# Cell reprogramming via direct somatic embryogenesis in an Atlantic Forest species vulnerable to extinction: polarity of Euterpe edulis stem segments induced with pyridinecarboxylic acid

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**Research Article**

**Keywords:** juçara, plant tissue culture, embryogenic inducers, auxin, cytokinin

**Posted Date:** January 20th, 2023

**DOI:** https://doi.org/10.21203/rs.3.rs-2484021/v1

**Version of Record:** A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on May 16th, 2023. See the published version at https://doi.org/10.1007/s11240-023-02521-7.

# Abstract

*Euterpe edulis* Martius, commonly known as juçara, has high economic value because its palm heart is considered a delicacy and its fruit, which is rich in antioxidants, is considered a “super fruit.” Because this endangered species can only be propagated via the seminiferous route, we aimed to analyze somatic embryogenesis of stem explants from *E. edulis* seedlings in response to their polarity and the type and concentration of growth regulators. Immature seeds were collected from a selected matrix in Pedra Menina (ES/MG, Brazil) and germinated *in vitro*. Six-month-old seedlings were segmented into four explants based on their polarity, and placed in culture medium supplemented with 50, 100, 150, 200, 250, 300, 450 or 600 μM picloram (PIC). After induction, the explants were transferred to maturation medium supplemented with 1-naphthaleneacetic acid (0.53 μM) and 2-isopentenyladenine (12.3 μM) and two maturation times (30 and 60 days) were evaluated. After 60 days of induction, proembryos appeared asynchronously directly from the stem segments. Upon transfer to maturation medium, a large number of somatic embryos and masses were observed at both times. The polarity of the explants did not influence their embryogenic induction, and all four stem segments could be used for somatic embryogenesis following treatment with 150 μM PIC. A large number of somatic embryos were generated during later stages of maturation. It is recommended to remove the explants from the maturation medium after 30 days to avoid oxidation.


# 1 Introduction

The palm tree *Euterpe edulis* Martius, better known as juçara, is an economically highly important species because its palm heart is considered a delicacy, and is the second-most exported non-timber product from the Atlantic Forest (Barroso et al. 2019). Owing to its intense exploitation since the late 1960s, this species is now listed as endangered (Schulz et al. 2016). Additionally, *E. edulis* fruits have attracted attention for their similarity to açaí berries of *Euterpe oleracea* Martius, whose nutritional properties they surpass owing to an abundance of compounds such as anthocyanins, and are thus considered a “super fruit” and a functional food (Schulz et al. 2015).

The valorization of *E. edulis* fruits instead of palm hearts could offer a sustainable way to mitigate the exploitation of palm hearts, as harvesting the heart of palm kills the plant, and harvesting the fruit increases economic income and rational use, thereby contributing to environmental awareness and thus its preservation (Biazotto et al. 2019; Schulz et al. 2021). Similar to açaí, *E. edulis* only grows via natural propagation through its seeds, as the species does not emit lateral shoots (Schulz et al. 2016). Therefore, it is necessary to develop propagation protocols that can provide high-quality, uniform, and large numbers of seedlings.

Somatic embryogenesis is the production of thousands of identical plants from a single explant (Ree et al. 2020), which can be any part of the plant. However, this technique remains challenging for many species, particularly *E. edulis*. The only somatic embryogenesis protocols for this species were developed first by Guerra and Handro (1988, 1998) and then by Saldanha et al. (2006, 2012), who used zygotic

embryos, young inflorescences, young leaves, and shoots of 150-day-old plants as explants, and 2,4-dichlorophenoxyacetic acid (2,4-D) as the main inducer. However, no protocol was satisfactory and it remains unclear as to which is the most responsive explant or embryogenic inducer and at what concentration.

Owing to a local hormonal gradient, the concentration of the inducer normally depends on the type of explant. Polarity is mainly dictated by auxin and cytokinin gradients; with auxin diminishing from apical tissues towards the base of the plant and posteriorly to the root tip (Sun et al. 2013), and cytokinin decreasing in the opposite direction.

Explants of stem segments from *E. edulis* seedlings are expected to be more responsive at the base, close to the collar. Therefore, the objective of this study was to analyze somatic embryogenesis of stem segments from *E. edulis* seedlings in relation to their polarity and to the type and concentration of growth regulators.

