase 1—induction of embryogenic calluses

Upon extraction, the immature leaflets were subjected to disinfection in aseptic solution (1% sodium hypochlorite) for 10 min. Then, they were rinsed eight times with distilled water in a laminar flow cabinet. The leaflets were sectioned with a scalpel into segments of approximately 2 cm² (Fig. 2a) and inoculated in polystyrene Petri dishes (90 × 15 mm) containing 30 mL of basal culture medium (BM) composed of salts and vitamins Y3 (Eeuwens 1978), 30 g L⁻¹ sucrose, 1 g L⁻¹ hydrolyzed casein, 100 mg L⁻¹ myo-inositol, 100 mg L⁻¹ arginine, 100 mg L⁻¹ asparagine, 100 mg L⁻¹ glutamine, jellied with 2.5 g L⁻¹ of Phytigel® (Sigma, USA) and different concentrations (9.0, 18.0 and 36.0 μM) of picloram. The pH of the culture media was adjusted to 5.7 ± 0.1 before they were autoclaved for 20 min at 121 °C and 1.5 atm. In the control treatment, no plant growth regulator was added.

For inoculation of explants, 5 explants of approximately 1 cm each were inoculated in petri dishes, each petri dishes consisted of a replication, and each explant an experimental unit, in total 20 plates were used per treatment. After

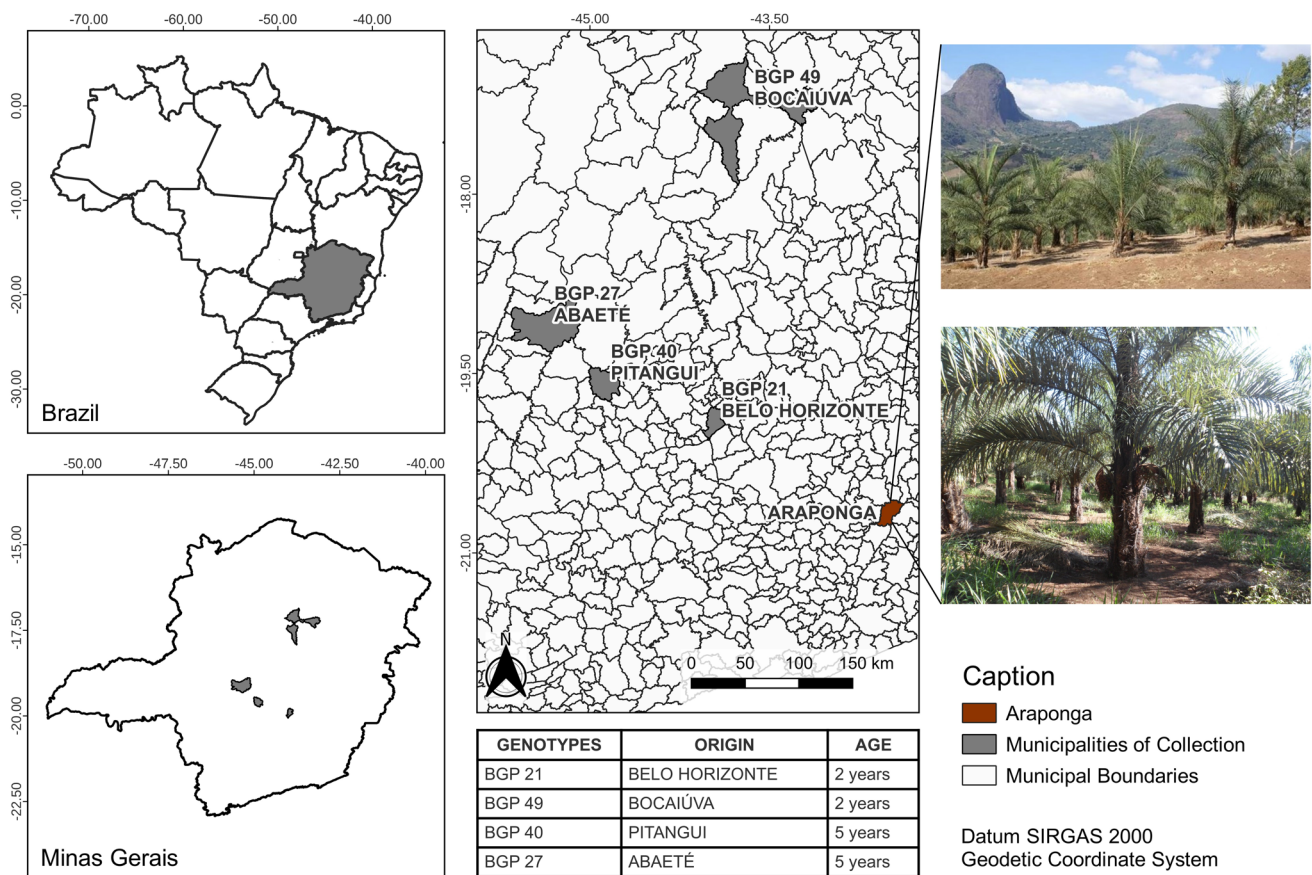


Fig. 1 Map with the geographic distribution of the collection area of *Acrocomia aculeata* genotypes in the state of Minas Gerais, Brazil

inoculation of the explants, the petri dishes were sealed with PVC film (Rolopac®) and kept in a growth room, at temperature of 27 ± 1 °C in the dark. Evaluations for the presence of biological contaminants were performed after 10 days of culturing. The development of calluses was monitored periodically and, after 60 days, showing callus formation explants were subcultured on a new culture medium containing the same composition, remaining for more 30 days under this condition. After this period, the percentage of responsive explants (number of explants per Petri dish with the presence of callus) and percentage of fully oxidized explants per Petri dish were evaluated.

