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HISTOLOGICAL STUDIES OF THE SOMATIC EMBRYOGENESIS PROCESS IN COCO CUMBÉ PALM (*Parajubea cocoides* BURRET), FROM CIGOTYC IMMATURES EMBRYOS

Estudios histológicos del proceso de embriogénesis somática en palma Coco Cumbé (*Parajubaea cocoides* Burret) a partir de embriones cigóticos inmaduros

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ABSTRACT. The aim of the present research was to determinate the type of tissue present in a sample obtained from an embryogenic callus of Coco Cumbé Palm, *Parajubaea cocoides* Burret and somatic embryos on globular stage. For this purpose, in order to the protocol established in the histotechnology analysis procedure manual of Patology Service from "Carlos Andrade Marín" Hospital (HCAM, according its acronyms in Spanish) was proceeded located in Quito, Pichincha, Ecuador. The results showed that the embryogenic calli are conformed by meristematic cells and although present cells with starch granules near to embryogenic cells, indicating embryos formation. In somatic embryos case, was verified that these were in globular stage, because of these showed a characteristic structure of the first development stage, named protoderm. Therefore was determined that calli present distinctive characteristics of embryogenic competence and viability of the somatic embryos.

Key words: calli, somatic embryos, plant histology, meristems

RESUMEN. El objetivo propuesto para este estudio fue determinar el tipo de tejido presente en una muestra obtenida de callos embriogénicos y de embriones somáticos en estado globular de Palma Coco Cumbé (*Parajubaea cocoides* Burret). Para esto se procedió según el protocolo establecido en el manual de procedimiento de análisis histotecnológico del Servicio de Patología del Hospital "Carlos Andrade Marín" (HCAM), ubicado en Quito, Pichincha, Ecuador. Los resultados mostraron que los callos embriogénicos están conformados por células meristemáticas y que además presentan células con gránulos de almidón, muy cercanas a las embriogénicas, indicador este, de la formación de embriones. En el caso de los embriones somáticos se comprobó que estos se encontraban en etapa globular, pues presentaban una estructura característica de la primera etapa de desarrollo embrionario denominada protoderma. Se determinó además que los callos presentan características distintivas de competencia embriogénica y viabilidad de los embriones somáticos.

Palabras clave: callos, embrión somático, histología vegetal, meristema

INTRODUCTION

Through the technique of plant tissue culture it is possible to massively propagate a wide variety of plants; however, there is now a greater interest in those plant species that have difficulties in reproducing, taking into account, in addition, their recalcitrance status, as well as the threat of extinction due to different factors (1). Such is the case of the Coco Cumbé palm (*Parajubaea cocoides* Burrett),a monoecious plant that forms a stipe,

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belonging to the family of the Aracaceae, native to the South American continent, used as ornamental in the cities of Ecuador, where it constitutes an emblematic and patrimonial species. The same is in a extinction state because of its reproductive difficulties and its maintenance requirements.

Currently in Ecuador, many efforts are made to rescue this species, through different multiplication techniques. The same have not been sufficient, since the reproduction of the initial material presents difficulties for different reasons; being essential to follow or trace the material propagated by different routes, such as embryogenic, because it is the most efficient; as well as for the maintenance of this species after having been obtained by its *in vitro* culture, so it is essential to carry out studies of its internal structures and thus to know its embryogenic viability. These analyzes are necessary to comply with an efficient micropropagation protocol, based on the microscopic observation of the structure of its tissues and cellular organs using techniques based on histological sections.

The histological studies provide a great contribution in the establishment of the sequence and dynamics of the vegetal material obtained *in vitro* and especially of the somatic embryogenesis process, where it is possible to better understand the morphogenic development and the structural and functional changes that occur during each of the stages that characterize the embryogenic structures (1, 2).

In performing the complementary tissue analyzes that make up the embryogenic structures of callus and somatic embryos, where zones with greater cell division activity are determined, as well as those with a high potential of explant response to the formation of embryogenic structures (3), only those that responded efficiently to the treatments and to the activity generated through plant growth regulators (PGRs) should be used.

