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In vitro morphogenesis in zygotic embryos and leaf sheaths of *Euterpe edulis* Martius (Arecaceae)

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ABSTRACT - *Different factors associated to somatic embryogenesis induction in* Euterpe edulis *were investigated in the present study. Zygotic embryos were inoculated in MS culture medium supplemented with 2.4-D (0, 30, 35, 40 mg L^{-1}), 3 mg L^{-1} 2iP, 0.5 g L^{-1} glutamine, 0.5 g L^{-1} activated charcoal; 30 g L^{-1} glucose or sucrose and gelled with 5 g L^{-1} Agar. Leaf sheaths were inoculated on MS culture medium supplemented with Picloram (72.3 mg L^{-1}) or 2.4-D (66.3 mg L^{-1}), 3 mg L^{-1} 2iP, glutamine (0; 0.29; 0.58; 1.17 g L^{-1}), 1.5 g L^{-1} activated charcoal and gelled with 2.5 g L^{-1} Phytagel®. Indirect somatic embryos were induced from zygotic embryos on culture medium with 40 mg L^{-1} 2.4-D and 30 g L^{-1} sucrose. The highest percentage of callus formation on leaf sheaths occurred in MS culture medium supplemented with 1.17 g L^{-1} glutamine and 66.3 mg L^{-1} 2.4-D.*

Keywords: heart of palm, tissue culture, growth regulators, somatic embryogenesis

Abbreviations: MS (Murashige and Skoog 1962); Morel (Morel and Wetmore 1951); *2.4-D (2.4-Dichlorophenoxyacetic acid); 2iP (2- isopentenyladenine); NAA (1-Naphthaleneacetic acid).*

INTRODUCTION

Euterpe edulis Martius species belongs to the Palmae (Arecaceae) family, has a 5 to 10 m tall main shoot and is popularly known as juçara, jiçara or ripa (Carvalho 2003). This species is found along the Brazilian coast from Southern Bahia to Rio Grande do Sul and in the upcountry forests of the states of Goiás, Mato Grosso do Sul, Minas Gerais and the Federal District (Carvalho 2003), predominantly in the mid forest strata (Reis et al. 2000).

The economic value of palm tree is related to heart-of-palm production, a food with high nutritional value consisting of the stem tip wrapped in adult leaves. Botanically it represents the apical shoot that is responsible for the palm tree development. The fruits can be used in the food industry as a source of juice, but to obtain the heart-of-palm the palm tree must be cut off (Carvalho 2003).

The natural palm tree populations have been drastically reduced through human action and are currently found only in restricted areas in the Atlantic Rain Forest biome. Extraction and illegal exploitation are threats to the sustainability of the natural palm tree populations (Reis and Kageyama 2000). However, this species has a great potential for management in a

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sustained production system because of its relatively short cycle. It is an important source of income for producers and maintains its ecological role in the ecosystem to which it belongs (Pereira 2000). The ecological importance of this species is related to the levels of interaction within the forest communities as its fruit is a food source for birds and mammals, such as rodents, marsupials, primates and bats (Reis and Kageyama 2000).

The propagation of *E. edulis* is fundamental to maintain and increase the remaining populations since natural regeneration has not been sufficient to replace them (Reis et al. 2000). The propagation of this palm tree occurs exclusively through seeds, which lose the germination capacity within few months (Carvalho 2003). A reduction in moisture content below 28% in palm tree seeds causes a loss of viability and consequently a drop in germination rates (Reis et al. 1999) od these recalcitrant seeds, making long term storage difficult.

As the palm tree has no natural vegetative propagation system (i.e. off-shoots) and does not respond to the conventional vegetative propagation methods (Guerra et al. 2000), biological techniques are suitable tools for its conservation and large-scale germplasm multiplication. Among these techniques, somatic embryogenesis is of particular interest, characterized by the development of bipolar structures with a root-shoot axis (somatic embryos) from haploid or diploid cells, which repeats the morphogenetic events of the development of a zygotic embryo (Guerra et al. 2000).