Phase 2—multiplication of embryogenic lines

The calluses obtained in the induction phase of somatic embryogenesis were separated into masses of approximately 5 mm in diameter. Due to the number of calluses obtained, it was possible to establish a total of 5 replicate per treatment, each replication consisting of a petri dish.

The calluses were transferred to polystyrene Petri dishes (90 × 15 mm) containing 30 mL of BM, supplemented with the same concentrations of picloram used in the induction

phase (9.0, 18.0 and 36.0 μM), with the addition or not of 1mM putrescine. Due to the lower callus induction frequency of explants obtained from BGP40 and BGP27 plants, only calluses from BGP49 and BGP21 genotypes were used in this phase. After inoculation, the Petri dishes were sealed with PVC film (Rolopac®) and kept in a growth room, at temperature of 27 ± 1 °C in the absence of light.

The Petri dishes were kept under this condition for 90 days. Subsequently, the embryogenic lines, according to classification of Corrêa et al. 2015 (lines that presented granular aspect, yellow coloration, multiplication capacity increased five times or more from the original size) were transferred to culture medium with the same composition. After 30 days, the embryogenic lines continued to be subcultured in BM culture medium supplemented with 18 μM picloram and 1 mM putrescine, being subcultured every 60 days, for a total period of 180 days.

Phase 3—regeneration and germination of somatic embryos

The embryogenic lines were kept for 45 days in a regeneration medium consisting of BM, supplemented with 0.1

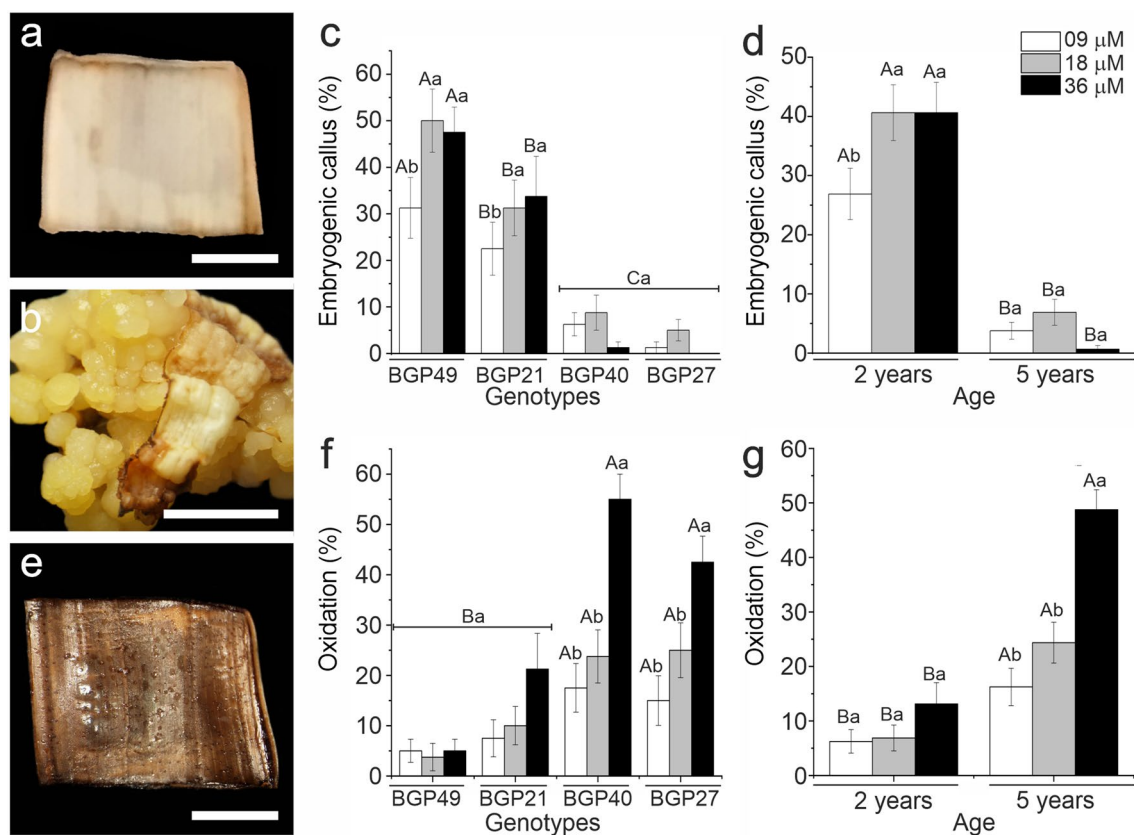


Fig. 2 Responsiveness of leaf explants of *Acrocomia aculeata* in somatic embryogenesis induction medium. Initial explant (a). Embryogenic calluses (b–d). General appearance of the embryogenic callus (b). Interactions between genotypes (BGP49 and BGP21, BGP40 and BGP27) and picloram concentrations (9.0, 18.0 and 36.0 μM) (c) and between genotype age (2 and 5 years) and picloram concentrations (d) on the percentage of embryogenic calluses. Oxidation of explants (e–g). Oxidized leaf explant (e). Interaction between genotypes and

