Research Article
doi: 10.1590/01047760202632013638
vol(32), 2026

CERNE

Embryogenic callus induction from *in vitro* culture of mature zygotic embryos of *Euterpe edulis* Martius (Arecaceae)

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SILVICULTURE

ABSTRACT

Background: *Euterpe edulis* Martius, a symbolic species of the Atlantic Forest in Brazil, popularly known as the Juçara palm tree, does not produce axillary or basal shoots, which prevents its vegetative propagation through conventional techniques. Plant tissue culture techniques have been proposed for the clonal propagation of superior genotypes of this species. The present study aimed to evaluate the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on the induction of embryogenic calluses from mature zygotic embryos of *E. edulis*. Mature zygotic embryos were inoculated in culture media supplemented with different concentrations (20, 40, 80, 160, 320, 480, and 600 mg L^{-1}) of 2,4-D, while the control treatment contained no 2,4-D. The experiments were conducted in a completely randomized design.

Results: After 70 days in the induction medium, three morphogenic responses were observed: seedlings, abnormal seedlings, and calluses. Increasing concentrations of 2,4-D reduced the formation of normal seedlings while enhancing callogenesis up to concentrations between 160 and 279 mg L^{-1}. At higher concentrations, embryogenic calluses or oxidized explants predominated. Morpho-anatomical evaluations confirmed the embryogenic identity of the calluses, which consisted of a meristematic center surrounded by a parenchymatic zone. Neutral polysaccharides and starch grains were mainly observed in the parenchymatic zone, which was not directly involved in somatic embryo regeneration.

Conclusion: This study demonstrates the formation of embryogenic calluses from *in vitro* cultures of zygotic embryos of *Euterpe edulis*. These findings contribute to the development of a complete regeneration protocol and may support the clonal propagation and conservation of this important Atlantic Forest palm species

Keywords: Auxin, somatic embryogenesis, Juçara palm tree, plant regeneration, morphoanatomy.

HIGHLIGHTS

2,4-D triggers somatic embryogenesis in *Euterpe edulis* Martius.
High doses shift seedling growth toward embryogenic callus.
Optimal embryogenic response ranged between 160 and 279 mg L^{-1} 2,4-D.
Histological and histochemical analyses supported the embryogenic identity of the calluses.

SILVA, V. P. L.; FERREIRA, T. A. S.; FERNANDES, J. C. T.; CRUZ, A. C. F.; MOTOIKE, S. Y.; ROCHA, D. I.Embryogenic callus induction from *in vitro* culture of mature zygotic embryos of *Euterpe edulis* Martius (Arecaceae). CERNE, v. 32, e103638, 2026. DOI: 10.1590/01047760202632013638

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Scientific Editor: Gilvano Ebling Brondani

Received: September 23, 2025
Accepted: December 01, 2025

Open Access
CERNE
ISSN 0104-7760
UFLA UNIVERSIDADE FEDERAL DE LAVRAS

INTRODUCTION

Euterpe edulis Martius (Arecaceae), popularly known as Juçara palm tree, has aroused growing scientific and economic interest due to its commercial relevance (Oliveira et al., 2022). This interest is mainly driven by the wide acceptance and appreciation of its palm heart in national and international markets, and by the productive and nutritional potential of its fruits, which serve as functional food due to their high antioxidant content (Carvalho et al., 2022).

Additionally, the commercial value of the fruit has increased in recent years, making it a vital non-timber forest product in the Atlantic Forest (Vianna et al, 2023). However, the intense pressure on this palm has caused social and environmental problems in regions where the species occurs, as harvesting its palm heart results in the plant's death (Oliveira et al., 2022). Alternatively, cultivation of this species for fruit production has reduced exploitation pressure (Meier et al., 2022).

Commercial production of this species remains limited, partly due to the lack of alternatives for vegetative propagation of superior *E. edulis* genotypes. This palm does not produce axillary or basal shoots, and seeds are its only means of conventional propagation (Vianna, 2025). However, seminiferal propagation in this species, besides being inefficient due to low seed viability, also leads to high genetic variability, resulting in non-uniform production (Soler-Guilhen et al., 2020; Regnier and Salatino, 2020). Thus, clonal propagation methods, such as somatic embryogenesis, are necessary and represent a good alternative for many species that are difficult to propagate by conventional methods (Mikuła et al., 2022).