In vitro regeneration through somatic embryogenesis has been documented in several palm tree species and morphogenetics in vitro was already reported for *E. edulis* by Guerra and Handro (1991a, b), Guerra and Handro (1998) and Guerra et al. (2000) and is considered the best form of in vitro regeneration of palm trees.

In this context, the present study aimed to optimize the culture conditions and describe the principal in vitro morphogenetic events in *Euterpe edulis* zygotic embryos and leaf sheaths to contribute to an enhanced somatic embryogenesis protocol for this species.

MATERIAL AND METHODS

Zygotic embryos

Unripe palm tree fruits with semi-gelatinous endosperm (Figure 1A) were collected from a *Euterpe edulis* stand of the Experimental Station of the State Agricultural Research Foundation (FEPAGRO) in Santa Maria-RS, Brazil.

In the Forest Biotechnology Laboratory of the Federal University of Santa Maria (Santa Maria-RS) the fruits were disinfected in a solution consisting of 1.5 g L^{-1} Agrimycin, 20% (v/v) sodium hypochlorite, 4 g L^{-1} Captan and 0.7 g L^{-1} Benalte for 15 minutes with agitation, in a flow chamber. They were then rinsed in bi-distilled sterile water and dried on Petri dishes lined with sterile filter paper discs .

The zygotic embryos (Figure 1B), excised in a laminar flow hood, were inoculated in test tubes (25 x 150mm) containing 10 ml MS culture medium (Murashige and Skoog 1962) supplemented with the Morel vitamins (Morel and Wetmore 1951), 5 g L^{-1} agar, 0.5 g L^{-1} glutamine, 3 mg L^{-1} 2iP, 0.5 g L^{-1} activated charcoal and different 2.4-D concentrations. The pH was adjusted to 5.8 before adding the gelling agent and then sterilized by autoclave at 127 °C and 1 kgf cm^{-2} during 15 minutes.

Four 2.4-D concentrations were tested for somatic embryogenesis induction (0; 30; 35; 40 mg L^{-1}) combined with glucose or sucrose (30 g L^{-1}). MS culture medium with the Morel vitamins and 2iP (3 mg L^{-1}) was used for somatic embryo maturation. Half-strength MS (MS/2) culture medium with Morel vitamins was used to convert the somatic embryos into plantlets, without adding NAA. The culture media were based on Guerra et al. (2000).

The percentage of callus induction was evaluated 90 days after inoculation and the culture period was photodocumented throughout culturing. No subcultures were employed until induction of somatic embryogenesis (240 days), maturation (30 days) and conversion of the somatic embryos (30 days).

A randomized block design was used with treatments that consisted of four 2.4-D concentrations with two carbohydrate sources in a 4x2 factorial design, totaling eight treatments in six replications. Each replication consisted of two test tubes, containing one embryo each.

During the induction step the cultures were kept in a growth chamber at 25±2 °C under total absence of light and at 25±2 °C under a 16 hour light period during maturation and germination of the somatic embryos. The