# 2 Material And Methods

# 2.1 Collection, disinfection, and establishment of *in vitro* cultures

*E. edulis* fruits were harvested from a 6.7 m tall plant with a diameter at breast height (1.30 m) of 13.4 cm, located in the district of Pedra Menina in the municipality of Dores do Rio Preto, Espírito Santo, Brazil. The plant was found growing near the Caparaó National Park, at 20°32'45.4" S, 41°49'35.7" W, and an altitude of 951 m. The matrix was selected based on the large diameter of its fruits (14.52 and 14.46 mm), percentage of emergence (96%), shoot dry mass (3.35 g), root dry mass (1.63 g), titratable acidity (1.37% citric acid), and anthocyanins (0.459 mg cyanidin 3-glycoside per 100 g fresh mass). The fruits were harvested ~ 164 days after anthesis.

After cleaning the fruits with water and neutral detergent, the tegument was removed and immersed in 2% ascorbic acid solution (Dinâmica). Subsequently, *E. edulis* seeds were immersed in 70% ethyl alcohol for 1 min, 2.5% sodium hypochlorite (Candura) for 10 min, and 3 g $L^{-1}$ amoxicillin (Germed) for 10 min in a laminar flow chamber, followed by three washes in distilled and autoclaved water after each disinfecting agent.

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

# 2.2 Induction

Six-month-old seedlings were obtained from immature seeds germinated *in vitro* (MELLO et al. 2021). The seedlings were segmented into four explants and classified according to their polarity (1–4), discarding the apex and the base. The segments were arranged horizontally on 90 × 15 mm polyethylene plates (Global Trade Technology, Brasil) with 20 mL culture medium (Fig. 1g–i).

The culture medium consisted of MS salts supplemented with 0.1 g $L^{-1}$ myo-inositol, 1 g $L^{-1}$ PVP, and 30 g $L^{-1}$ sucrose. The pH was adjusted to 5.7 ± 0.1 prior to the addition of agar (5.5 g $L^{-1}$). After autoclaving, the medium was supplemented with filtered 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram; PIC) (Sigma), at concentrations of 50, 100, 150, 200, 250, 300, 450, or 600 µM.

The plants were kept in a growth room at 27 ± 2°C in the dark for 60 days, and oxidation (%), explants displaying an embryogenic center during longitudinal growth (%), number of proembryos, and explant mass (g) were analyzed.

## 2.2.1 Histological and structural analyses

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

## 2.2.2 Experimental design

The design was completely randomized and performed in a 4 × 8 factorial scheme (polarities × inducer concentrations), with four replications of four explants. Data were subjected to analysis of variance and Tukey's test ($P < 0.05$) using R software (R Core Team 2020).

# 2.3 Maturation

Induced explants were transferred to MS culture medium, supplemented with 0.1 g $L^{-1}$ myo-inositol, 1 g $L^{-1}$ PVP, and 30 g $L^{-1}$ sucrose. The pH of the medium was adjusted to 5.7 ± 0.1 prior to the addition of agar (5.5 g $L^{-1}$). The medium was supplemented with 0.53 µM 1-naphthaleneacetic acid (NAA; Sigma) and 12.3 µM 2-isopentenyladenine (2-iP; Sigma) (Guerra and Handro 1998).

The plants were kept in a growth room at 27 ± 2°C in the dark for 60 days. At 30 and 60 days, the number of embryos and explant mass (g) were analyzed.

The design was completely randomized according to an 8 × 2 factorial scheme (PIC concentrations × evaluation time), with four replications of four explants. Data were subjected to analysis of variance and Tukey's test ($p < 0.05$) using R software (R Core Team 2020) when there was no model adjustment.

# 3 Results

## 3.1 Induction

Seven days after transfer to the induction medium, seedling segments (Fig. 2a, b) started to grow longitudinally (59.37%) with an average oxidation of 40% (Fig. 2c, d). After 60 days, embryogenic structures (proembryos) appeared directly from the non-oxidized stem segments (Fig. 2e, f). Somatic embryos emerged asynchronously and could be observed at different stages; they included globular embryos emerging from the explant, scutellar embryos attached to the explant by the suspensor or detached from it, as well as the coleoptilar with protoderm and differentiated ground meristem (Fig. 2g–j).

No significant interaction was observed between polarity and PIC concentration. Similarly, no significant difference was detected between polarities. Instead, the percentage of explants with an embryogenic center during longitudinal growth, number of proembryos, and explant mass responded significantly to PIC concentration (Fig. 3).

PIC concentrations of 150, 200, and 450 μM resulted in more embryogenic centers in the stems of *E. edulis* seedlings (Fig. 3a); whereas 150 μM PIC stimulated the most proembryos (Fig. 3b).

