

## Notas Científicas

### Somatic embryogenesis and in vitro plant regeneration from pejobaye adult plant leaf primordia

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**Abstract** – The aim of this work was to evaluate a protocol for plant regeneration by means of somatic embryos obtained from isolated adult pejobaye leaf primordia, and to describe histological origin of embryos and morphogenetic response. Explants were cultivated in modified MS medium. Mesophyll parenchymatous cells originated meristemoids (preembryonic complex formation) induced with 7.1  $\mu\text{M}$  BAP in the first two subculture periods. After polarized structures with 12.9  $\mu\text{M}$  NAA and 3.55  $\mu\text{M}$  BAP were formed in the third subculture, somatic embryos developed and regenerated normal plants. The mesophyll parenchymatous cells display high capacity of direct response to the auxin and cytokinin.

**Index terms:** *Bactris gasipaes*, histology, micropropagation, peach palm.

### Embriogênese somática e regeneração de plantas in vitro a partir de primórdios foliares de pupunheiras adultas

**Resumo** – O objetivo deste trabalho foi avaliar um protocolo de regeneração de plantas por meio de embriões somáticos, obtidos a partir de primórdios foliares de pupunheiras adultas e identificar a origem histológica dos embriões e descrever as etapas morfológicas. Os explantes foram cultivados em meio MS modificado. Células parenquimáticas do mesófilo originaram meristemóides com BAP (7,1  $\mu\text{M}$ ) nos dois primeiros períodos de subcultura. A polarização das estruturas ocorreu com ANA (12,9  $\mu\text{M}$ ) e BAP (3,55  $\mu\text{M}$ ) no terceiro período de subcultura. Meristemóides se desenvolveram em embriões somáticos, regenerando plantas normais. As células parenquimáticas do mesófilo apresentam elevada capacidade de resposta direta à auxina e à citocinina.

**Termos para indexação:** *Bactris gasipaes*, histologia, micropropagação.

In Brazil, heart-of-palm agroindustry has been exploring the pejobaye as an alternative to be used in the market due to its rusticity, precocity, tillering and excellent product quality.

The species is considered rustic for developing well in low fertility soils, even in the poor soils of the Amazon. The extraction of its heart-of-palm can be performed after 18 or 24 months, depending on the amount of fertilizer used. Moreover, its capacity of tillering makes successive cuts possible for many years, due to the natural sprouting.

Besides being an excellent alternative for the heart-of-palm agroindustry, pejobaye is also important because of its fruits are used for making a kind of flour, which has been consumed for a long time as a food complement in

the North of Brazil, mainly in Rondônia, where areas planted with pejobaye are distributed throughout the State.

However, there are a lot of difficulties in the culture of this species: the occurrence of parthenocarpy and the deficient pollination make pejobaye propagation difficult and, therefore, the use of micropropagation seems to be a promising tool for its culture, mainly for allowing the cloning of selected plants in different regions of the country. Almeida (1994) had success in pejobaye micropropagation from shoot tip of in vitro germinated seedlings, and Almeida & Kerbauy (1996) were able to revert floral buds to adventitious buds, but the process was excessively slow to supply the current demand of seedlings.

Among the techniques to obtain *in vitro* seedlings, somatic embryogenesis stands out for its importance in the propagation of clones of superior genotypes. Moreover, it is very useful on studies related to the physiology of embryonic development, genetics and biochemistry, for obtaining transgenic plants (Prakash & Varadarajan, 1992) and producing synthetic seeds (Schultheis et al., 1990).

The objective of this work was to evaluate a protocol for plant regeneration through somatic embryos obtained from isolated adult pejobaye leaf primordia and to identify the histological origin as well as to characterize the developmental response.

One hundred and twenty 10 mm-leaf-primordia were used as explants, which were taken from the heart-of-palm shoot tips of 60 adult pejobaye (ten years old) cultivated in the experimental area of the Vegetable Production Department of Esalq in Piracicaba, São Paulo State, Brazil (22°45'S, 47°40'W).

The pejobaye origin was the INPA, Manaus, AM germplasm collection in progenies from the Peruvian Amazon (Yurimaguas). After isolated, shoot tips were washed in running water for four hours, and then superficially disinfected with sodium hypochlorite (0,4% of active chlorine) for 30 minutes and rinsed five times in distilled and sterilized water. They were then immersed in 70% (v/v) ethanol for 1 min and rinsed five more times in distilled and sterilized water.