picloram concentrations (f) and between genotype age and picloram concentrations (g) on the percentage of oxidized explants. Different uppercase letters indicate differences between genotypes/age at the same picloram concentration by Tukey test ($p < 0.05$). Different lowercase letters show differences between picloram concentrations for the same genotype/age by Tukey test ($p < 0.05$). Error bars indicate the standard error. Scale bars = 0.5 cm

μM 2,4-D, 1 mM putrescine (Corrêa et al. 2015, 2016) and 3.0 g L^{-1} activated charcoal (Sigma, USA). The Petri dishes were sealed with PVC film (Rolopac®) and kept in a growth room, at temperature of 27 ± 1 °C in the dark for 60 days. After this period, the 90 somatic embryos obtained from the embryogenic lines ($n = 40$) were individualized and inoculated in a test tube (150 \times 25 mm) containing 10 mL of the germination medium. The culture medium for germination consisted of basal medium supplemented with 0.54 μM NAA, 1 mM putrescine (Corrêa et al. 2015, 2016) and 3.0 g L^{-1} activated charcoal (Sigma, USA). The test tubes were kept in a growth room at 27 ± 1 °C with photoperiod of 16 h/day and irradiance of ± 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by LED tubular lamps (18 W, Arapeva LED Lighting). After 90 days, the number of fully formed plantlets, that is, those with shoots and roots, at least 5 cm long, was recorded.

Anatomy and histochemistry of calluses and embryogenic lines

Samples of embryogenic calluses obtained in the induction phases (9.0 and 18.0 μM picloram) and multiplication of embryogenic lines (0.1 μM 2,4-D, 1 mM putrescine) were collected, fixed in FAA₅₀ (formaldehyde: acetic acid: 50% ethyl alcohol in the proportion of 5: 5: 90) for 24 h, and stored in 70% ethyl alcohol. Subsequently, the fixed samples were dehydrated in an increasing series of ethanol and embedded in methacrylate (Histo-resin, Leica). With a rotary microtome (RM2265, Leica Microsystems Inc., Buffalo Grove, IL), transverse and longitudinal sections with 5 μm thickness were stained with toluidine blue (O'Brien and McCully 1981) for structural characterization, PAS (periodic acid-Schiff's reagent; Feder and O'Brien 1968) for total polysaccharides and ruthenium red (Johansen 1940) for

pectin detection. Observations and photomicrographs were performed in an Olympus Provis AX70 photomicroscope, equipped with a U-photo system.

Statistical design

The experiment on phase 1 was carried out in a completely randomized design, in a $4 \times 4 \times 2$ factorial scheme, consisting of 4 genotypes (Fig. 1), 3 concentrations of the picloram (9.0, 18.0 and 36.0 μM) and two ages (2 and 5 years). Twenty replicates per treatment were used, each replicate consisting of one Petri dish containing four leaflets explants. The callus data obtained by explant in each replication were transformed according to the square root of $(x + 0.5)$ and subjected to analysis of variance. The three factors alone and the genotype \times picloram concentration and age \times picloram concentration interactions were tested. As not all genotypes tested were present in the different age classes, the genotype \times age and genotype \times picloram concentration \times age interactions were not tested. The means were compared by Tukey test at 5% probability level, using Genes statistical software (Cruz 2013).

The experiment on phase 2 was carried out in a completely randomized, in a $2 \times 3 \times 2$ factorial scheme: calluses of BGP49 and BGP21 plants, 3 concentrations of picloram (9.0, 18.0 and 36.0 μM) and presence (1 mM) or absence of putrescine. The treatments consisted of 5 replicates per treatment, each replicate consisting of a petri dish with masses of approximately 5 mm in diameter. Each replicate was represented by one Petri dish with four callogenic masses of approximately 5 mm in diameter. In the experiment on phases 2 and 3, only descriptive evaluations were performed.

Results

Induction of embryogenic calluses

Genotype, age and picloram concentration significantly influenced the development of embryogenic calluses of *A. aculeata* (Fig. 2). Regardless of the picloram concentration tested, the genotype BGP49 proved to be superior in the formation of embryogenic calluses, followed by the genotype BGP21 (Fig. 2c). For both genotypes (BGP49 and BGP21), picloram concentrations of 18.0 and 36.0 μM induced greater responsiveness of the explants (Fig. 2c). The calluses obtained showed a bright nodular aspect, with beige to light yellow color and with globular structures along their entire surface (Fig. 2b).