## 3.2 Maturation

After 60 days in induction medium, the explants containing globular embryos of different sizes (Fig. 4) were transferred to maturation medium. Thirty days later, somatic embryos grew significantly in weight and number (Fig. 4), albeit in an asynchronous manner.

Stem segments of *E. edulis* seedlings treated with different concentrations of PIC were transferred to maturation medium supplemented with 0.53 μM NAA and 12.3 μM 2-iP, and were evaluated after 30 and 60 days. At both times, the highest number of somatic embryos (≅ 19) was detected in stem segments induced with 150 μM PIC (Fig. 5a).

Although PIC concentration and maturation time had a significant effect on the number of somatic embryos (Fig. 5a), the mass of explants showed a significant dependence on maturation time only when analyzed individually (Fig. 5b, c). Accordingly, the mass was greater after 60 days of maturation, but the embryos showed oxidative characteristics.

# 4 DISCUSSION

In our study, somatic embryogenesis was only observed in competent cells from stem segments of *E. edulis* (Arecaceae) seedlings induced by the auxinic growth regulator PIC (Fig. 2). Morphogenesis occurred via direct embryogenic induction and was particularly evident at 150 μM PIC (Fig. 3).

The type and concentration of plant growth regulators, such as the auxins 2,4-D, 4-chlorophenoxy acetic acid (4-CPA), dicamba, and PIC, influences the transition of somatic cells to totipotent embryogenic cells (Fig. 2e; Phillips; Garda 2019). The acquisition of embryogenic competence depends largely on dedifferentiation, a process by which existing transcription and translation profiles are erased, silenced or altered to allow cells to establish new developmental programs (Fehér et al. 2003). Each species responds differently to auxinic plant growth regulators. In the present study, all *E. edulis* explants became oxidized and died after 60 days when exposed to 2,4-D and 4-CPA (25–600 µM) or dicamba (100–600 µM) (data not shown). Only PIC (50–600 µM) elicited a response at concentrations up to 450 µM.

Somatic embryogenesis in *E. edulis* was first attempted in 1988 with the induction of zygotic embryos from immature fruits upon treatment with 2,4-D (> 50 mg $L^{-1}$), which caused embryos to arise directly from the tissue surface (Guerra and Handro 1988). Ten years later, the same authors used 50–100 mg $L^{-1}$ 2,4-D to induce somatic embryos directly from the surface of the cotyledonary node or from subepidermal tissues of *E. edulis* (Guerra and Handro 1998). However, the same procedure also led to the formation of proembryogenic tissue in the region of the floral primordium and cell proliferation in the vascular parenchyma of leaves. Eight years later, somatic embryos were induced indirectly from zygotic embryos in a culture medium supplemented with 40 mg $L^{-1}$ 2,4-D (Saldanha et al. 2006). The present study follows the work of Oliveira et al. (2022), who induced indirect somatic embryogenesis in zygotic embryos of immature *E. edulis* fruits by adding PIC (300 and 125–175 µM), and confirms the occurrence of direct somatic embryogenesis in *E. edulis* seedlings.

In *E. oleracea*, immature zygotic embryos were induced first with 225 µM PIC (Scherwinski-Pereira et al. 2012) and then with 450 µM PIC (Freitas et al. 2016), which yielded 44.9% and 84.7% embryogenic calli, respectively.

The maturation medium used in the present study was MS medium supplemented with 0.53 µM NAA and 12.3 µM 2-iP (Guerra and Handro 1998; Scherwinski-Pereira et al. 2012; Freitas et al. 2016), which is 4.9 times and 2 times lower, respectively, than the values (2.6 µM NAA and 24.6 µM 2-iP) used previously by Guerra and Handro (1988). Therefore, the auxin:cytokinin ratio in the present study was 2.44 times lower than the one utilized by Guerra and Handro (1988), but sufficient for the maturation of ~ 19 somatic embryos from each seedling stem segment previously induced with 150 µM PIC. With a total of four stem segments per seedling, it was possible to produce an average of 76 embryos.

Young leaves of *Coffea canephora* seedlings preconditioned *in vitro* with 0.54 µM NAA and 2.32 µM kinetin for two weeks showed a significant increase in free indole-3-acetic acid, its conjugate, and indole-3-butyric acid. This form of preconditioning enabled subsequent induction of somatic embryos in liquid medium supplemented with 5 µM 6-benzylaminopurine, leading to the appearance of the first globular embryogenic structures after 21 days (Ayil-Gutiérrez et al. 2013). Calabuig-Serna et al. (2021) reported a lower rate of microspore-derived embryos of *Solanum melongena* L. following continuous use of 2.68 µM NAA plus 2.21 µM 6-benzylaminopurine; however, the spores exhibited a dramatically higher endogenous auxin:cytokinin ratio. According to these authors, such high ratio exerted a negative effect on

embryogenic progression, with globular embryos transforming into undifferentiated calli instead of transitioning to the cordiform phase. A 20% reduction in NAA and 6-benzylaminopurine decreased the number of embryos, but produced structures that were anatomically similar to embryos.