The cultures were kept in a growth room at  $26\pm 1^{\circ}\text{C}$  in light conditions ( $42\ \mu\text{mol m}^{-2}\text{s}^{-1}$ ), with a photoperiod of 16 hours. The explants were placed in test tubes (20 cm x 25 mm) with MS culture medium (Murashige & Skoog, 1962), in which they remained for four weeks, and were subcultivated three times, totalizing 16 weeks in the same medium and environmental conditions, supplemented or not with growth regulators, naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) in the following concentrations: in the initial culture (first four weeks), without growth regulators; in the first and second subculture periods (second and third month of culture), the explants were kept in culture medium with  $7.1\ \mu\text{M}$  BAP and, after that (third subculture period, four months), they were transferred to a culture medium added with  $12.9\ \mu\text{M}$  NAA and  $3.55\ \mu\text{M}$  BAP. The pH value of all the culture media was adjusted to 5.8 before the addition of 0.45% (v/v) Agar (Merck).

Five samples of the plant tissue from each subculture were collected and fixed in a glutaraldehyde and

formaldehyde solution (Karnovsky, 1965), and then dehydrated in an alcoholic series [10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% (v/v)], remaining in each concentration for ten minutes; finally, samples were included in hydroxyethyl methacrylate resin (Leica-Historesin), following the manufacturer recommendations.

The blocks with the samples were cut in a rotating microtome in  $5\ \mu\text{m}$  thick transversal and longitudinal sections. The sections were colored with 0.05% (v/v) toluidin blue in phosphate-buffered and citric acid (Sakai, 1973) and set in histological blades with synthetic resin (Entellan). Later, histological sections were analyzed and the images were obtained in an optic microscope connected to an image capture system.

The cleaning and surface-sterilize procedure appeared to be efficient. However, active oxidation was observed in the first of the four weeks of culture, probably due to high mitotic index tissues and consequent formation of derived cells, in which the presence of phenol components (lignin precursors) is frequently observed during the stage of the cell secondary wall production in the differentiation process.

Thus, explants were not eliminated, although they were apparently dead, because it was clear that they were gradually increasing in size. During the first subculture (5 to 8 weeks in MS medium supplemented only with  $7.1\ \mu\text{M}$  BAP), they duplicated, showing that the oxidative process was restricted to the covering tissue, and that the mesophyll parenchymatous cells remained alive, metabolically active and in division (Figure 1 A). Apart from the increase in the explants volume, characterized by the growth of typical mesophyll parenchymatous cells, no external or internal morphologic alteration was observed in this period, including the formation of calli.

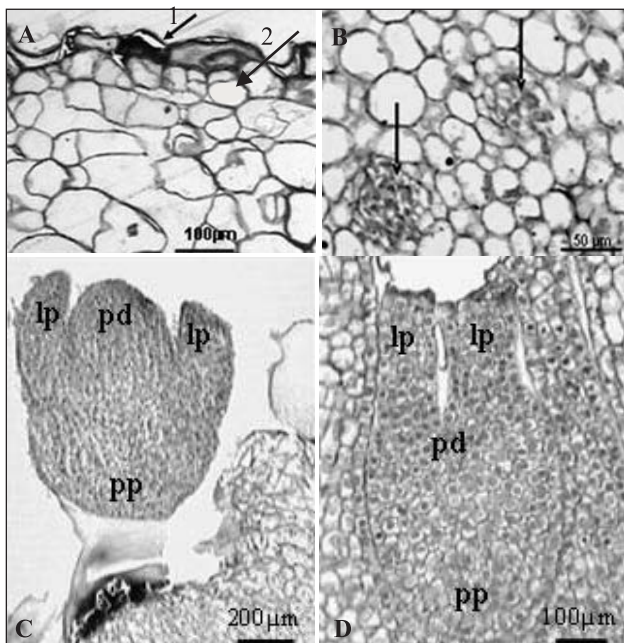
After ten weeks of culture, that is, during the second subculture, in a culture medium supplemented only with  $7.1\ \mu\text{M}$  BAP, the volume increase in the explants caused a rupture in the oxidized epidermis, making visible a chlorophyll-containing tissue in the leaf primordium base. By means of histological cuts of this material, the presence of clusters of small isodiametric cells with a high nucleus/cytoplasm relation was verified, which is typical of the meristemoids or preembryonic complex formation (Figure 1 B).