For the age and picloram concentration interaction, there was a greater formation of calluses in the two-year-old explants (BGP49 and BGP21) when grown in basal medium supplemented with 18.0 and 36.0 μM picloram (Fig. 2d).

Five-year-old genotypes (BGP40 and BGP27) showed lower responsiveness, regardless of the picloram concentration, with no difference in responsiveness between them (Fig. 2c, d). In the absence of plant growth regulators, there were no calluses in any of the genotypes evaluated (data not shown).

Regarding oxidation, genotypes BGP40 and BGP27 had a higher number of fully oxidized explants when grown in culture medium supplemented with 36.0 μM of picloram (Fig. 2e, f). The interaction between the percentage of oxidation and genotype age was significant, and 5-year-old explants showed higher percentage of oxidized explants (Fig. 2g) and also lower percentages of embryogenic calluses (Fig. 2d).

Somatic embryo development and callus multiplication

Callus already formed, after 100 days of these to multiplication media, consisting of the same picloram concentrations plus 1 mM putrescine, differentiation of somatic embryos was observed (Fig. 3). In the media supplemented with 9.0 μM picloram plus 1 mM putrescine, the development of somatic embryos was observed in 93.75% of the embryogenic calluses of the BGP49 genotype. These somatic embryos were white in color (Fig. 3a) and were tightly fused to each other and to the calluses that originated them (Fig. 3b).

The formation of embryogenic lines was observed only in embryogenic masses from the genotype BGP49 subcultured in culture medium supplemented with 18.0 μM picloram plus 1 mM putrescine. The embryogenic lines showed light beige color with translucent aspect (Fig. 3c). The histological sections showed the formation of globular embryos from the proliferation of cells of the central meristematic region observed in embryogenic calluses (Fig. 3d). At this stage, the embryos showed typical globular structure, well-defined protoderm, delimiting the homogeneous mass of cells (Fig. 3d). The embryonic cells were intensely stained with Toluidine Blue and showed characteristics similar to those of meristematic cells, such as dense protoplasm, bulky nuclei containing a single evident nucleolus, high nucleus:cytoplasm ratio and poorly vacuolated cells (Fig. 3e).

The calluses multiplied in medium supplemented with 36.0 μM picloram in the presence (or absence) of putrescine showed translucent calluses, with a watery appearance and brown color after 100 days of culturing (Fig. 3f), making it impossible to perform histological sections. The calluses multiplied in culture medium containing 9.0 and 18.0 μM of picloram, in the absence of putrescine, only increased in size, with no differentiation of somatic embryos (data not shown).

After three cycles of multiplication of the embryogenic lines in Y3 medium supplemented with 18.0 μM