# 5 Conclusions

The present study demonstrates that embryogenic induction is independent of explant polarity in *E. edulis*. Accordingly, all four stem segments derived from each seedling could be used for somatic embryogenesis, producing an average of 76 somatic embryos per zygotic embryo.

Somatic embryogenesis occurred directly from the stems of *E. edulis* seedlings upon induction with 150 µM PIC, an auxinic growth regulator. Subsequent supplementation with 0.53 µM NAA and 12.3 µM 2-iP during maturation led to an average of 19 somatic embryos per explant.

Despite the greater mass of explants obtained at 60 days of maturation, the embryos showed traces of oxidation. Therefore, it is recommended that explants be removed from maturation medium after 30 days.

# Declarations


### Funding

This study was funded by the Fundação de Amparo à Pesquisa e Inovação do Espírito Santo (FAPES) (EDITAL FAPES/CNPq N° 05/2017 - PRONEM - PROGRAMA DE APOIO A NÚCLEOS EMERGENTES. The agreement was registered with SICONV under n° 794009/2013, Process FAPES n° 72660945), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Finance code 308365/2019-4).


### CRediT authorship contribution statement

**Tamyris de Mello**: investigation, methodology, writing – review, and editing. **Ludmila Nayara Freitas Correia** and **Clovis Eduardo Nunes Hegedus**: writing, review, and editing. **Edilson Romais Schmildt** and **Adésio Ferreira**: formal analysis. **Wagner Campos Otoni**: supervision, writing – original draft, writing – review, and editing. **Rodrigo Sobreira Alexandre**: conceptualization, supervision, project administration, funding acquisition, writing – original draft, writing – review, and editing.

### Declaration of Competing Interest

The authors declare no conflict of interest.


### Acknowledgments

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa e Inovação do Espírito Santo (FAPES) for funding and Editage

(www.editage.com) for English language editing.

