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Somatic Embryogenesis and Plant Regeneration in Different Organs of *Euterpe edulis* Mart. (Palmae): Control and Structural Features

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Somatic embryogenesis and further plant regeneration were observed using zygotic embryos, young inflorescences and young leaves of *Euterpe edulis* (Palmae) as explants. Both for the cultures of zygotic embryos and inflorescences, activated charcoal in the medium was essential for the establishment of viable cultures. Embryogenesis was induced by using a gelled basal medium with MS or Euwens salts supplemented by high 2, 4-D levels (50-100 mg L^{-1}). The embryogenic process was direct without a callus stage. For further development, cultures with globular or post-globular embryos were transferred to the basal medium with 2-iP (2.5 mg L^{-1}) and NAA (0.1 mg L^{-1}). To convert embryos to plantlets, cultures were transferred to a third medium in which sucrose and salts were reduced to the half-strenght of the basal medium, without growth regulators. In the case of leaves, cultures were initiated on filter paper bridges on a liquid medium, with either 2, 4-D or NAA (10-20 mg L^{-1}). The developmental stage of each explant was critical for the induction of embryogenesis. The histological study of embryogenic cultures revealed that in the case of zygotic embryos, somatic embryos arise directly from the surface of the cotyledonar node, or from subepidermal tissues. In the inflorescences, a pro-embryogenic tissue is formed at the floral primordium region; in the leaves, the first morphogenic event is cell proliferation in the vascular parenchyma.

Key words: *Euterpe edulis* — Micropropagation — Somatic embryogenesis — Tissue culture

Euterpe edulis Mart. is a single stemmed neotropical palm indigenous to rain forests in the Brazilian southern and southeastern coastal region. Economic importance is related to the growing apical bud surrounded by young leaves, which constitutes the "palmito" (heart of palm), an edible product where increasing exploitation and demand is leading to the rapid disappearance of natural populations. From the ecological point of view, *E. edulis* is an understory species, its seedlings requiring shade to survive and develop. As a consequence, the commercial exploitation of this species requires the maintenance, or even enlargement of the existing vegetation. In addition this species plays a very essential role in the attraction and maintenance of the fauna in the forest during a large part of the year, by supplying flowers and fruits for insects and other animals (Fantini *et al.* 1992). Thus it is reasonable to assume that a sustainable yield management system could provide for not only the long term exploitation of a non-timber forest product, but also the conservation of existing biological diversity. As the propagation of *E. edulis* is done only through seeds, the use of tissue cultures could be advantageous for the mass clonal propagation of elite plants, as well as for germplasm conservation.

In general, two basic procedures have been used for palm tissue culture: a) the regeneration of plantlets through somatic embryogenesis, or direct/indirect organogenesis via callus; b) the reversion of young flower meristems to a vegetative state (Tisserat 1987). Several species of high economic interest among them *Cocos nucifera, Elaeis guineensis* and *Phoenix dactylifera* were propagated by tissue cultures (see George 1996). In previous reports, we have described the success of using zygotic embryos and young inflorescences of *E. edulis* as explants to obtain somatic embryogenesis (Guerra and Handro 1988, 1991). The aim of this work is to better describe the process of somatic embryogenesis in tissue cultures of *E. edulis*, including in leaves, as well as certain structural features of this process.

Material and Methods

Explants

Three kinds of explants were employed: a) *embryos*- Zygotic embryos were excised either from mature seeds (red

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fruits, embryos ca. 3.5 mg), or from immature ones (green fruits, embryos ca. 1.5 mg), after submitting the fruits to a heavy sterilization: 12 hr under agitation in a solution with 4% sodium hypochloride, 0.15% streptomycin and 0.01% Merthiolate, followed by 5 min of washing in 70% ethanol. After fruit washing in sterile water, embryos were rapidly excised and innoculated to avoid browning. b) *inflorescences*-In the adult plant of *E. edulis* ca. 10 young inflorescences at different development stages (1-40 cm long) are sheltered by leaf sheathes. These inflorescences are in general free of contamination, and were removed after cleaning the leaf sheathes with 70% ethanol. Inflorescences ranging from 5-10 cm were used to obtain 1 cm long sections to start cultures. c) *leaves*-From seedlings obtained aseptically through *in vitro* culture of mature embryos, the inner, yellowish-white young leaves were isolated. Explants 0.5-1.0 cm long from the sheath and the lamina were then innoculated on the medium.