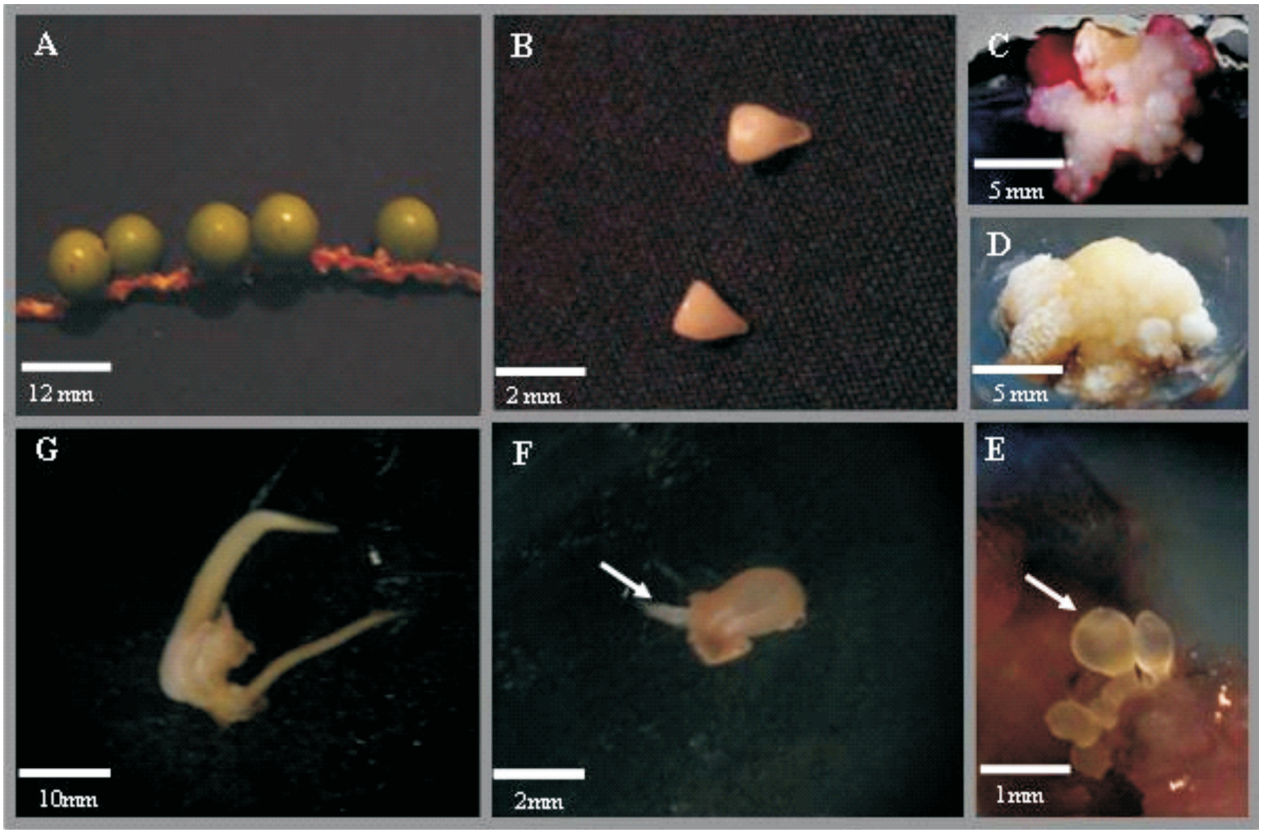

Figure 1. In vitro somatic embryogenesis of immature zygotic embryos of palm tree (*Euterpe edulis* Mart.). A) unripe fruit. B) excised zygotic embryos. C) Compact embryogenic callus with a yellowish coloring and intense anthocyanin pigmentation in the cotyledon node region. D) haustorial tissue with white color and spongy appearance. E) somatic embryos (arrow) developed on friable embryogenetic tissue in MS culture medium with 3 mg L^{-1} 2iP and 40 mg L^{-1} 2.4-D 240 days after inoculation. F) development of the root on somatic embryo after 280 days. G) somatic plantlet with root-shoot axis after 300 days

results of callus formation expressed in percentage were transformed into arcsin $\sqrt{x}$ and submitted to analysis of variance at the level of 5% significance.

Leaf sheaths

Leaf sheaths, approximately 7 cm long, were isolated from in vitro-grown plantlets. The zygotic embryos were excised from fruits of palm trees of an arboretum at the Agrarian Science Center of the Federal University of Santa Catarina, Florianopolis, Santa Catarina. For in vitro germination MS/2 culture medium supplemented with Morel vitamins, 30 g L^{-1} sucrose, 1.5 g L^{-1} activated charcoal and 7 g L^{-1} agar was used. The cultures were kept in a growth chamber at 25±2 °C and 16 hour light period and 50-60 µM m^{-2} s^{-1} light intensity.

Transversal sections of the leaf sheaths (1±0.5mm) were inoculated in 10 cm diameter Petri dishes containing 30 ml MS culture medium supplemented with Morel vitamins, 30 g L^{-1} sucrose; 1.5 g L^{-1} activated charcoal; 2.5 g L^{-1} Phytagel; 3 mg L^{-1} 2iP and different glutamine concentrations (0; 0.29; 0.58; 1.17 g L^{-1}) combined with Picloram (72.3 mg L^{-1}) or 2.4-D (66.3 mg L^{-1}).