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# Figures

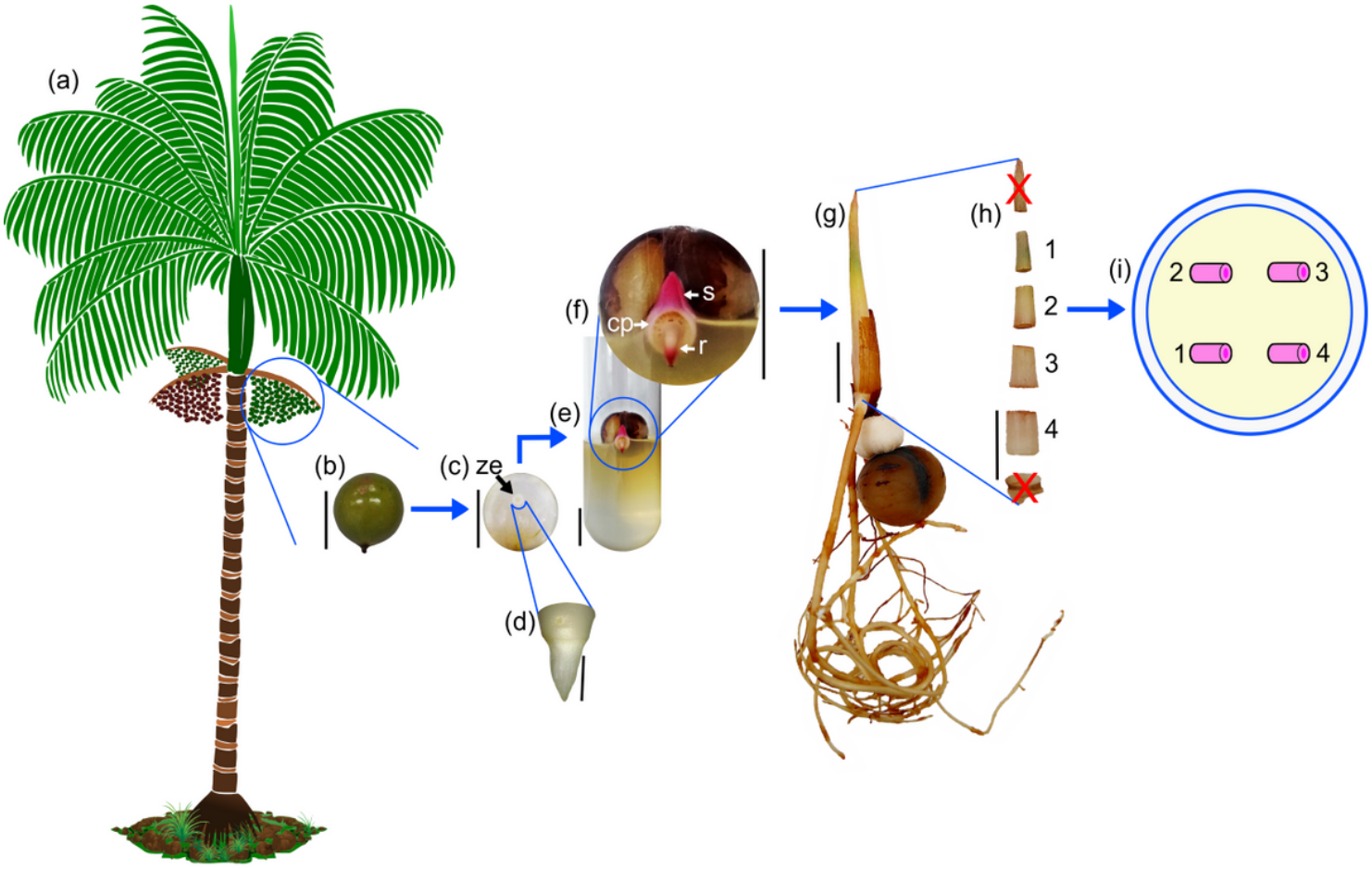
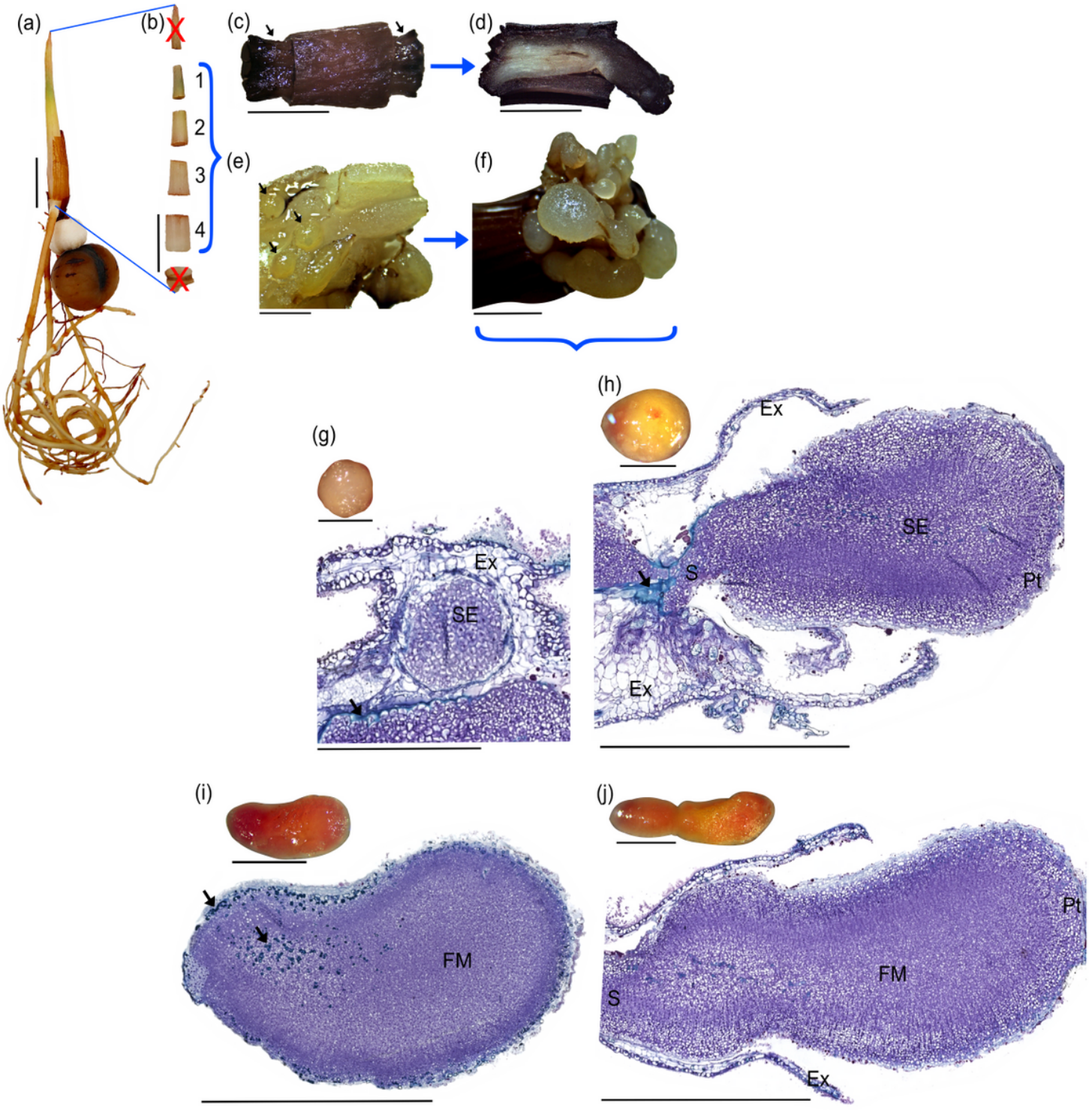