Culture medium

The basal medium was constituted of mineral salts according to Euwens (1976), vitamins after Morel and Wetmore (1956) and sucrose (30 g L^{-1}). MS salts (Murashige and Skoog 1962) were also tested, with similar results. Activated charcoal (1.5-3.0 g L^{-1}) was the only effective compound to prevent browning. Auxins (2, 4-D: 2, 4-dichlorophenoxiacetic acid, NAA: 1-naphthaleneacetic acid) and a cytokinin (2-iP: 2-isopentenyladenine) were added at different stages of the culture (see results). The pH of the medium was adjusted to 5.6. When necessary the medium was gelled with agar (6-8 g L^{-1}). In the case of inflorescences, explants were initially cultured in liquid medium before being transferred to the gelled medium. Cultures of young leaves were also initiated in a liquid medium, on filter paper bridges.

Culture conditions

All cultures were initiated in the dark to prevent browning. The dark period varied from 48hr (zygotic embryos) to 20days (inflorescences). After this period cultures were transferred to a light/dark cycle, 16/8 hr, photonic flux ca 45 μM m^{-2} sec^{-1}. The temperature of incubation was 26±1 C.

Histology

During the period of culture, at regular intervals, samples were fixed in FAA 50 (formalin: acetic acid: 50% ethanol, 5 : 5 : 90) (Sass 1951) for histological studies. The material was dehydrated in a tertiary-butyl-alcohol series and then embedded in paraffin. Twelve micrometer-thick sections were obtained and stained with safranin and fast green.

Results

Zygotic embryos

The establishment of viable cultures and their further progression occurred only in a gelled medium containing activated charcoal. In preliminary experiments, several auxins were tried in an attempt to induce embryogenesis