Somatic embryogenesis is defined as a process in which a bipolar structure, similar to a zygotic embryo, develops from somatic cells without vascular connection to the original tissue (Elhiti and Stasolla, 2022). For the regeneration process to be initiated, plants typically require an induction stimulus, most often triggered by stress conditions or the supplementation of plant growth regulators, particularly auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), which represents the most widely employed approach (Mikuła et al., 2022).

Somatic embryogenesis has been successfully studied and established as a pivotal biotechnological strategy for the efficient clonal propagation of palm species (Oliveira et al., 2022; Bueno et al., 2025). Different explants can be used for somatic embryo formation, including young leaves, zygotic embryos, and inflorescences (Guerra and Handro, 1998). However, *in vitro* culture of zygotic embryos is the main pathway for inducing somatic embryogenesis in *E. edulis* (Carvalho et al., 2022; Oliveira et al., 2022). Nevertheless, the formation of somatic embryos and subsequent plant regeneration is still not uniform, showing asynchrony in the development and maturation of tested materials (Ferreira et al., 2022), highlighting the need to improve this regeneration process.

To elucidate the mechanisms and sites of action of plant growth regulators, detailed morphoanatomical analyses are essential, as they provide insights into the cellular dynamics triggered in exposed tissues (Campos et al., 2020). Investigations in *Cocos nucifera* L. (Mu et al., 2024) and *Elaeis guineensis* Jacq. (Panggabean et al., 2023) have significantly advanced the understanding of cellular processes underlying somatic embryogenesis induction, offering valuable models for other species.

Given the limited knowledge of somatic embryogenesis in *Euterpe edulis*, the irregular development of somatic embryos, and the limited understanding of the cellular mechanisms driving embryogenic callus induction, further investigations on this species are crucial to optimize embryogenic systems capable of achieving complete and uniform plant regeneration. Accordingly, the present study aimed to evaluate the effects of different concentrations of 2,4-D on the induction of somatic embryogenesis from *E. edulis* zygotic embryos cultured *in vitro*, as well as to characterize the morphoanatomical features of the resulting embryogenic calluses.

MATERIALS AND METHODS

Plant material

Mature zygotic embryos of *E. edulis* were used as explants. The fruits were collected from two mother plants present in the collection of nutraceutical palms at the Federal University of Viçosa, located in the municipality of Araponga, Minas Gerais, Brazil. The fruits were washed with a solution of water and 10% neutral detergent, agitated for 10 minutes, and then depulped with a sieve. The seeds were soaked in a solution of water and 10% neutral detergent, then transferred to a 0.5% sodium hypochlorite (NaClO) solution and agitated for 10 minutes, followed by nine rinses with autoclaved distilled water, in a laminar flow hood. After rinsing, the zygotic embryos were extracted from the seeds with tweezers and a mini-vice number 1 and subsequently transferred to Petri dishes containing germitest paper moistened with sterile water. After this step, they were transferred again to a container with 0.1% NaClO solution, agitated for 10 minutes, and rinsed three times with autoclaved distilled water. Subsequently, the material was inoculated into test tubes containing induction medium.

Effect of 2,4-D on somatic embryogenesis induction

To evaluate the effect of 2,4-D supplementation on somatic embryogenesis induction, mature zygotic embryos of *E. edulis* were inoculated into test tubes (2.5 cm × 15 cm) containing 20 mL of culture medium composed of MS basal salts (Murashige and Skoog, 1962), B5 vitamins, 0.5 g L^{-1} casein, 0.5 g L^{-1} glutamine, 30 g L^{-1} sucrose, 1.5 g L^{-1} activated charcoal, 2.5 g L^{-1} phytagel, supplemented with 3.0 mg L^{-1} isopentenyladenine (2iP), and different 2,4-D concentrations (20, 40, 80, 160, 320, 480, and 600 mg L^{-1}). In the control treatment, no 2,4-D was added. The medium was adjusted to pH 5.7 and autoclaved for 20 minutes at 121 °C under 1.5 atm. The cultures were maintained in a culture room at 28 °C in the dark for 70 days. After this period, the percentage of responsive explants, germination, callogenesis, and oxidation

was evaluated. All treatments were maintained under these conditions before being collected for microscopic analyses.