Exogenous growth regulators are directly involved in the induction of embryogenic cells and in the formation of somatic embryos by modifying the cell polarity and interference of the pH gradients of the cytoplasm and cell wall of the plant material after an asymmetric division, which implies that the explant cells change their expression pattern and generate embryogenic structures.

Auxins are responsible for the reprogramming of gene expression, followed by a series of cellular divisions that induce both the disorganized growth to produce calli and the polarized growth leading to the formation of globular somatic embryos (4).

The knowledge and study of the histogenesis of the callus formed is important, because it allows establishing relations between regulator influences of the vegetal growth on the cellular organization of the fabrics that have these structures, to the normal tissue organization of a structure present in another type of plant organ (2).

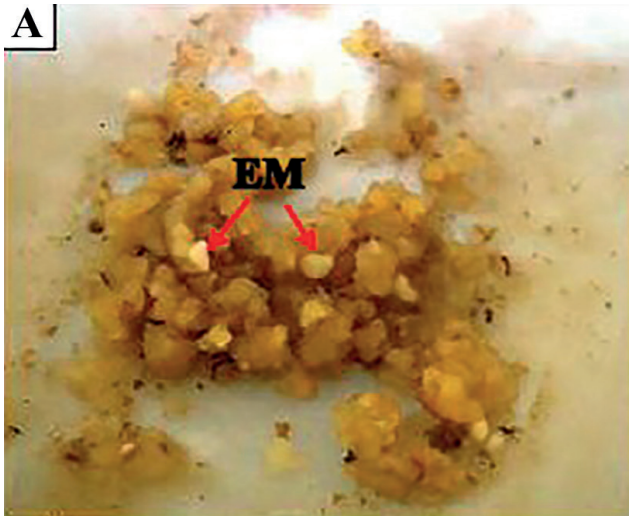
By performing histological sections, the viability of the cells present in tissues that form the embryogenic zones derived from the meristematic centers can be determined, the percentage of polarity and asymmetric division can be established and, above all, the formation of somatic embryos that will later represent a new plant, taking into account the similarity of these with the patterns that occur in zygotic embryogenesis (5). Based on these premises, the following objectives were set: to determine the different types of tissues present in a sample obtained from an embryogenic callus and the somatic embryos in the globular state, to establish their embryogenic viability in the production of seedlings of Coco Cumbé palm (*Parajubaea Cocoides* Burret).

MATERIALS AND METHODS

Histological analyzes were performed in the Laboratories of the Pathology Service of the Carlos Andrade Marín Hospital (HCAM, according its acronyms in Spanish), located in the sector of Miraflores Bajo, San Juan parish, Quito canton, Pichincha province, Ecuador. Samples of embryogenic calli and somatic embryos (Figure 1), which were maintained in a semi-solid culture medium containing salts and vitamins MS (6), modified with extra thiamine (1 mg L^{-1}), were taken as starting material (6 g L^{-1}) and different concentrations of indole acetic acid (AIA), benzylaminopurine (BAP) and kinetine (Kin) (1,0; 2,0 and 3,0 mg L^{-1}), which were obtained from zygotic embryos from the Coco Cumbé palm (*Parajubaea cocoides* Burret), Arecaceae native to Ecuador (5, 7), which is also called “coquito or cumbe” and it is very typical in the street landscapes of Ecuador cities (8).

For the accomplishment of this study the protocol established in the procedure manual of histological analysis of the Pathology Service of the same hospital, elaborated in the year 2015, was followed[A].

[A] Hospital «Carlos Andrade Marín». *Manual de Procedimiento de Análisis Histológico*. 2014, Quito, Ecuador, 131 p., Servicio de Patología del Hospital «Carlos Andrade Marín» (HCAM).