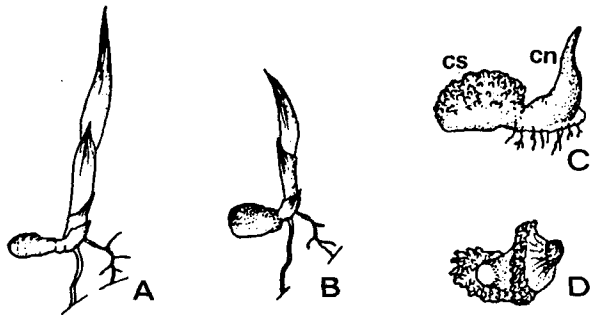
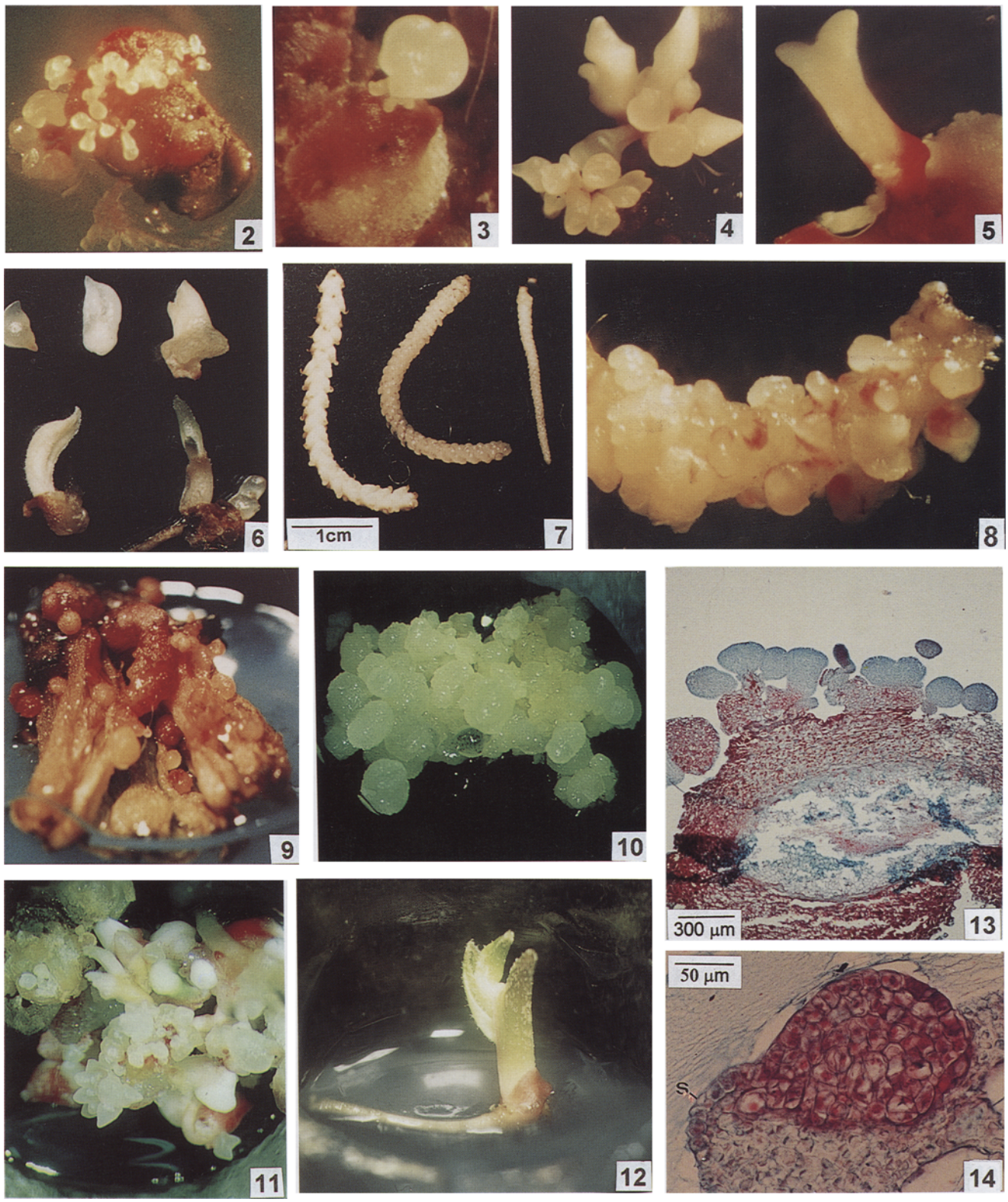


Fig. 1. Effect of 2, 4-D on zygotic embryos cultured *in vitro*, after 2 months in culture. A-control, B-10 mg L^{-1}, C-50 mg L^{-1}, D-100 mg L^{-1}. cs-cotyledonary sheath (haustorium), cn-cotyledonary node.

(NAA, picloram and 2, 4-D) but only 2, 4-D was effective. When zygotic embryos are cultured in increasing concentrations of 2, 4-D, distinct morphogenetic responses occur as shown in Fig. 1, with the progressive inhibition of germination, and the development of granular structures on the cotyledonar node, with high 2, 4-D levels (50-100 mg L^{-1}). These granules developed in translucid structures that characterize the initial stages of somatic embryogenesis (Figs. 2-3). If the cultures are kept in this medium, the matrix tissue produces new embryos which remain at the globular stage without reaching the bipolar stage, and a continuous and non-synchronous embryogenic process continues. Under these conditions the embryogenic potential remains unchanged for periods longer than one year. The transference of post-globular embryos to the same basal medium free of activated charcoal and supplemented with 2-iP (2.5 mg L^{-1}) and NAA (0.1 mg L^{-1}) resulted in the progression to a bipolar developmental stage (Figs. 4-5). When these somatic embryos were transferred to a third medium with sucrose and mineral salts reduced to the half strenght of the basal medium, they were converted to plantlets presenting the same pattern observed in the germination of zygotic embryos (Fig. 6). This embryogenic model is direct, without previous callus formation, where embryos arise directly on the surface of the cotyledonar petiole (Figs. 3, 5). Plants that reached 10 cm high with good rooting (ca 5 cm long), could be transferred to pots, kept in a humid chamber for 1-2 weeks and then transferred to the greenhouse. Regarding the embryogenic potential of mature zygotic embryos when compared with immature ones, it was noted that the latter constitutes a high frequency model in which the onset of embryogenesis occurs 30 days after inoculation, with plantlet production in 180 days. The process with mature embryos was slower, the beginning of the embryogenic process occurring only 90 days after the onset of cultures.