Morphoanatomical analyses

The embryogenic calluses obtained were collected and fixed in FAA 50 (formaldehyde, acetic acid, and 50% ethyl alcohol). To characterize the micromorphology of the calluses by scanning electron microscopy (SEM), the fixed samples were dehydrated in an increasing alcohol series. The samples were analyzed and photographed using a Leo 1430VP Scanning Electron Microscope, and all collected images were digitally processed.

For light microscopy analysis, the fixed samples were dehydrated in an increasing alcohol series and embedded in Leica Historesin according to the manufacturer's recommendations. The materials were sectioned longitudinally at five micrometers (µm) using an automatic rotary microtome (RM2265–Leica) equipped with a disposable glass blade and subsequently stained with toluidine blue (0.5%) at pH 4.0 for 5 min. The slides were mounted with Permount® and visualized under a light microscope (Olympus-AXE 70) coupled to a photomicrography system (Olympus U-Photo).

For the detection of pectins, neutral polysaccharides, and starch, histochemical tests were carried out using Schiff reagent (PAS) (Feder and O'Brien, 1968) and Lugol solution (Johansen, 1940), respectively.

Statistical analysis

The experiment was conducted in a completely randomized design, with eight 2,4-D concentrations (0, 20, 40, 80, 160, 320, 480, and 600 mg L^{-1}). Each treatment consisted of five replicates, each containing four test tubes with one mature zygotic embryo per tube. The morphogenic responses observed in each treatment, such as percentage of normal and abnormal seedlings, callus, and non-responsive and/or oxidized explants, were subjected to descriptive analysis. The percentage of calluses and oxidation was tested for normality and homogeneity using the Shapiro-Wilk and Bartlett tests. The data were then subjected to analysis of variance (ANOVA) and evaluated by regression using Sisvar 5.6 software.

RESULTS

2,4-D concentrations dictate different morphogenic pathways

After 70 days in induction medium, three different morphogenic responses were observed: seedlings, abnormal seedlings, and calluses (Fig. 1a, b). Germination of zygotic embryos and consequently seedling formation were mainly observed in the control treatment and at the lowest concentration of 2,4-D (20 mg L^{-1}) (Fig. 1b). At concentrations of 40 and 80 mg L^{-1} 2,4-D, most of the seedlings formed were abnormal (Fig. 1b). These seedlings generally showed reduced root systems and callus formation in this region (Fig. 1a). The coleoptile, although visible, appeared smaller than in normal seedlings (Fig. 1a), although this parameter was not measured. Callogenesis was observed from the treatment supplemented with 20 mg L^{-1} 2,4-D, with the increase in concentration resulting in a higher percentage of calluses up to 160 mg L^{-1}. At concentrations higher than 160 mg L^{-1} 2,4-D, there was a sharp decrease in callogenesis (Fig. 1b). In the treatment supplemented with 160 mg L^{-1}, only calluses were observed, which constituted 66.7% of the morphogenic responses obtained (Fig. 1b). The highest percentages of non-responsive explants were observed in the control treatment and in the treatment supplemented with 600 mg L^{-1} 2,4-D, where most explants were oxidized (Fig. 1b, d).

The concentration of 2,4-D influenced somatic embryogenesis induction (Fig. 1f). The frequency of callogenesis was adjusted to a quadratic model, whose optimum point was approximately 279 mg L^{-1} 2,4-D (Fig. 1c). The oxidation rate was adjusted to a linear model, showing that the increase of 2,4-D resulted in a proportional increase in oxidation rate (Fig. 1d). The embryogenic calluses, characterized by nodular structures (Fig. 1e), differentiated into somatic embryos (Fig. 1f).