A randomized block design was used with treatments consisting of two sources of auxin and four glutamine concentrations in a 2 x 4 factorial design, totaling eight treatments in three replications. Each plot consisted of three 10 cm diameter Petri dishes. The cultures were kept in a growth chamber at 25 °C ± 2 without light. The assessments were made by counting callus induction (nodules) at the base of the explants and callus occurrence with anthocyanin 60 days after inoculation. The results were transformed into arcsin $\sqrt{x}$ and submitted to analysis of variance and Tukey´s test at 5% probability.

RESULTS AND DISCUSSION

Zygotic embryos

In the present study, somatic embryogenesis in zygotic embryos of *Euterpe edulis* pursued an indirect model with callus formation followed by the induction of embryogenetic tissues. After 90 days of culture the zygotic embryos inoculated in culture medium supplemented with 40 mg L^{-1} 2.4-D (with glucose or sucrose) produced a compact yellowish callus and intense anthocyanin pigmentation in the cotyledon node region (Figure 1C), but without difference s in callus formation among the tested carbohydrate sources [glucose ($\overline{X}$= 46%) and sucrose ($\overline{X}$ = 42%)] on callus induction. These results are in agreement with those reported by Guerra et al. (2000), who observed that the germination of *Euterpe edulis* zygotic embryos cultivated in MS culture medium containing high 2.4-D levels was inhibited and the embryonic tissues expanded. The other treatments only presented an intense formation of white haustorial tissue (corresponding to the cotyledon blade), with a spongy appearance as morphogenetic responses (Figure 1D). In the control treatments that corresponded to primordial leaf and root emission and haustorial development normal germination of the zygotic embryos was observed throughout.

After 240 days the cultures kept in culture medium supplemented with 0.5 g L^{-1} activated charcoal and 40 mg.L^{-1} 2.4-D presented embryogenetic characteristics resulting in globular and translucent structures (Figure 1E), especially in the callus regions in contact with the culture medium. The induction of direct somatic embryo formation in *Euterpe edulis* was observed in the cotyledon tissues of zygotic embryos inoculated in MS culture medium with activated charcoal (1.5 g L^{-1}) and 100 mg L^{-1} 2.4-D (Guerra et al. 2000). The characteristics of the somatic embryos observed in the present study were similar to those reported by Guerra and Handro (1998).

The disagreement over the most suitable 2.4-D concentration to induce palm tree somatic embryogenesis between this study (40 mg L^{-1}) and that of Guerra et al. (2000) (100 mg L^{-1}) may be attributed to the difference in the activated carbon concentration added to the culture medium; in the present study 0.5 g L^{-1} activated charcoal was used while Guerra et al. (2000) used 1.5 g L^{-1}. Activated charcoal can adsorb various substances present in the culture medium, including the growth regulators (Eymar et al. 2000). Correlations have been detected between the activated charcoal levels present in the culture medium and the total free and available 2.4-D (Toering and Pullman 2005); the activated charcoal adsorbed a significant part of the 2.4-D present in the culture medium.

The factors that affect the induction of somatic embryogenesis include the physiological state of the mother plant, the developmental stage of the explants, the culture medium composition and the type, balance and concentration of growth regulators, especially the auxins (Guerra and Handro 1998). In the present study, the intense formation of haustorial tissue at low 2.4-D concentrations suggested the fundamental role of auxins in the supression of germination and acquisition of embryogenic competence of the explant.

In the present study the embryogenetic cultures containing globular somatic embryos and friable embryogenetic tissue were transferred to MS culture media without activated charcoal and supplemented with 3 mg L^{-1} 2iP 240 days after inoculation. With the transference to this culture medium, the globular somatic embryos progressed to bipolar stages in 30 days. Thereafter, the somatic embryos were transferred to MS culture medium with MS/2 with 1.5 g L^{-1} activated charcoal without plant regulators and after 10 days in this culture medium the mature somatic embryos presented root emergence (Figure 1F), followed by shoot growth and complete plantlet formation 300 days after inoculation (Figure 1G).