Inflorescences

Successful cultures of young inflorescences (Fig. 7) occurred only when they were placed in a liquid medium with activated charcoal and kept the first 15 days in the dark, to avoid the intense browning. After this treatment, rachyl



Figs. 2-3. Globular somatic embryos developed on the cotyledonary node, after 60 days in culture. Figs. 4-5. Bipolar stages of somatic embryos, 100 days. Fig. 6-Sequence of development of somatic embryos observed in cultures of immature zygotic embryos. Fig. 7. Young inflorescences of *E. edulis* used to prepare explants. Centre and right stages are the best to obtain embryogenic cultures. Fig. 8. Pro-embryogenic nodes and somatic embryos in an inflorescence segment, after 60 days in culture. Fig. 9. Pro-embryogenic nodes in leaves after 100 days in culture. Fig. 10. Mass of globular embryos originated 60 days after transfer of the primary culture shown in Fig. 9 to a new medium. Fig. 11. Further stage of the embryos in the culture shown in Fig. 10. Fig. 12. Plantlet developed after the isolation of the germinated embryos shown in Fig. 11. Fig. 13. Transection at the cotyledonary node showing embryogenesis from pro-embryogenic tissue at the epidermal surface (70 days old cultures). Fig. 14. Longisection of a somatic embryo at the globular stage showing a suspensor-like structure(s).