ME = mature embryos

Figure 1. Mature somatic calli and embryo of Coco Cumbé palm (*Parajubaea cocoides* Burret), at six weeks, in embryogenic culture medium

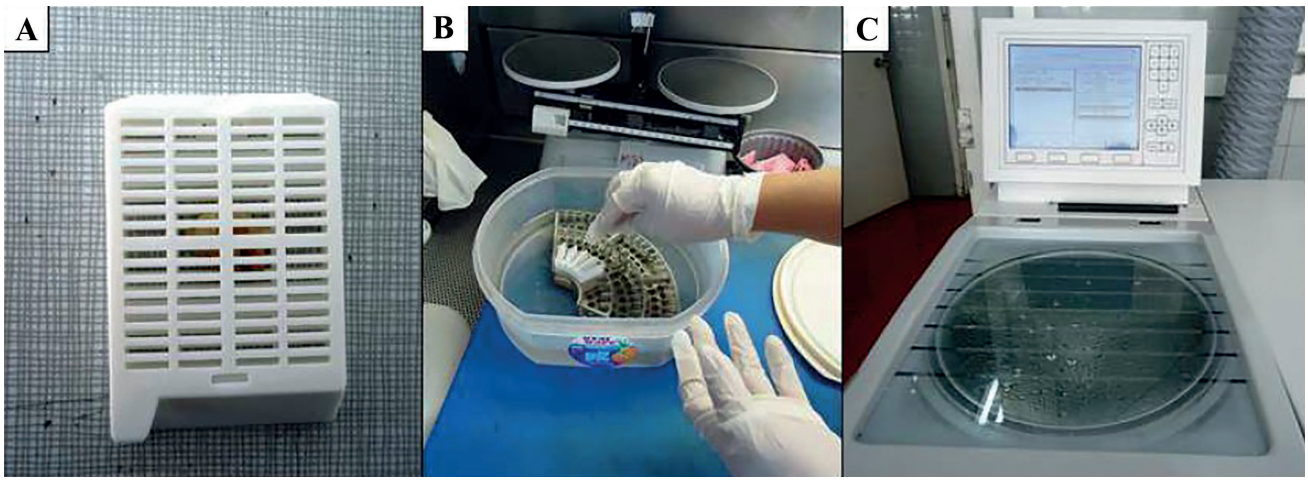
Firstly, explants were obtained from cuts of embryogenic calli, 1,5 x 1,5 cm and 3 mm thick. These samples were immersed in a fixative solution (10 % buffered formol, pH = 7) for two hours. They were then placed in the branded tissue processor (Thermo CIENTIFIC SHANDON PATHCENTRE) (Figure 2) and the steps set forth in the protocol in Table I were followed, where the samples were exposed to different concentrations of alcohol and xylol.

Table I. Protocol for processing tissue samples from embryogenic calli

Component	Time spent
Alcohol 75 %	1 hour
Alcohol 80 %	1 hour
Alcohol 90 %	1 hour
Alcohol 100 %	1 hour
Alcohol 100 %	1 hour
Alcohol 100 %	1 hour
Xylol 100 %	2 hours
Xylol 100 %	2 hours

Paraffin blocks were made in a brand dispenser (Thermo SCIENTIFIC, Histo Star), whose function is to expend this material through nozzles (Figure 3). Subsequently, the tissues found in these blocks were made with 3 µm of semi-fine sections, using a brand microtome (Thermo SCIENTIFIC SHANDON), then placed in water at a temperature of 40 °C; they were collected and spread on a sticky slide (Figure 4).

The samples were placed in a Dako Cover Stainer, for subsequent dewaxing, hydration and tissue staining (Figure 5). For this they were subjected to a temperature of 60 °C, followed by the steps shown in Table II. Once the samples were stained with methylene blue, photomicrographs were made using a fluorescence optical microscope (NIKON ECLIPSE 80i), which uses a fluorescent marker DAPI (4 ‘, 6-diamino-2-phenylindole) (9).



(A) Samples cut and placed in a basket, (B) samples submerged in a fixative solution, (C) samples placed in the tissue processor (Thermo CIENTIFIC SHANDON PATHCENTRE)

Figure 2. Samples from areas with meristematic cells of embryogenic calli of Coco Cumbé palm (*Parajubaea cocoides* Burret)

Figure 3. Paraffin dispenser (A) (Thermo SCIENTIFIC, Histo Star), (B) paraffin blocks cooling, (C) paraffin blocks with embryonic tissue