**Figure 1**

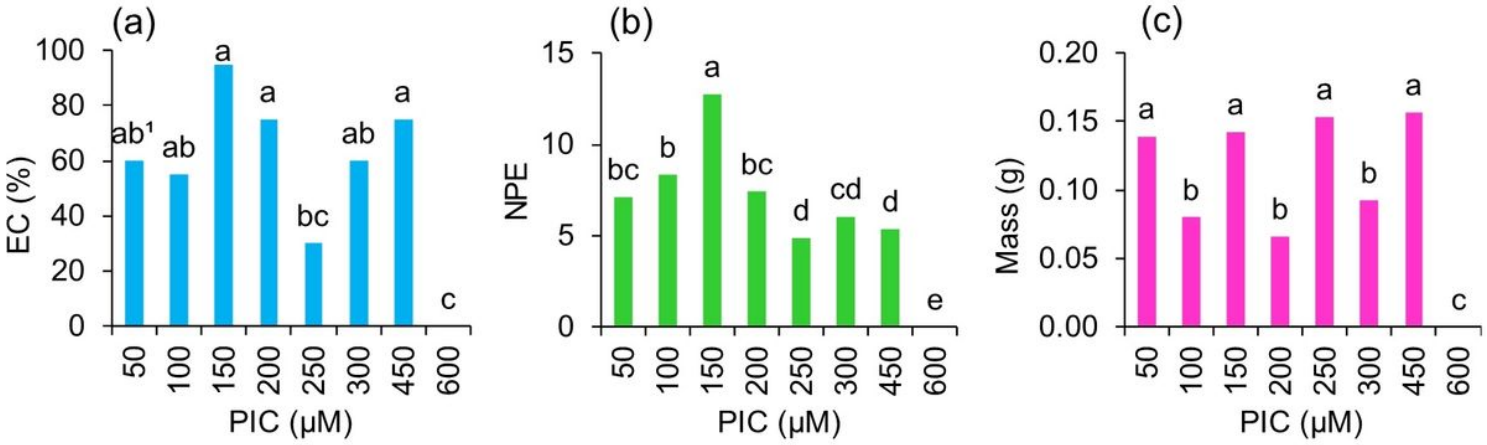
Schematic summary of embryogenic induction in *Euterpe edulis* seedling sheath segments. (a, b) Illustration of the palm tree, highlighting a bunch of immature fruits (a) and an immature fruit 180 days after anthesis (b). *In vitro* germination showing the seed without tegument (c), zygotic embryo (ze) detached from the seed (d), protrusion of the primary root (e), and germinated seed (cp, cotyledonary petiole; s, sprouting; r, primary root) (f). Six-month-old seedlings (g) and excision of the stem segments according to their polarity (h), followed by transfer of the explants to a Petri dish containing culture medium (i). Bar: 1.0 cm (b, c, e, f, g, h), 2.0 mm (d).




## Figure 2

Embryogenic induction of *E. edulis* after 60 days. Origin of explants (a) and their segmentation (b). Oxidized explants after longitudinal growth (black arrows) (c) and internal region (d). Direct emergence of somatic proembryos (black arrows) in the longitudinal growth region of the explant (e). Asynchronous emergence of somatic embryos (f), globular embryos (g), scutellar embryos attached to the explant (h), scutellar embryos not connected to the explant (i), and coleoptilar (j). Ex, explant; SE, somatic embryo; S,

suspensor; Pt, protoderm; FM, fundamental meristem; black arrows (phenolic compound). Bar: 1 cm (a, b), 5 mm (c, d), 2 mm (e, f, g, h, i, j).