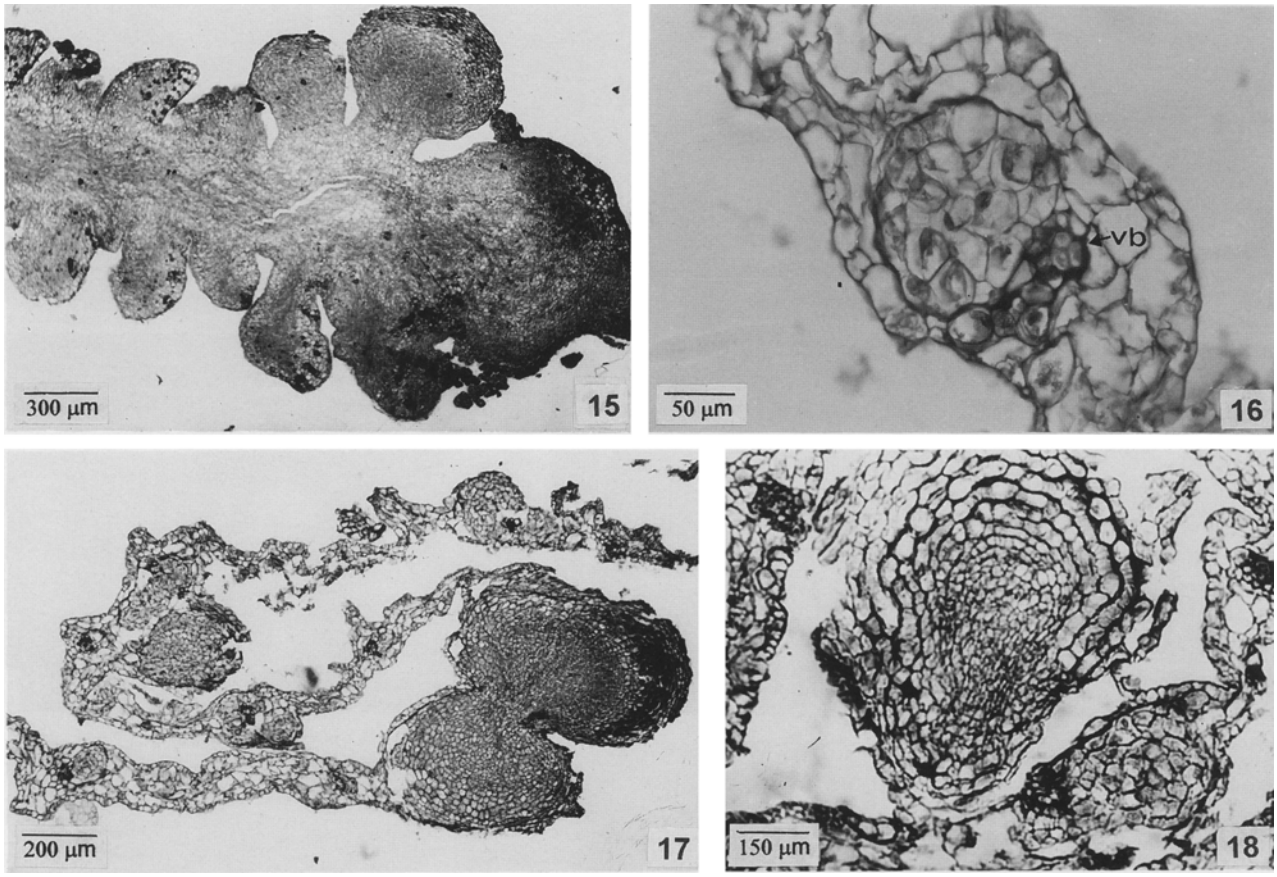


Fig. 15. Longisection of the rachyl after 30 days in culture with 2, 4-D. Note high cell proliferation in the region of floral primordia. Fig. 16. Cell proliferation in the leaf vascular parechyma (vb-vascular bundle). Fig. 17-Leaf transection showing several pro-embryogenic nodes. Fig. 18. An embryo developed from the embryogenic node on a leaf.

segments 1.0 cm long were transferred to a gelled medium. As occurred for zygotic embryos, only the addition of 2, 4-D (50 mg L^{-1}) plus 2-iP (3.0 mg L^{-1}) induced embryogenesis in this type of explant. The sequence observed in cultured inflorescences showed that in the first place rachyls elongated and thickened, especially at the floral primordia (Fig. 8). Cell proliferation resulted in pro-embryogenic tissues, where translucid globular embryos arose after 60 days. The embryogenic regions were isolated and transferred to the MS medium supplemented with 2-iP (5 mg L^{-1}) plus NAA (0.1 mg L^{-1}), leading to a progressive, continuous and asynchronous high frequency embryogenic system. In some cultures the production of globular embryos was continuous for one year. The transfer of embryogenic regions to a medium without growth regulators produced plantlets, similar to what was described for the culture of zygotic embryos. Sharp differences were found in the morphogenetic responses in relation to the developmental stage of the inflorescences. Highly differentiated inflorescences showed intense oxidation and eventual development of flowers, but never embryogenesis. Repetitive somatic embryogenesis was observed either in inflorescences or in zygotic embryos-derived cultures when subcultured in the MS medium supplemented with 2, 4-D or NAA (5-10 mg L^{-1}) and 2-iP (3 mg L^{-1}), the new embryos arising from the surface of primary globular somatic embryos, suggesting a protodermic origin.