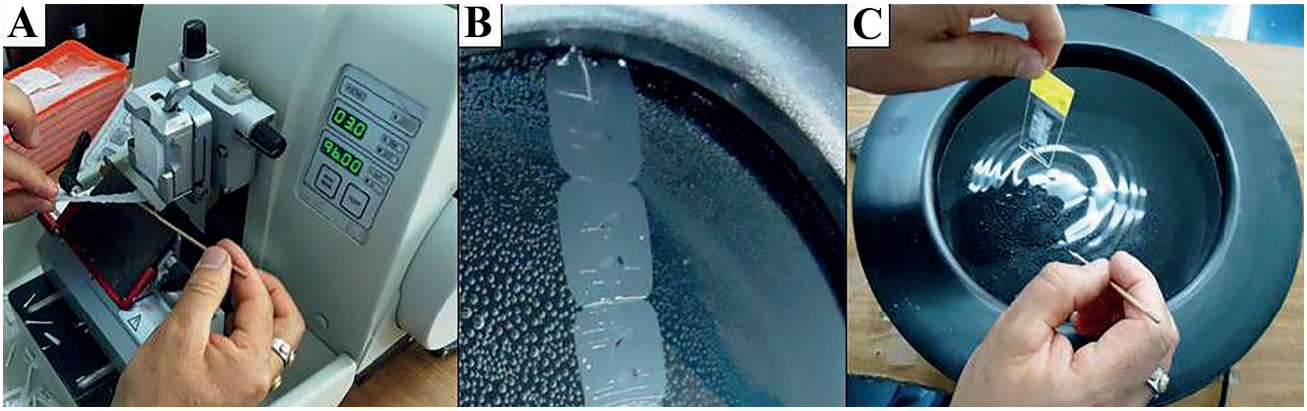
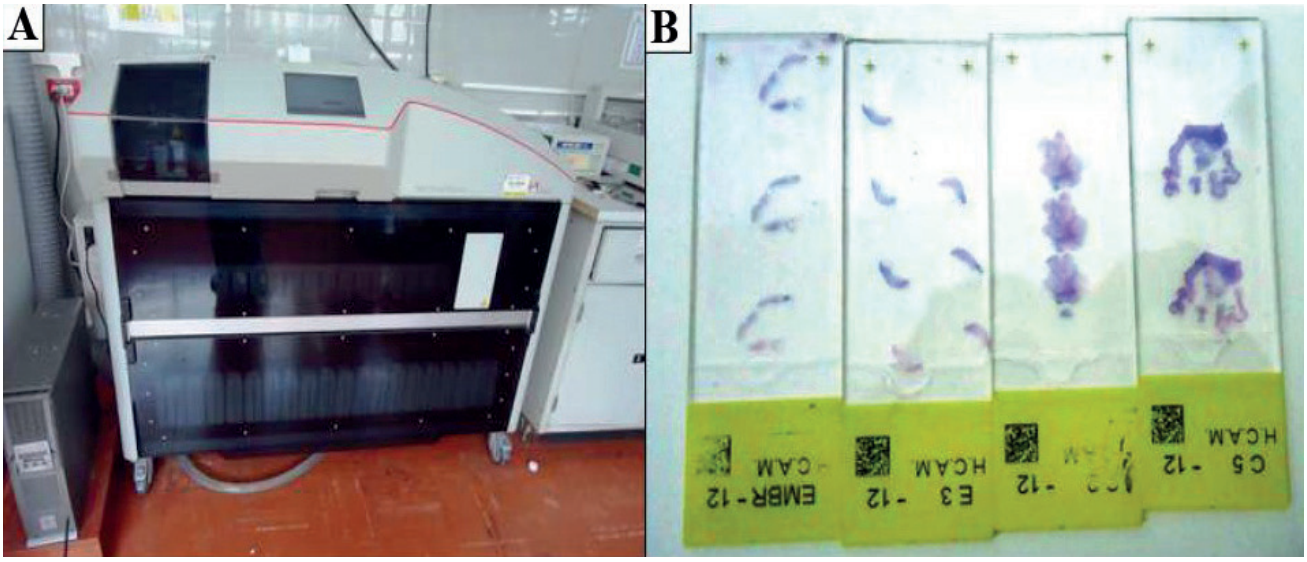