Histological zonation of embryogenic calluses

The embryogenic calluses of *E. edulis* consisted of numerous nodular structures with a globular shape (Fig. 2a). These structures were mainly formed in the haustorium region of the zygotic embryos used as explants (Fig. 2b). The nodules varied in size and had an irregular surface (Fig. 2c). Anatomically, these structures showed a well-defined histological zoning, consisting of a meristematic center surrounded by a parenchymatic zone (Fig. 2d). The meristematic center was composed of small, isodiametric cells, with a high nucleus-to-cytoplasm ratio, a prominent nucleus containing more than one nucleolus, and numerous regions of heterochromatin (Fig. 2e). In contrast, the parenchymatic zone was composed of elongated cells, with retracted protoplasts and a low nucleus-to-cytoplasm ratio (Fig. 2f), highlighting the differences in metabolic activity of these distinct regions of the nodules.

Histochemical analyses of embryogenic calluses

Neutral polysaccharides were identified in the embryogenic calluses of *E. edulis* using the histochemical periodic acid-Schiff (PAS) reaction (Fig. 3a, b). The cell walls of the parenchymatic zone of the embryogenic calluses were intensely stained compared with the cells of the meristematic center, indicating differences in the abundance of these components along the callus (Fig. 3a, b).

Cytoplasmic compounds of peripheral cells of the nodular structures were also stained by PAS (Fig. 3b), a characteristic not observed in the cells of the meristematic center. This observation was confirmed by the histochemical Lugol test (Fig. 3c, d). Starch grains were observed only in the peripheral cells surrounding the meristematic centers of pro-embryogenic structures (Fig. 3d).