Leaves

Somatic embryogenesis was observed when non-chlorophyllous leaves were cultured *in vitro*. To initiate cultures, very young leaves were put on filter paper bridges in a basal liquid medium, with either 2, 4-D or NAA (10-20 mg L^{-1}) plus 2-iP (3.0 mg L^{-1}). The leaf blade expands, and after 30 days several nodular proliferations appeared, which were translucid or red-pigmented (Fig. 9). The transfer of primary cultures to a gelled medium supplemented with 2-iP (0.5 mg L^{-1}) and 2, 4-D (1.0 mg L^{-1}) resulted in the production of an embryogenic tissue where masses of globular embryos arose (Fig. 10). After a further transfer to a medium with 2-iP (2.5 mg L^{-1}) plus NAA (0.1 mg L^{-1}) the embryos reached bipolar stages (Fig. 11). Cultures were then transferred to the basal medium free of growth regulators, in which embryos developed into plantlets and could be isolated (Fig. 12). After reaching a height of 10 cm, they were transferred to pots.

Structural aspects

When zygotic embryos where cultivated with a high level of 2, 4-D, germination was inhibited and embryo tissues

expanded. Two characteristic models lead to somatic embryogenesis. In the first, after 60 days in culture, cell tissues with pro-embryonary features are found in the sub-epidermic region of the cotyledonar node. These tissues may extrude, forming bunches of somatic embryos. This pattern of pro-embryogenic induction was observed when fully mature zygotic embryos were used as explants. In the second and most common pattern embryos arise directly from the epidermic tissue of the cotyledonar node of immature zygotic embryos (Fig. 13), frequently connected by suspensor-like structures (Fig. 14). Another variation is the occurrence of several embryos from a pro-embryogenic tissue developed on the surface of the cotyledonar node of zygotic embryos. The initiation of somatic embryos occurs in a large extension of the epidermal tissue. In the case of inflorescences, intense cell proliferation occurs at the region of floral primordia, leading to the formation of a characteristic pro-embryogenic tissue (Fig. 15) from which embryos arise directly. In the leaves, the first morphogenic event is a cell proliferation neighbouring the vascular parenchyma (Fig. 16) that gives origin to a meristematic node, which enlarges and bursts the tissues of the explant (Fig. 17). In these nodes some embryos were observed (Fig. 18), but a conspicuous embryogenic process occurs only after transfer of the primary cultures to another medium as described above.

Discussion

Our results showed that a high frequency, asynchronous and continuous model of somatic embryogenesis can be obtained in cultures of zygotic embryos and young inflorescences of *E. edulis*, leading to whole plants which are able to grow in the field. The induction and expression of this route was dependent on the kind and the developmental stage of the explant, and on the type and concentration of auxin in the primary medium, followed by successive transfers to a medium adequate for each critical step of the process.

The success of tissue culture of palm species was demonstrated to be associated to the use of explants in physiological conditions which allow for following a morphogenic program. According to Blake (1983), to induce *in vitro* morphogenesis in palms, an adequate culture medium that leads to a rapid callus initiation and the determination of an embryogenic route in such way that embryos could develop when transferred to another medium, is necessary. This complete morphogenetic sequence was first demonstrated for palms by Rabechault and Martin (1976) in cultures of *Elaeis guineensis* through sequential variations in the type and concentrations of growth regulators. Our results showed that the standard procedures suggested by Tisserat (1984a, 1984b, 1987), regarding to the culture medium, were successfully applied. This author concluded that MS organic salts (Murashige and Skoog 1962), complemented with sucrose, myo-inositol, thiamine, activated charcoal, and growth regulators (2, 4-D, NAA and 2-iP), constitute the best medium conditions to elaborate a protocol for plant regeneration in palms from different explants through somatic embryogenesis.

The characteristics of the *in vitro* system of *E. edulis* showed in this work comprises two important aspects for studies of plant morphogenesis: first, the induction of an embryogenic program in which the events were modulated leading to the regeneration of complete plants similar to the development and germination process of a zygotic embryo; second, the tissues of *E. edulis* cultured *in vitro* expressed this route directly, without the intermediary callus stage, thus preventing eventual anomalies and difficulties of the direct model. In other palm species where production of plants through zygotic embryogenesis was successful (*Elaeis guineensis*, *Phoenix dactylifera*, *Chamaedorae costaricana*, *Cocos nucifera* and *Veitchia merrilii*), the process was indirect, from the callus (George 1996). The induction of direct embryogenesis in *E. edulis* is fundamental when the possibility of exploring this process for micropropagation is considered.