Leaf sheaths

Callus induction from leaf sheaths of in vitro-grown plantlets showed a significant influence ($p<0.05$) of the auxin source (i.e., Picloram and 2.4-D).

The morphological analyses showed that cell proliferation in the leaf tissues started in regions adjacent to the vascular bundles, resulting in nodules (Figure 3D) after 30 days in culture medium. These morphogenetic events were observed in young leaves of the same species by Guerra and Handro (1998). However, this pattern of in vitro morphogenetic response did not seem to be exclusive to the monocotyledons because the embryogenetic tissues

Table 1. Effect of different auxin sources on callus induction and anthocyanin accumulation in explants from palm tree *Euterpe edulis* leaf sheaths cultivated in vitro, 60 days after inoculation

Auxin source	Callus formation (%)	Callus with anthocyanin (%)
2.4 - D (66.3 mg L^{-1})[a]	28.4 a*	3.1 b*
Picloram (72.3 mg L^{-1})[a]	20.3 b	10.7 a

* Means followed by different letters differ by the Tukey test at 5%. Coefficient of variation (CV%) for callus formation: 15.7% and coefficient of variation (CV%) for callus with anthocyanin accumulation: 9.5%. [a]Equivalent to 300 μM.

were formed from parenchyma cells with high mitotic activity in *Coffea arabica* leaf explants (Quiroz-Figueroa et al. 2002).

In the present study, the addition of 66.3 mg L^{-1} 2.4-D into the culture medium resulted in a higher rate of callus induction in leaf sheaths compared to the rates observed in the culture medium supplemented with 72.3 mg L^{-1} Picloram, with significant differences by Tukey's test (Table 1). These results reinforce those reported by Guerra and Handro (1998) who pointed out that 2.4-D was essential for callus induction in palm leaf sheaths. However, other studies on palm trees reported that Picloram promoted similar morphogenetic responses (Goh et al. 2000, Karun et al. 2004).

The percentage of friable calluses showing the presence of anthocyanin was 10.7% in response to the culture medium supplemented with Picloram and 3.1 % in response to 2.4-D (Table 1). During *Euterpe edulis* somatic embryogenesis induction in zygotic embryos there was intense anthocyanin pigmentation on the cotyledon, from where the somatic embryos appeared (Guerra and Handro 1991a). Anthocyanin accumulation may be related to factors such as light, nitrogen source in the culture medium, sugar source, osmotic stress, temperature and growth regulator sources (Ikram-ul-Haq and Zafar 2004). Anthocyanin production in embryogenetic cultures of *Gossypium hyrsutum* was a strong indicator of physiological stress caused by adding different nitrogen sources to the culture medium (Ikram-ul-Haq and Zafar 2004).

Somatic embryogenesis induction in monocotyledon from leaf explants has been reported in several species. Leaf blade expansion and the appearance of nodular structures were observed on *Euterpe edulis* leaf bases inoculated in MS culture medium supplemented with 2.4-D, ANA and 2iP after 30 days in culture medium (Guerra et al. 2000). Calluses were induced in the *Phoenix dactylifera* only when the first leaves were inoculated for 12 weeks in culture