## Figure 3

Induction of somatic embryogenesis in juvenile *E. edulis* explants after PIC treatment. (a) Percentage of explants with an embryogenic center (EC) during longitudinal growth. (b) Number of proembryos (NPE). (c) Mass of explants. [1]Means not followed by the same lowercase letter between concentrations differ from each other by Tukey's test ($P < 0.05$).

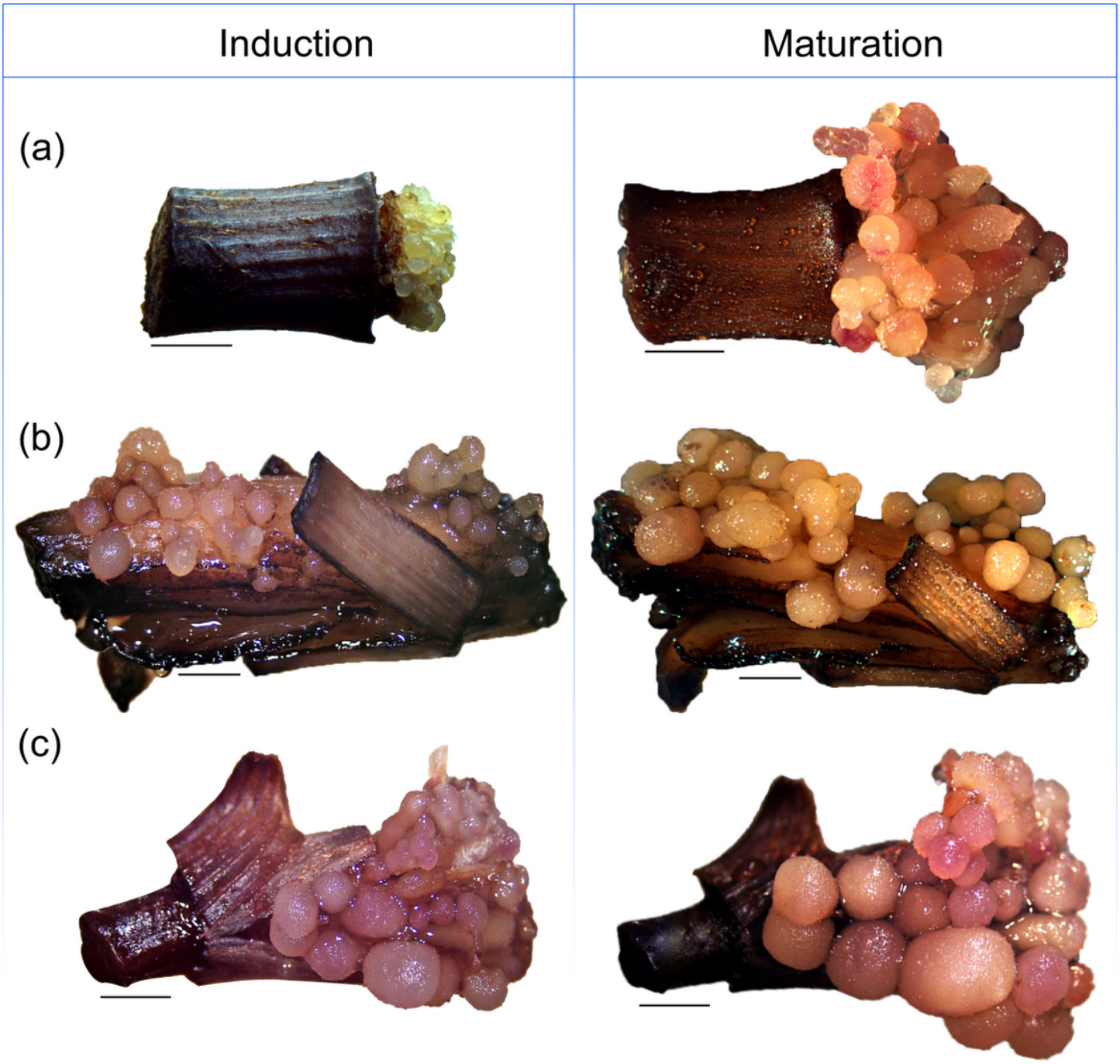



**Figure 4**

Maturation of somatic embryos derived from stem segments of *E. edulis* seedlings. Explants were previously induced with 100 µM PIC (a), 150 µM PIC (b), and 450 µM PIC (c). Explants are shown post-induction (left column) and after 30 days in maturation medium (ANA (0.53 µM) + 2iP (12.3 µM)) (right column), with explants from induction medium with PIC 100 µM (a), 150 µM (b) and 450 µM (c). Bar: 2.0 mm.