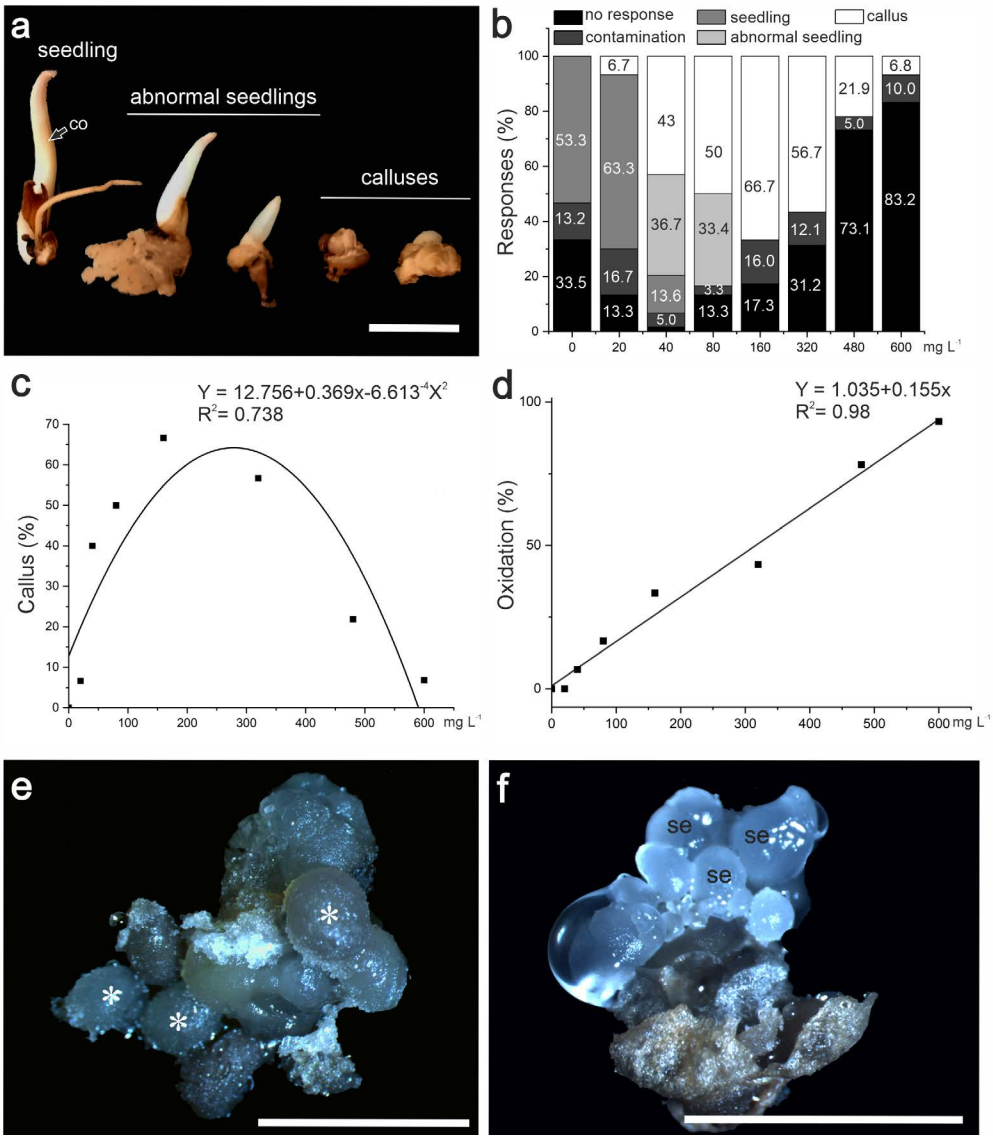

Figure 1: Effects of 2,4-D supplementation on somatic embryogenesis induction in *Euterpe edulis*. a: Morphogenic responses of explants exposed to different concentrations of 2,4-D. b: Percentage of morphogenic responses observed in each treatment. c: Percentage of embryogenic callus d: Percentage of oxidized explants e, f: Embryogenic callus. Note the presence of nodular structures (*) and somatic embryos (SE). Scale bars = a: 20 mm; e, f: 0,5 mm. Abbreviation: co coleoptile.

DISCUSSION

In this study, we evaluated the effect of 2,4-D supplementation on the induction of somatic embryogenesis from *in vitro* culture of *E. edulis* zygotic embryos. The use of 2,4-D was based on the results of Saldanha et al. (2006), Saldanha and Martins-Corder (2012), and Oliveira et al. (2022), who successfully reported the formation of embryogenic calluses in *E. edulis*. The presence of 2,4-D in the culture medium was essential for inducing the embryogenic process. 2,4-D is the primary plant growth regulator used to induce this process due to its rapid response and efficiency (Karami et al., 2023). This auxinic molecule acts as a chemical signal, reprogramming the cellular development of a tissue in a specific region (Yan and Pan, 2023). The effect of 2,4-D on the formation of embryogenic calluses is closely related to the acquisition of embryogenic competence (totipotency), attributed to its ability to stimulate cell division, regulate the expression of genes related to embryonic development, and induce the cellular reprogramming necessary for the formation of somatic embryos (Passamani et al., 2020; Fan et al., 2022).

However, the precise concentration of auxin in the culture medium is crucial for somatic embryogenesis induction (Oliveira et al., 2023; Jayarathna et al., 2023). Here, the highest observed callogenesis occurred at 160

mg L^{-1} 2,4-D, whereas the model estimated an optimum near 279 mg L^{-1}, both higher than those used in previous studies (Saldanha et al. 2012; Oliveira et al. 2022). Saldanha et al. (2006) successfully induced somatic embryogenesis in immature zygotic embryos of *E. edulis* using 100 mg L^{-1} of 2,4-D. More recently, Oliveira et al. (2022) successfully induced somatic embryogenesis in immature zygotic embryos of the same species by supplementing with 4.49 mg L^{-1} of 2,4-D, during which embryogenic calluses were first formed, characterizing indirect somatic embryogenesis. This low auxin dosage was likely effective because immature zygotic embryos are more responsive to somatic embryogenesis induction (Yang et al., 2021; Trabelsi and Jedidi, 2022), due to their lower degree of differentiation. Another possible factor explaining the higher concentration of 2,4-D found in this study could be the amount of activated charcoal present in the medium. Activated charcoal can adsorb several substances present in the culture medium, including plant growth regulators (Sharma et al., 2022). We must also take into account the genotypic characteristics of each plant material, since the explants used in the present study were derived from only two mother plants. The success of establishing *in vitro* plant culture is influenced not only by culture medium composition and growth conditions but also by genotype, since genotype-dependent responses are common in *in vitro* regeneration processes (Corrêa et al. 2015; Nadarajan et al. 2023). Further studies using materials sourced from different populations should be conducted to determine the most precise concentrations of 2,4-D required to induce somatic embryogenesis from zygotic embryos of *E. edulis*.