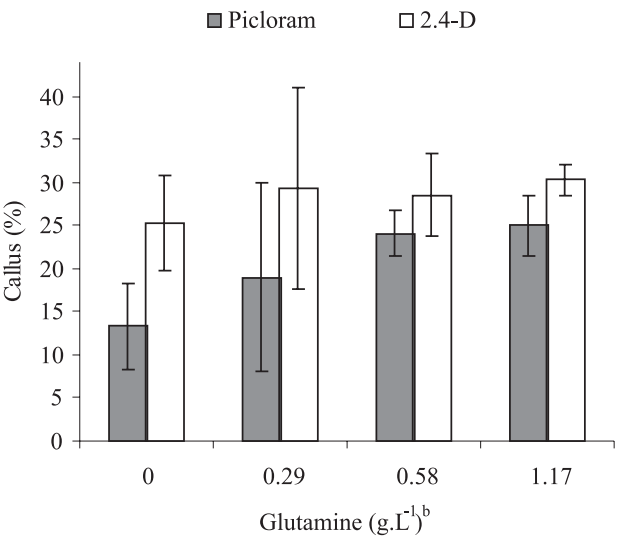

Figure 2. Percentage of callus induction in explants derived from palm tree *Euterpe edulis* leaf sheaths in MS culture medium supplemented with different concentrations of glutamine and different auxin sources [Picloram (72.3 mg L^{-1}) or 2.4-D (66.3 mg L^{-1}), at 60 days. [b] Values of 0; 0.29; 0.58; 1.17 g L^{-1} equivalent, respectively, to 0, 2, 4 and 8 mM glutamine. The treatment means are presented with standard deviation (±)

medium with 2.4-D and 2iP (Al-Khayri and Al-Bahrany 2004). Embryogenetic cultures were observed in *Areca catechu* when young leaves were inoculated in MS culture medium supplemented with 2.4-D (Karun et al. 2004).

Additionally, it was observed in the present study that the MS culture medium supplemented with 1.17 g L^{-1} glutamine combined with 66.3 mg L^{-1} 2.4-D resulted in a greater percentage of callus induction (Figure 2). The nitrogen present in the culture media seemed to be determinant in the success of somatic embryogenesis (Guerra et al. 1999). Supplementing the culture medium with an organic nitrogen source has been an important factor for somatic embryogenesis induction in forest species (Kirby et al. 1987). In the present study, increased glutamine concentrations in the culture medium resulted in an increased callus induction rate. In other embryogenetic systems, such as *Feijoa*

sellowiana (Dal Vesco and Guerra 2001), glutamine supplemented to the LPm culture medium increased the number of somatic embryos derived from immature zygotic embryos. Amino acids are being the most used organic nitrogen sources in culture media to induce somatic embryogenesis, and in some cases, were fundamental for callus and somatic embryo induction (Kirby et al. 1987). Glutamine as organic nitrogen source, in addition to being a precursor to the synthesis of other amino acids, is also one of the first precursors in the chain reaction for polyamine synthesis, mediated by ornithine synthesis (Taiz and Zeiger 2002). The polyamines have various functions in the plant kingdom and are frequently derived from amino acid descarboxilation. The polyamine levels and growth rates of plant tissues were positively correlated, suggesting that they were involved in the control mechanisms of cell division (Bagni and Biondi 1987). Furthermore, the results demonstrated a positive polyamine influence (exogenous and endogenous) on in vitro morphogenesis. Exogenous polyamine application significantly increased the somatic embryogenesis rate in calluses derived from *Elaeis guineensis* zygotic embryos (Rajesh et al. 2003). In the present study the supplementation of the culture medium with glutamine may have resulted in an increased polyamine metabolism, influencing the in vitro morphogenesis of the explants.

Different morphogenetic responses were observed among leaf sheaths removed from different positions of the plantlets. Explants obtained from the subapical region presented a swelling after 30 days in MS culture medium with 2.4-D (66.3 mg L^{-1}) or Picloram (72.3 mg L^{-1}). Furthermore, intense callus proliferation was observed at the base of these explants (Figure 3C) after 60 days in culture. Explants without chlorophyll pigmentation presented nodule induction. When the explants were removed from chlorophyll tissues and were therefore more differentiated, intense oxidation was observed after 30 days of culture. It has been reported that in vitro plant regeneration depends on the plant material source, and usually young explants are more responsive than those derived from adult tissues (Raemakers et al. 1995), in agreement with observations in the present study. Different callus initiation rates were observed in segments extracted from different portions of *Cocos nucifera* embryonic axles, probably in response to the regenerative competence of the explant tissues (Gomes et al. 2004).