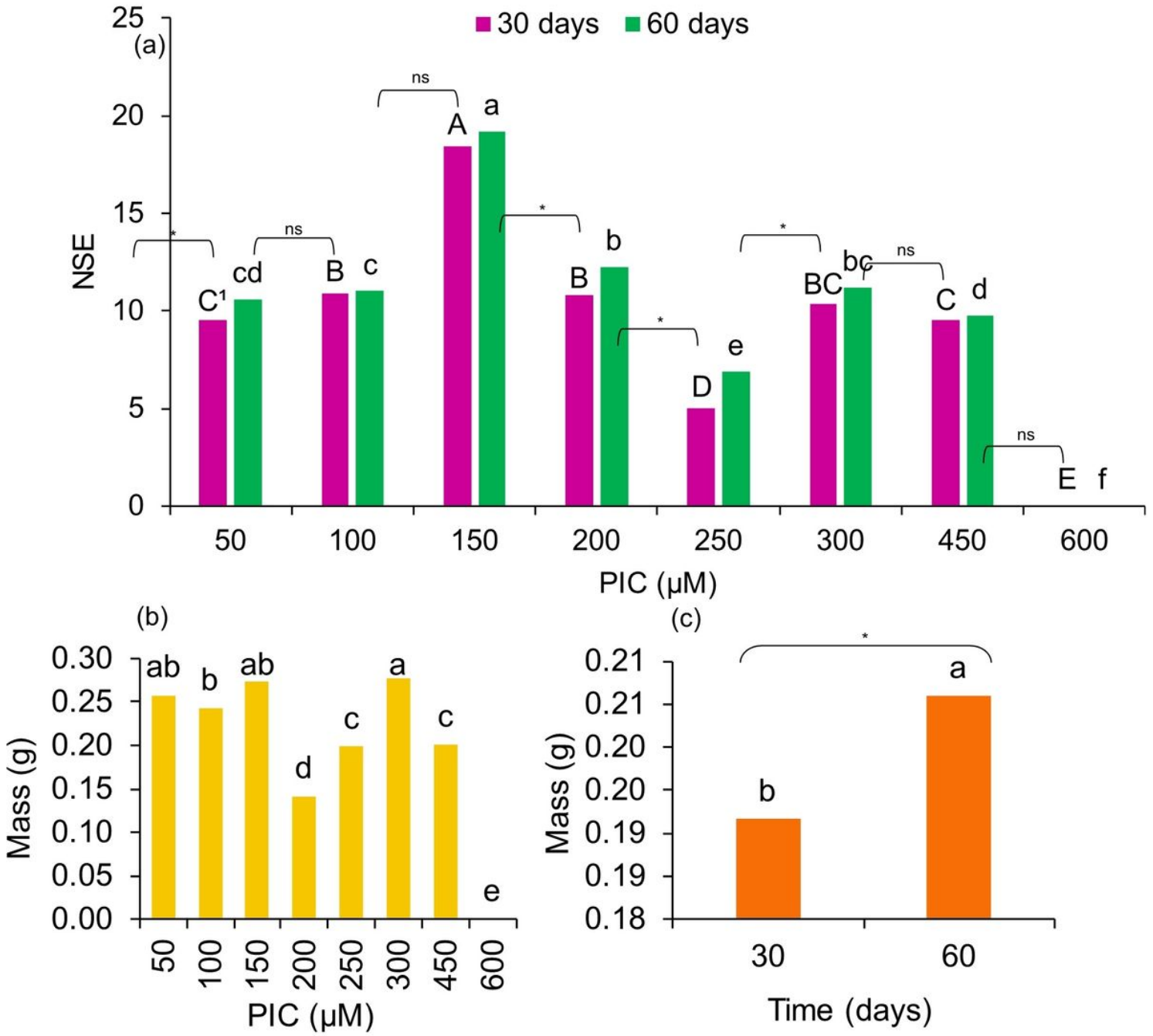



**Figure 5**

Maturation, after 30 and 60 days, of *E. edulis* somatic embryos previously induced with different concentrations of PIC. (a) Number of somatic embryos (NSE). (b) Mass of explants in relation to the PIC concentration. (c) Mass of explants in relation to maturation time. [1]Means not followed by the same capital letter (30 days) and lowercase letter (60 days) between concentrations and time do not differ significantly from each other based on Tukey's test ($P < 0.05$). *$P < 0.05$; ns, not significant by the F-test between maturation times.