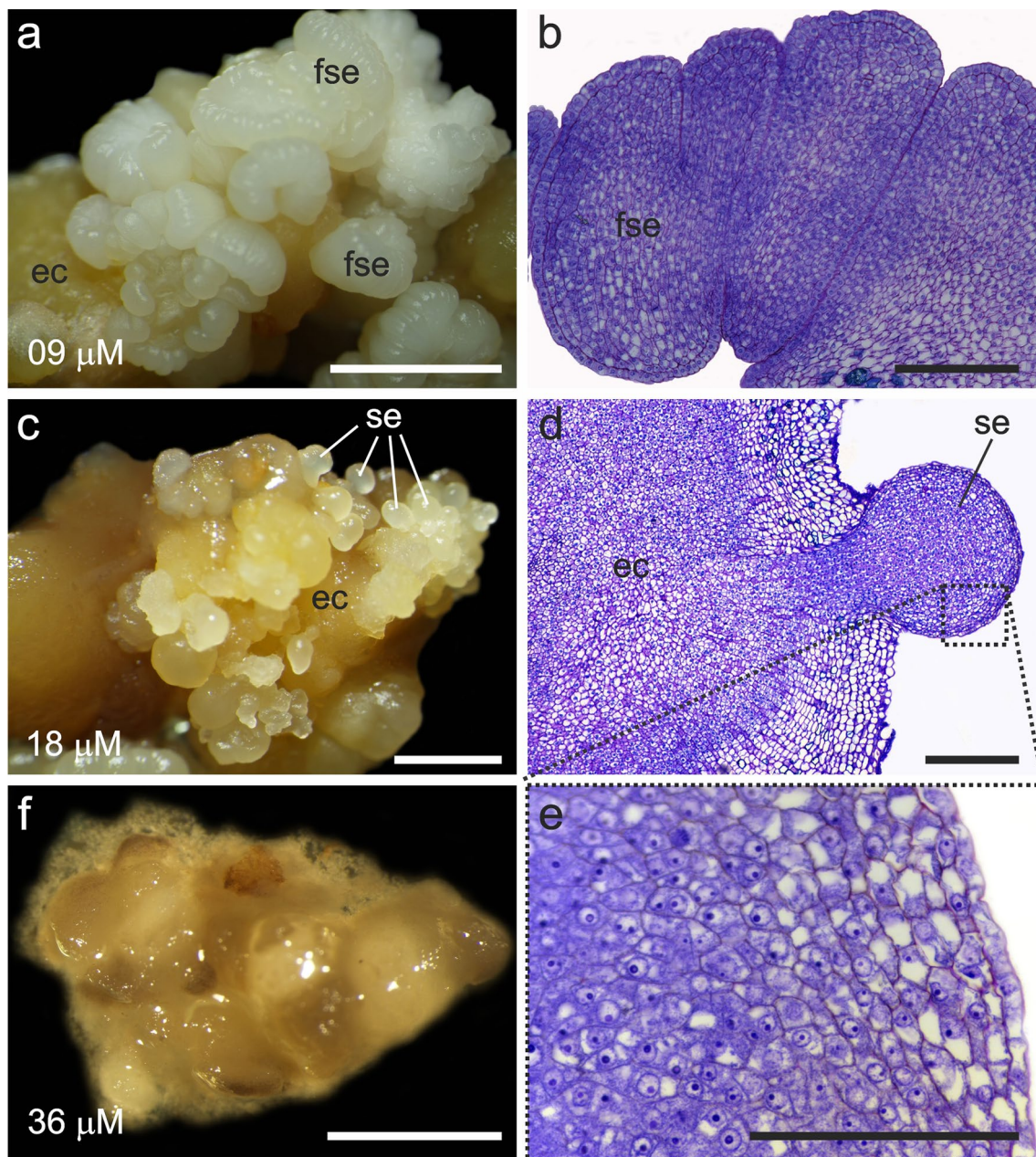


Fig. 3 Embryogenic calluses of BGP49 genotype after 100 days in multiplication medium. Development of somatic embryos in medium supplemented with 9.0 μM picloram plus 1000 μM putrescine (**a**, **b**). Note the intense fusing of embryogenic structures (**fse**) (**b**). Development of somatic embryos in medium supplemented with 18.0 μM picloram plus 1,000 μM putrescine (**c–e**). Cross-section of the

embryogenic callus (**ec**) that originated the globular somatic embryos (**se**) (**d**). Note the meristematic-like features of somatic embryo cells (**e**). Callus with unstructured aspect after multiplication in medium supplemented with 36.0 μM picloram and 1,000 μM putrescine (**f**). Abbreviations: *ec* embryogenic callus, *fse* fused somatic embryo, *se* somatic embryo. Bars: **a**, **c**, **f**=0.5 cm; **b**, **d**, **e**=100 μm

picloram plus 1mM putrescine, every 60 days of culturing, 40 masses composed of globular embryos were obtained (Fig. 5a), indicating that this methodology allows the mass multiplication and in vitro preservation of these embryogenic lines. They remained in a continuous process of multiplication, without losing embryogenic capacity for 12 months.

Histochemical analysis

Polysaccharides were present throughout the embryogenic structure (Fig. 4a–c). However, the peripheral regions to the meristematic center were more intensely stained than the central region of the embryogenic callus (Fig. 4a). Noteworthy, starch grains were also detected only in the outskirts of

the embryogenic calluses (Fig. 4b), a region that is apparently not directly involved with the regeneration of somatic embryos. In the central region of embryogenic calluses, with intense cell division, starch grains were not observed (Fig. 4c).

The positive reaction to ruthenium red stain showed the presence of compounds of pectic nature were abundant in the cell walls of the somatic embryos of *A. aculeata* (Fig. 4d, e) and, unlike polysaccharides, were evenly distributed along the somatic embryo (Fig. 4e).

Regeneration and germination of somatic embryos

The somatic embryos regenerated from the embryogenic lines showed a white color (Fig. 5a–c) and were individualized with the aid of a scalpel and transferred to the germination medium (Fig. 5d, e). At times some embryos detached easily, without the need to use the scalpel. Somatic embryos were collected from all embryogenic lines obtained and, after 90 days in germination medium (Fig. 5e), 100% of

somatic embryos were converted into plantlets (Fig. 5f) with well-developed shoots and roots (Fig. 5g).

Discussion

The genotypic influence is likely related to the high genetic diversity within the population due to the reproductive biology of the species (Lanes et al. 2016). This species, despite being monoecious and self-compatible, has a mixed reproductive system, consists in the fact that the natural occurrence of both crossings and self-pollinations (Lanes et al. 2016; Abreu et al. 2012). A similar behavior was reported in *Elaeis guineensis*, after a high amplitude of variation (~90%) in the efficiency of the induction of somatic embryogenesis was observed in 32 different accessions, some of which did not even respond to the hormonal stimuli present in the culture medium (Corrêa et al. 2015). Ooi et al. (2012) suggested that the success of somatic embryogenesis of *E. guineensis* may be associated with the expression of the putative gene AUX/IAA (*EgIAA9*) and that the presence of