Figure 4. Obtaining cuts from embryogenic callus samples of Coco Cumbé palm (*Parajubaea cocoides* Burret). Using the microtome (A) (Thermo SCIENTIFIC SHANDON); (B) microcutting placed in warm water; (C) microcutting collected and spread on a sticky slide



(A) Dako Cover Stainer, (B) plates with samples stained with methylene blue

Figure 5. Dewaxing, hydration and staining of the tissue from embryogenic callus of Coco Cumbé palm (*Parajubaea cocoides* Burret)

Table II. Procedure for dewaxing, hydration and staining of tissue from embryogenic calli

Component	Time
Xylol 100 %	30 minutes
Xylol 100 %	30 minutes
Alcohol 100 %	30 minutes
Alcohol 100 %	30 minutes
Alcohol 90 %	30 minutes
Alcohol 90 %	30 minutes
Alcohol 70 %	30 minutes
H_2O destilled	30 minutes
H_2O destilled	30 minutes
Hematoxylin-eosin	3 minutes
Ammoniacal H_2O	2 seconds
Destilled H_2O	1 minute

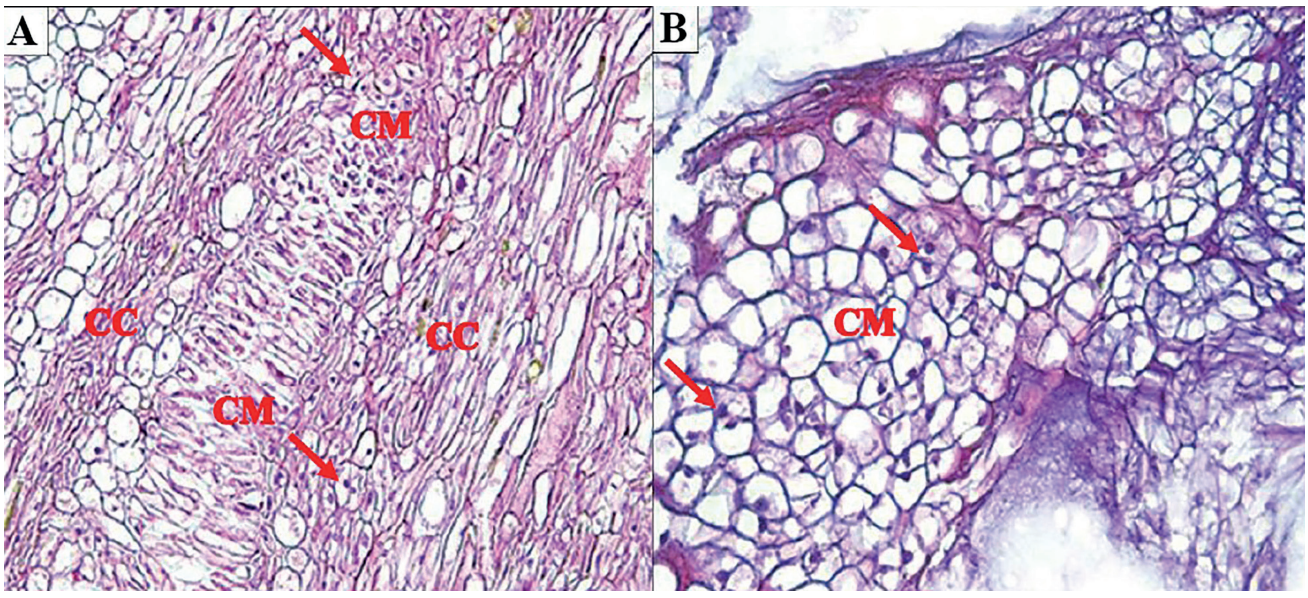
RESULTS AND DISCUSSION

The histological sections showed that the samples from the embryogenic callus of Coco Cumbé (*Parajubaea cocoides* Burret) had a very heterogeneous cellular composition, where a greater quantity of meristematic tissue was composed by isodiametric cells, with dense cytoplasm and a large dark nucleus, Characteristic of embryogenic calli and to a lesser extent vascular tissue was found (10). The meristematic tissue was found both in the inner and peripheral portions of callus, where mitotic activity was more intense and visible (Figure 6).