The formation of somatic embryos from calluses is known as indirect somatic embryogenesis (Ramírez-Mosqueda, 2022). This regeneration pathway is common in palm species and is the main route described for *Cocos nucifera* L. and *Elaeis guineensis* Jacq. (Bueno et al., 2025), and *Euterpe precatoria* Mart. (Ferreira et al., 2022). In general, the use of zygotic embryos as explants for somatic embryogenesis induction tends to proceed indirectly, with the formation of embryogenic calluses before somatic embryos.

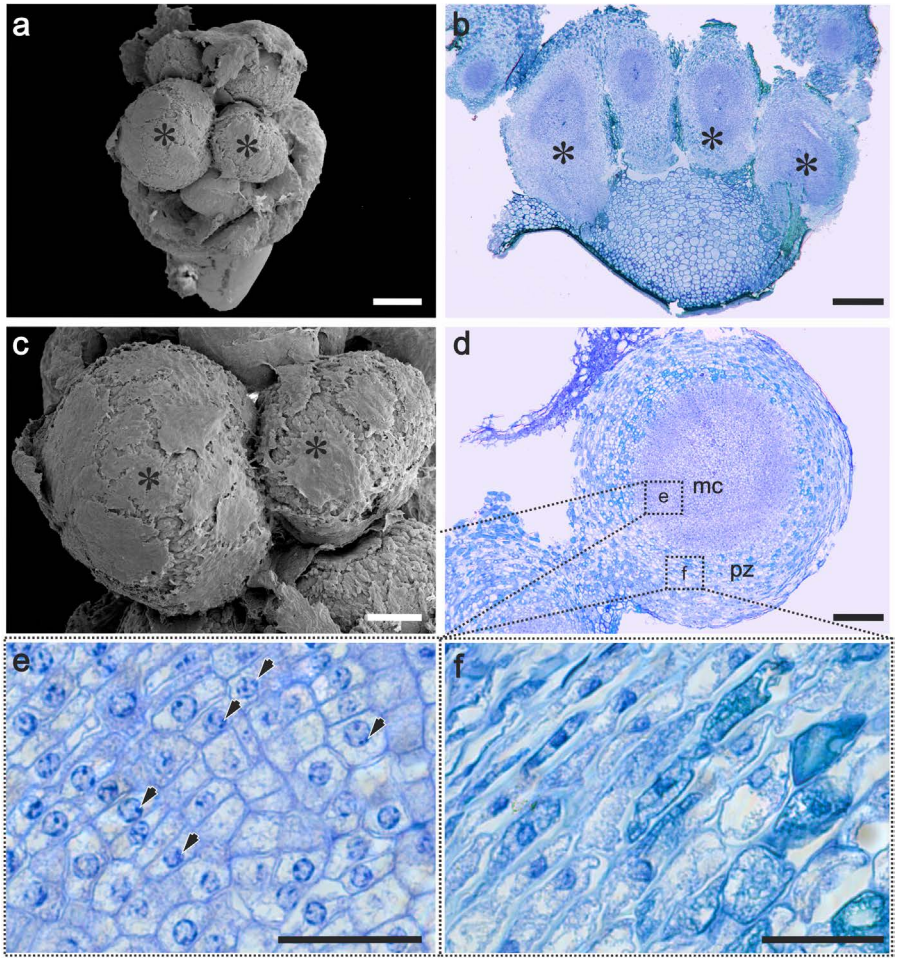

Figure 2: Anatomical aspects of embryogenic calluses of *Euterpe edulis*. a: Calluses with nodular structures (*) forming on their surface. b: Cross-section of embryogenic callus with nodular structures (*) stained with Toluidine Blue. c: Calluses with nodular structures (*) forming on their surface (in detail). d: Nodular structure of the embryogenic callus, showing