The results obtained in the present study showed that somatic embryo initiation from *Euterpe edulis* zygotic embryos depended on high 2.4-D concentrations in the culture medium. In this study, the optimal 2.4-D concentration for somatic embryogenesis induction in *Euterpe edulis* zygotic embryos was lower compared with other studies that have reported this morphogenetic procedure in palm tree in vitro regeneration. The higher callus induction rate in leaf sheaths was related to the auxin source (2.4-D) and addition of high glutamine concentrations (1.17 g L^{-1}) to the culture medium.

The development of a somatic embryogenesis protocol for the species is extremely important to establish programs for *E. edulis* conservation and breeding. The results obtained here show the potential of using this morphogenetic route for such programs, as complete plantlet regeneration was possible through somatic embryogenesis.

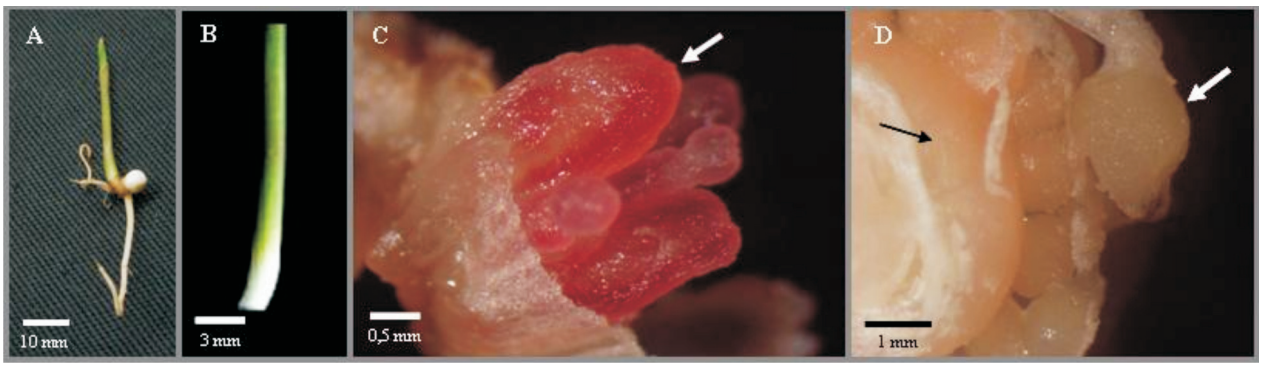

Figure 3. In vitro morphogenesis in palm tree *Euterpe edulis* leaf sheaths. A) The plantlet used to remove the leaf sheaths (explants). B) Explant source from in vitro-grown plantlet. C) nodules with intense anthocyanin pigmentation (arrow) 60 days after inoculation. D) Meristem tissue with swollen appearance (narrow black arrow) and nodule with yellowish appearance (wide white arrow) after 60 days.

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Morfogênese *in vitro* de embriões zigóticos e bainhas foliares de *Euterpe edulis* Martius (Arecaceae)

Resumo – *Diferentes fatores associados à indução da embriogênese somática em* Euterpe edulis *foram abordados no presente trabalho. Embriões zigóticos foram inoculados em meio MS suplementado com 2,4-D (0, 30, 35, 40 mg L^{-1}), 3 mg L^{-1} de 2iP, 0,5 g L^{-1} de glutamina, 0,5 g L^{-1} de carvão ativado; 30 g L^{-1} de glicose ou sacarose e geleificado com 5 g.L^{-1} de ágar. Bainhas foliares foram inoculadas em meio MS suplementado com Picloram (72,3 mg L^{-1}) ou 2,4-D (66,3 mg L^{-1}), 3 mg L^{-1} de 2iP, glutamina (0; 0,29; 0,58; 1,17 g L^{-1}), 1,5 g L^{-1} de carvão ativado e geleificado com 2,5 g.L^{-1} de Phytagel. Embriões somáticos indiretos foram induzidos a partir de embriões zigóticos em meio suplementado com 40 mg L^{-1} de 2,4-D e 30 g L^{-1} de sacarose. A maior porcentagem de formação de calos em bainhas foliares ocorreu em meio MS suplementado com 1,17 g L^{-1} de glutamina e 66,3 mg L^{-1} de 2,4-D.*

Palavras-chave: palmiteiro, cultura de tecidos, reguladores de crescimento, embriogênese somática.

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