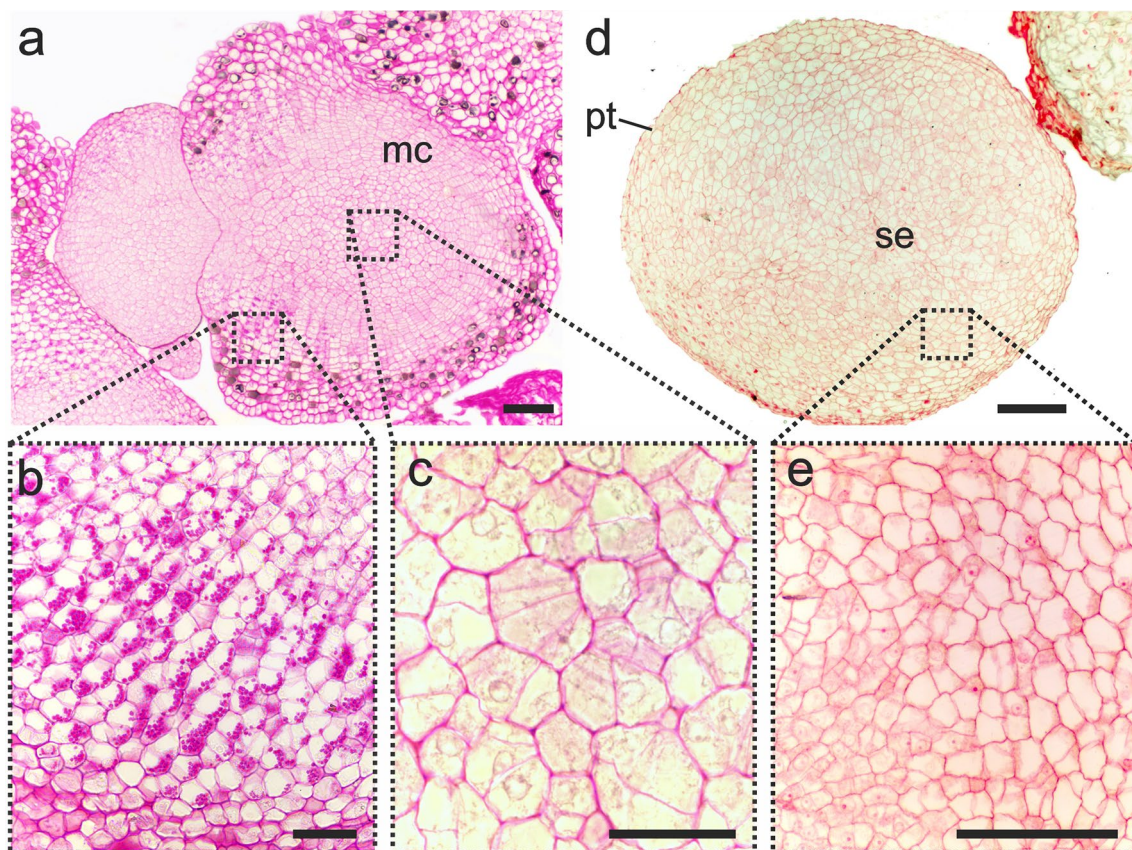


Fig. 4 Histochemical analyses of embryogenic calluses of BGP49 genotype after 100 days in multiplication medium supplemented with 18.0 μM picloram plus 1,000 μM putrescine. Positive reaction for polysaccharides evidenced by pink color (a–c). General view of embryogenic callus (a). Note the presence of starch grains in the most

peripheral cells of embryogenic calluses (b) and the absence of these compounds in the central region (c). Positive reaction for pectins in somatic embryos (se) evidenced by red color (d, e). Abbreviations: *mc* meristematic center, *pt* protoderm, *se* somatic embryo. Bars: a = 100 μm ; b–e = 50 μm