the meristematic center (mc) and the parenchymatic zone (pz). e: Heterochromatin in the nuclei of the meristematic center (mc) cells (arrows). f: Cells of the parenchymatic zone (pz). Scale bars = a, b 500 µm; c, d 250 µm; e, f 50 µm.

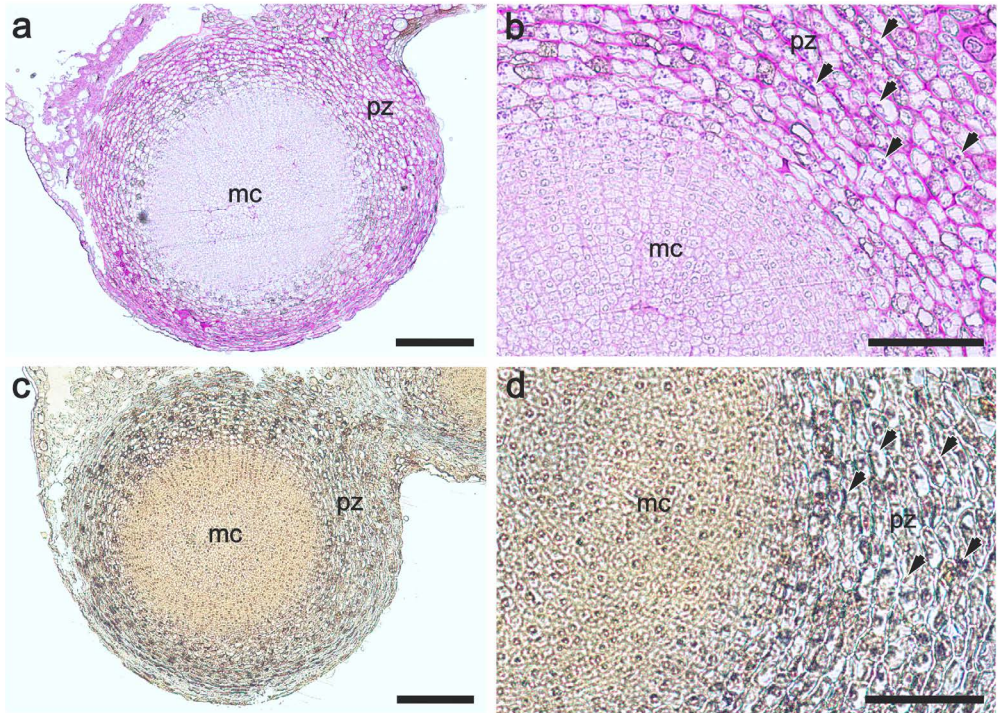

Figure 3: Histochemistry of *E. edulis* embryogenic callus. a: Presence of neutral polysaccharides, evidenced by the dark pink due to the positive reaction to the PAS test with Schiff's reagent. b: Presence of a greater amount of neutral polysaccharides in the parenchymatic zone (pz) cells, suggesting the presence of a polysaccharide matrix (black arrows). c: Presence of starch in pz cells evidenced by a purplish coloration due to the Lugol test. d: More detailed image showing the presence of starch grains in the amyloplasts of pz cells (black arrows). Scale bars = a, c: 400 µm; b, d: 100 µm. Abbreviation: mc meristematic center.