A critical point for establishing a sucessful *in vitro* culture is the selection of the explant. Palms seem to follow the general behavior of Monocots, where callus initiation occurs from organs and tissues with high meristematic activity (Tisserat 1984a). This observation is also true for palms where most of the results were obtained when embryonary tissues were used (see George 1996). In *E. edulis*, both zygotic embryos and inflorescences showed to be appropriate to obtain somatic embryogenesis, and the choice of the correct developmental stage of the explant was fundamental for success. In general, immature tissues were the more convenient.

According to Blake (1983) the *in vitro* system of palms follows a pattern where auxins are required for callus initiation and cytokinins are required for somatic embryos. Among the auxins 2, 4-D seems to be critical. According to Vasil (1982), embryogenic competence is acquired during the first stages of culture in the presence of 2, 4-D. About 60% of the successful cases of somatic embryogenesis in Dicots are associated with 2, 4-D; in Monocots this number is still higher, and high concentrations have been used, reaching 100 mg L^{-1} (Evans *et al.* 1981). In some cases stronger auxins like picloram were used to induce embryogenic competence, as in the case of *Elaeis guineensis* (Teixeira *et al.* 1995). In our work embryogenic cultures were obtained in the presence of high 2, 4-D and low 2-iP levels, but the expression of the model was possible in a second culture stage with the absence of 2, 4-D, addition of NAA and increase of 2-iP. This primordial role of 2, 4-D in the embryogenic route was also described for *Elaeis guineensis* (Ahée *et al.* 1981, Tisserat 1981, Teixeira *et al.* 1994) and *Cocos nucifera* (Blake and Hornung 1995). All these results confirm that this morphogenetic model occurs as a response to a series of procedures where the type and concentration of adequate growth regulators in a correct sequence of subcultures are fundamental.

Regarding the origin of somatic embryos, two basic patterns were observed in the cultures of zygotic embryos and young inflorescences: either the neo-formed structures developed directly from the surface tissues of the

cotyledonar node, with or without a suspensor, or from the inner tissues, from the proliferation of meristematic cell aggregates, similar to the process described by Hu and Sussex (1971), for *Ilex aquifolium*. These meristematic cell aggregates were defined by Haccius (1978) as pro-embryonary cell complexes, and are characteristic of direct embryogenesis. In *E. edulis*, as shown in this work, the embryogenic process in immature zygotic embryos originated from protodermic cells, while in full mature embryos the embryogenic induction occurs from subepidermic cell clusters. Similar patterns of somatic embryo differentiation were observed in *Feijoa sellowiana* (Canhoto and Cruz 1996). In young leaves, the process seems to be similar to that described for leaf segments of *Saccharum officinarum*, where division centers occurred adjacent to the vascular bundles (Ho and Vasil 1983). In this species these meristematic aggregates leads to an embryogenetic callus. A similar origin for embryogenic tissues was verified in leaves of *Elaeis guineensis* (Schwendiman *et al.* 1988) and *Cocos nucifera* (Buffard-Morel *et al.* 1992). In these cases leaf explants developed nodular structures within the leaf tissues, this pattern being the same as observed in our work.

In *E. edulis* competent cells of selected explants must be stimulated by 2, 4-D to follow an embryogenic program. This model is of relatively rare occurrence: a similar model was described by Maheswaran and Williams (1985, 1986) for *Trifolium repens*, where embryogenic initiation was mediated by characteristic pro-embryogenic cell complexes as described by Haccius (1978).

The relevance of the *E. edulis* model for somatic embryogenesis as herein described is based on two aspects: a) the model seems to be quite adequate for studying experimental embryogenesis; b) the regeneration of plants seems to involve only organized meristematic tissues. This favors genetic stability, and envisages the success of protocols for cloning elite progenies, both for commercial exploitation and conservation/improvement of the still remaining natural germplasm. Studies are now in progress in order to establish competent and synchronous embryogenic cell suspensions to improve the regenerative protocol, as well as to determine the embryogenic competence from different natural populations.

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