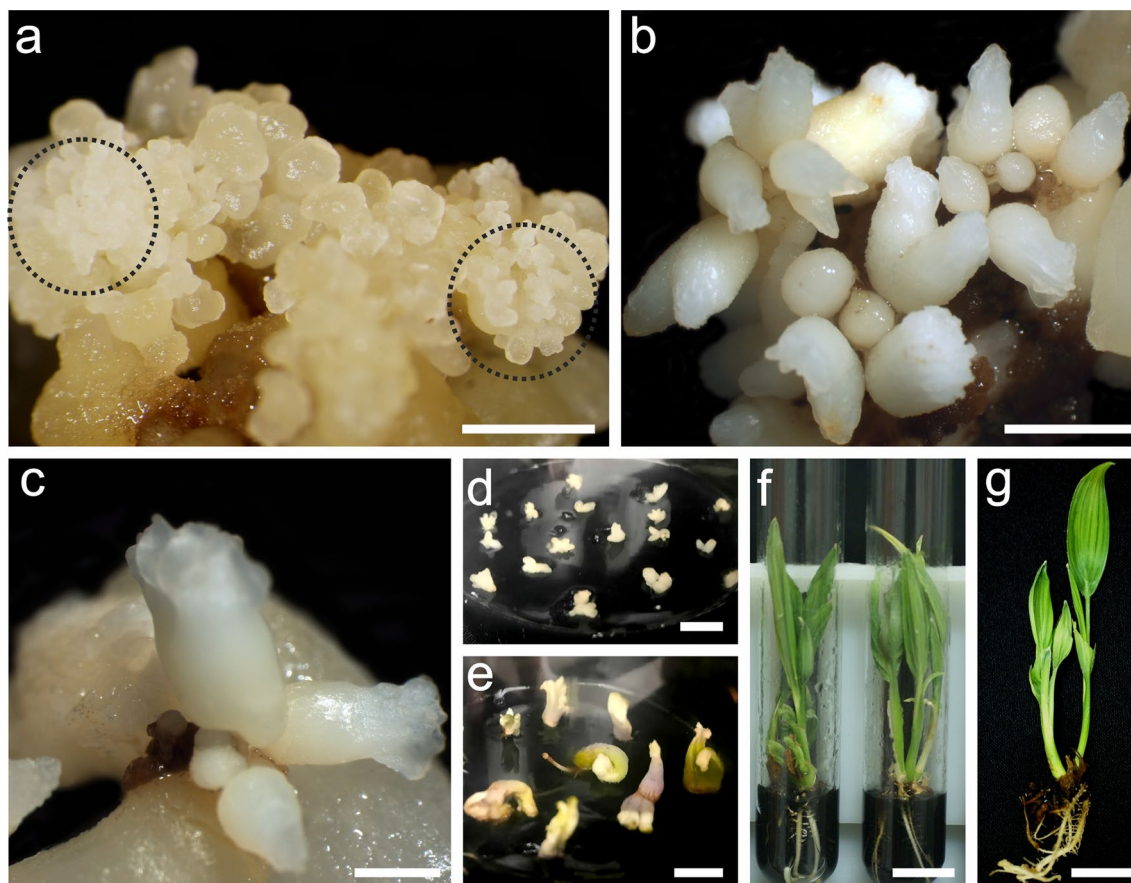


Fig. 5 Germination of somatic embryos of BGP49 genotype. Embryogenic lines after three multiplication cycles in BM culture medium supplemented with 18.0 μM picloram plus 1000 μM putrescine (circles indicate some globular somatic embryos (a)). Somatic embryos transferred to the germination medium (b–d). Somatic embryos after

60 days of culture in germination medium (e). Plantlets obtained from the germination of somatic embryos in BM medium supplemented with 0.54 μM ANA, 1000 μM putrescine and 3.0 g L^{-1} activated charcoal (d). Complete plantlet with more than one stem (e). Bars: a–c = 2 mm; d–g = 1 cm

this gene in some genotypes may be an important marker in the selection of plants to be cloned. However, this information is not available for *A. aculeata*, so more studies are needed to understand the genotype-dependent effect of this species regarding the induction of morphogenic processes.

It is important to highlight that the effect of the genotype on the response to somatic embryogenesis is not something exclusive to palm trees, other woody species present variations between the results in studies with different genotypes, such as cocoa, a very scientific species, in which many studies have demonstrated this phenomenon (Quainoo and Dwomo 2012; Kouassi et al. 2017; Pancaningtyas et al. 2021).

Younger genotypes (BGP49 and BGP21) were more responsive to the formation of embryogenic calluses, highlighting that, in addition to genotype, the juvenility of the plant tissue influences the regenerative capacity of the material. The ability of cells to acquire embryogenic competence depends on their physiological stage and

initial differentiation (Gueye et al. 2009; Rocha et al. 2016; Shahzad et al. 2017). The endogenous level of auxins, as well as the structural characteristics of explants, such as differentiation and lignification, can also influence in vitro responsiveness (Almeida et al. 2012). The intense presence of fibers and sclereids in the vascular bundles, for example, has been pointed out as cause for the low induction of nodular calluses in distal leaflets explants of *Phoenix dactylifera* (Gueye et al. 2009). It was interpreted similarly in the present study, since the adult genotypes (BGP40 and BGP27) had lower rates of callus induction. Explants obtained from these genotypes were probably in a more advanced physiological stage, which may have contributed to a lower frequency of callus formation and a higher rate of oxidation. However, it is important to mention that divergent responses are not expected along the proximal-distal axis of the same leaflets (Meira et al. 2020). According to these authors, the responsiveness of leaf explants of *A. aculeata* removed from

the distal and proximal portions was similar after 9 months of in vitro culture.

Although 2,4-D is the most commonly used auxin to induce somatic embryogenesis in palm trees (Gueye et al. 2009; Ledo et al. 2002), the picloram concentrations evaluated in the present study were efficient for the induction of embryogenic calluses of *A. aculeata*. Picloram is a synthetic auxin derived from picolinic acid and has been successfully employed in the induction of somatic embryogenesis in palm trees: *A. aculeata* (Moura et al. 2009), *Areca catechu* (Karun et al. 2004), *Bactris gasipaes* (Steinmacher et al. 2007a, b, 2011), *E. guineensis* (Scherwinski-Pereira et al. 2010; Balzon et al. 2013; Silva et al. 2013), *P. canariensis* (Huong et al. 1999) and *Syagrus oleracea* (Silva-Cardoso et al. 2019). The higher efficiency of picloram, compared to 2,4-D, for the induction activity in *A. aculeata* has been previously reported (Meira et al. 2020). However, the picloram concentration used by these authors (450 μM) was significantly higher than the best concentrations (18.0 and 36.0 μM) evaluated in the present study. Considering that Meira et al. (2020) have used explants of adult plants and that the best results obtained here were observed in juvenile plants, we suggest considering the chronological age of the mother plant supplying explants to determine the most effective picloram concentration to be added in the culture medium. In addition, high exogenous auxin levels interfere with the polar auxin gradient that is established during embryogenesis, which may prevent correct apical-basal embryo patterning. Low concentrations of picloram were also efficient in inducing somatic embryogenesis from zygotic embryos of *Passiflora edulis* (Silva et al. 2015) and *Picea abies* (Hazubska-Przybył et al. 2020), especially when combined with cytokinin. However, the comprehension of mechanisms underlined somatic embryogenesis induction with other auxins than 2,4-D remains limited and should be expanded as auxins play a key role in the embryogenic induction process (Hazubska-Przybył et al. 2020).