In this study, nodular structures began to form around the zygotic embryos. The formation of these structures was also reported in somatic embryogenesis induction in *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. (Andrade et al., 2024), *Euterpe oleracea* (Pereira et al., 2012), *Elaeis guineensis* Jacq. (Panggabean et al., 2022), and *Euterpe precatoria* (Ferreira et al., 2022), where these structures were classified as nodular embryogenic calluses. These calluses showed a histological conformation with a meristematic center surrounded by parenchymatic cells. The same cell arrangement pattern was observed in *Syagrus oleracea* Mart. (Silva-Cardoso et al., 2020) and *Elaeis guineensis* (Panggabean et al., 2022), demonstrating that this is not an exclusive feature of embryogenic calluses of *E. edulis*, but rather a conserved structural aspect during *in vitro* regeneration of these structures in plant palm species. Morphoanatomical analyses are indispensable for accurately discerning the identity of morphogenic responses in palms. As highlighted by Silva-Cardoso et al. (2020), the early formation of globular somatic embryos may have been overlooked or misinterpreted as callus tissue, mainly due to the extended exposure of explants to induction media containing supraoptimal auxin concentrations, which can impair rather than promote somatic embryogenesis.

Histochemical tests identified neutral polysaccharides using Schiff's reagent in PAS tests and starch grains using Lugol's reagent. The presence of neutral polysaccharides is a common feature of embryogenic events. These compounds were also observed in embryogenic structures of *Syagrus oleracea* Mart. (Silva-Cardoso et al., 2020) and *Acrocomia aculeata* (Andrade et al., 2024). In this study, a polysaccharide matrix was evidenced between parenchymatic cells, along with thickening of their cell walls. The origin of these compounds is likely linked to alterations in the cell wall, which may have disrupted the middle lamella (Ferreira et al., 2020; Ferreira et al., 2022).

The presence of starch grains is associated with their synthesis and storage by amyloplasts, specialized cells that perform this function. Starch grains provide energy for the initiation of cell divisions and the successive formation of embryogenic cells and pro-embryos, decreasing as embryogenic areas differentiate (Silva-Cardoso et al., 2020). Therefore, starch is fundamental for morphogenic processes. In this study, these reserve structures were found in the parenchymatic zone of embryogenic calluses, acting as an energy source that supports somatic embryogenesis. Studies in *Syagrus oleracea* Mart. (Silva-Cardoso et al., 2020) and *Elaeis guineensis* Jacq. (Vilela et al., 2021) showed similar patterns, where cells surrounding the meristematic center also contained starch grains as an energy source for embryogenic development.

Apparently, this starch tends to be remobilized toward the meristematic center, the region of intense cell division activity, closely linked to the acquisition of embryogenic competence (Vilela et al., 2021).

In summary, this study highlights the crucial role of 2,4-D in inducing somatic embryogenesis in *E. edulis*, showing that concentrations between 160 and 279 mg L^{-1} are most effective. The formation of nodular embryogenic calluses, associated with the presence of polysaccharides and starch grains, reflects the complexity of the cellular processes involved, including the acquisition of embryogenic competence and the provision of energy for cell divisions. The use of zygotic embryos as explants has shown promise, though further studies on optimal concentrations of 2,4-D and the molecular mechanisms underlying cell reprogramming are needed to further optimize results.

CONCLUSION

The results indicate that 2,4-D is an effective inducer of embryogenic callus in *Euterpe edulis*, with concentrations between 160 - 279 mg L^{-1}, favoring the formation of these structures. Histological and histochemical analyses supported the embryogenic identity of the calluses. These findings represent an initial advance in understanding the induction phase of somatic embryogenesis in this species. Further research is necessary to confirm embryo maturation, conversion, and plantlet development, which will be essential steps toward establishing efficient clonal propagation and conservation protocols for *E. edulis*.

ACKNOWLEDGEMENTS

The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, the Conselho Nacional de Desenvolvimento Científico e Tecnológico (Brasília, Brazil; Grant No. 308217/2025-0), and the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Belo Horizonte, Brazil; Grant No. RED-00225-23) for financial support.

AUTHORSHIP CONTRIBUTION

Project Idea: VPLS; SYM; DIR

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Database: VPLS; TASF; JCTF

Processing: VPLS; TASF; ACFC; DIR

Analysis: VPLS; TASF; ACFC; DIR

Writing: VPLS; TASF; ACFC; DIR

Review: TASF; ACFC; DIR

DATA AVAILABILITY

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

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