In the third and last subculture (13 to 16 weeks), the presence of  $12.9\ \mu\text{M}$  of ANA and  $3.55\ \mu\text{M}$  of 6-BAP induced the development of somatic embryos in 82% of

the explants from the polarization of the meristematic centers (meristemoids), which could be confirmed by histological analysis (Figure 1 C and D).

The distal polar region of these embryos presented chlorophyll, while in the proximal polar region, only one root was formed, similarly to the observed in the development of zygotic embryos (Figure 2 A, B and C). According to Arnold et al. (2002), somatic embryogenesis is defined as a process in which a bipolar structure, similar to that of the zygotic embryo, develops from a non-zygotic cell. However, it is important to note that, differently from somatic organogenesis, there is no vascular connection between the embryo and the original tissue.

It was possible to confirm direct somatic embryogenesis, since no callus was observed in the

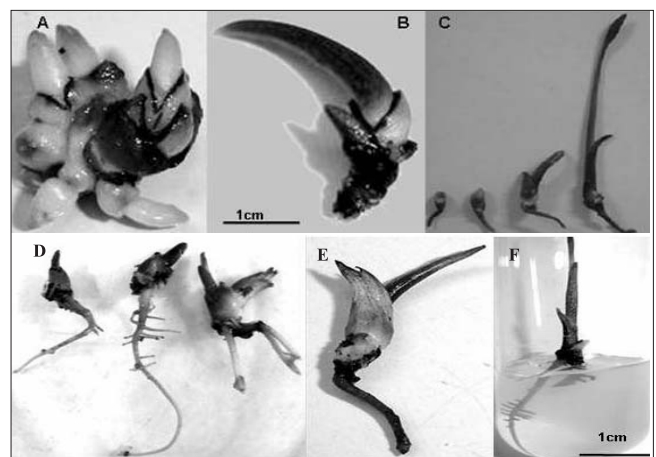


**Figure 1.** Histological analysis of the somatic embryos differentiation from adult pejibaye leaf primordium. A) Transversal section of the plant tissue from first subculture (second month of culture) showing oxidized epidermic cells (arrow 1) and mesophyll cells metabolically active and in division (arrow 2); B) Transversal section of the plant tissue from second subculture (third month of culture) showing mesophyll with the meristemoids or preembryonic complex formation (arrows); C) Transversal section of polarized somatic embryos separated from the original tissue from thirty subculture (fourth month of culture); D) Transversal section of polarized somatic embryos with explant tissue from thirty subculture (fourth month of culture). pd: distal polar region; pp: proximal polar region; lp: leaf primordium.

histological analyses, due to the polarization of the meristematic centers (meristemoids) and to the combined presence of auxin and cytokinin during the third subculture, which was the phase of greatest morphogenic response of the meristematic centers. Moreover, the auxin/cytokinin relation applied was adequate, so that cells had their embryogenic ability activated, and without need for auxinic shocks, that normally lead to the callus formation, an associated somaclonal variation occurrence.

Somatic embryos regenerated plants that presented in vitro normal growth, both in the aerial part and root system, comparable to the development of plants obtained from zygotic embryos (Figure 2 D, E and F). However, these plants hardly acclimatized to the field, and showed 12% of survival in soil after three months.

Adult pejibaye leaf primordium presents a high direct morphogenetic response capacity to the auxin and cytokinin treatment, which induces the development of



**Figure 2.** Somatic embryos and seedling developed from adult pejibaye leaf primordia. A) Embryos still adhered to the explant, with visible aerial part without roots (fifth month of culture); B) Somatic embryo separated from the original tissue, showing the aerial part formed and beginning of the root system formation (sixth month of culture); C) Development sequence of the somatic embryo (fifth to eighth month of culture); D) Somatic embryos separated from the original tissue, showing the different root system formed and beginning of the aerial part formation (sixth and seventh month of culture); E) Somatic embryo with similar development of the aerial part and root system (seventh month of culture); F) Pejibaye seedling regenerated through somatic embryogenesis induced in mesophyll parenchymatous cells of the adult plant leaf primordium (eighth month of culture).

somatic embryos regenerating normal plants, without the need for using auxin shock. Histological analyses showed that mesophyll parenchymatous cells originated small meristematic cell clusters (meristemoids or preembryonic complex formation), apparently induced by the presence of 7.1  $\mu$ M BAP in the two first subculture periods.

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