The proliferation of embryogenic lines is essential for the continuous supply of embryos and, consequently, for determining the efficiency and large-scale use of the proposed regeneration system. In our study, multiplication cycles and, consequently, the obtaining of embryogenic lines were observed when putrescine was added together with 18.0 μM of picloram. Polyamines have been linked to the regulation of several physiological processes, including the differentiation of somatic embryos (Mustafavi et al. 2018; Rakesh et al. 2021). Putrescine supplementation apparently induces the accumulation of proteins that play important roles in protecting cells against an in vitro stress environment, conferring protection on the DNA molecule against damage caused by reactive oxygen species (ROS) (Ha et al. 1998) and in controlling DNA methylation (Brooks et al. 2010), which can contribute to the formation and maturation of somatic

embryos (Reis et al. 2016). In *E. guineensis*, exogenous supplementation of putrescine in the culture medium contributed to the multiplication and maintenance of pro-embryogenic masses and to the regeneration of somatic embryos (Corrêa et al. 2015, 2016; Granja et al. 2018), which is consistent with the result obtained in the present study.

The formation of somatic embryos occurred through the proliferation of cells from the meristematic center located in the internal region of the embryogenic callus. This somatic embryo development pattern has also been described in *C. nucifera* (Fernando et al. 2003), *A. aculeata* (Moura et al. 2009d) *E. guineensis* (Balzon et al. 2013; Silva et al. 2013) and, apparently, is conserved among the species of the family. The starch grains, observed in the cells adjacent to regions with intense cell division, may be mobilized in the cortical region of the callus probably functioning as an additional carbon and energy source (Smith and Denyer 2003) for the growth and development of somatic embryos (Smith and Denyer 2003; Silva-Cardoso et al. 2020).

Somatic embryos were composed of small cells with dense cytoplasm, large nucleus and a single evident nucleolus. These cellular characteristics are considered markers of embryogenic competence (Verdeil et al. 2007; Rocha et al. 2016) and reveal the regenerative potential of the embryogenic structures observed here. Pectins were evenly distributed along the cell walls of somatic embryos with ruthenium red dye. Some authors have suggested a role of pectin polymers in promoting cell wall remodeling during the somatic embryogenesis induction (Rocha et al. 2016; Pérez-Pérez et al. 2019; Kurczynska and Godel-Jędrychowska 2023). According to Goh et al. (2001), pectin deposition is a consequence of modifications of the middle lamella and primary cell wall and may indicate cell quality for subsequent development.

The germination of somatic embryos occurred in culture medium supplemented with activated charcoal, NAA and putrescine, which resulted in the regeneration of dozens of plantlets. This stage was characterized by the exchange and reduction in the concentration of synthetic auxin in the culture medium, while maintaining the putrescine concentration used in the multiplication phase, in addition to supplementation with activated charcoal. Activated charcoal is often added to the medium in order to adsorb and minimize the toxicity of the growth regulator or of substances with deleterious effects, such as phenolic compounds (Sáenz et al. 2010; Guedes et al. 2011). The conditions used in the present study for the germination of somatic embryos of *A. aculeata* were based on the culture medium established to obtain complete plantlets of oil palm (Corrêa et al. 2015), also using NAA and putrescine at the same concentrations described in the present study. Polyamines have been demonstrated to play an important role in adventitious root formation promoting root elongation and growth by increasing

root cell division (Tang and Newton 2005). Putrescine is involved in root cell wall phosphorus remobilization in a nitric oxide-dependent manner which could be released to the cytoplasm contributing to cellular processes such as cell division (Jing et al. 2022).

In summary, was reported here a complete system of regeneration, via somatic embryogenesis, from the in vitro culture of leaflets explants of *A. aculeata*, and determined the influence of genotypes and their chronological age on the induction of embryogenic calluses, with juvenile donor-plant being more responsive. We believe that the results obtained here may provide basis for the genetic improvement of this species, contributing to the cloning and propagation of elite genotypes of *A. aculeata*, establishment of clonal commercial plantations, and consequent maximization of the oil production of this potential oil palm.

Author contribution statement APSA and SYM designed the research; APSA, VQ, DDCM and TRC established in vitro cultures; ACFC, EATP and DIR assisted with histological evaluations; DDCM and TRC performed statistical analyses; APSA, SYM, KNK, TRC and DIR wrote the paper.

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

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors consent to the publication of this manuscript.

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