The histological characteristics found in embryogenic callus samples from this palm species show the presence of meristematic cells in different stages of division, which are specific to embryogenic structures found in localized sites, where it is performed greater meristematic activity of the cells. These results are corroborated by those described in other varieties of palms, such as *Acrocomia aculeata* (1), *Cocos nucifera* L. (9) and *Euterpe oleracea* (11).

The presence of conducting cells or tracheal elements could also be observed by using a fluorescent substance called (DAPI), which allows the staining of live and fixed cells, which may be close to the meristematic or embryogenic cells. In this way, the observation of small structures is accessed and facilitated, through the cell membrane (Figure 7).

Studies have been carried out on species such as *Acrocomia aculeata* (12) and *Bactris gasipaes* Kunth (13), through which it has been verified that these elements were formed from predetermined cells to later differentiate into xylem, taking into account that these cells are in more advanced stages of cell differentiation, where the auxin used (AIA) favorably influenced its maturation. This same situation favored the differentiation of the tracheal elements in embryogenic calli; this is due to the connection of these calli with the origin explant.



CM = meristematic cells CC = conductive cells

(A) histological cut of embryogenic callus of Coco Coconuts (Parajubaea cocoides Burret) 10x, (B) dividing cells with clearly visible nuclei 40x

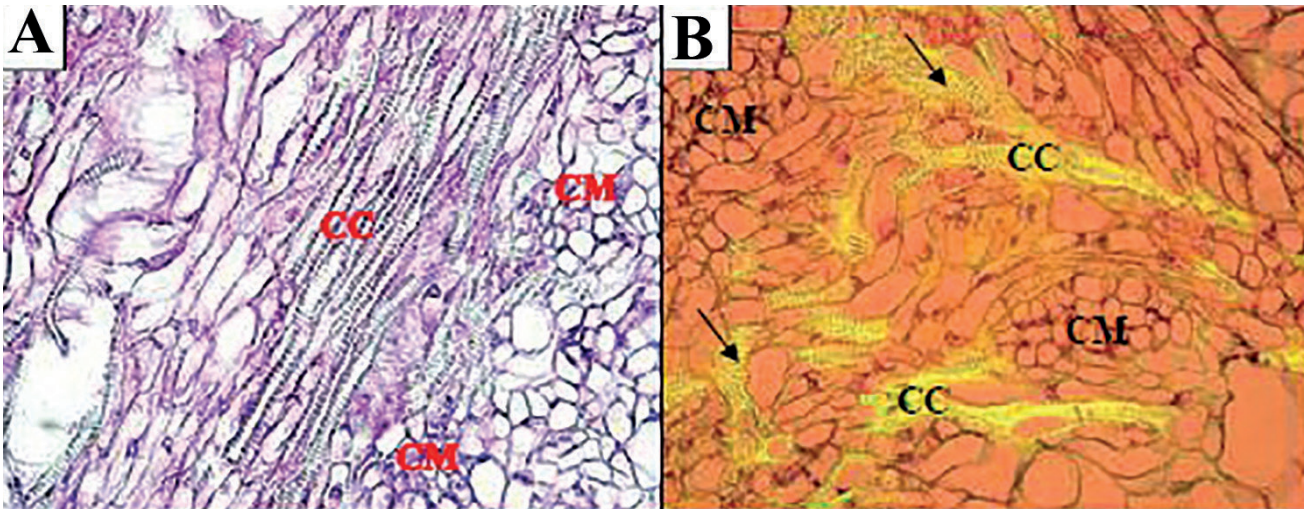
Figure 6. Cutting microphotographs

The culture conditions in which the elements described above were developed directly influenced cell differentiation, where the presence, concentration and activity of the growth regulators employed were determinant.

As shown in Figure 8, the meristematic cells present in some histological sections made in the embryogenic calluses of this plant species were forming zones, where a greater cell division or mitotic activity was carried out, which gives rise to a greater formation embryogenic tissue and somatic embryos.

Through the meristematic activity, it is shown that the embryos obtained were of multicellular origin; although a deeper and more complete histological study would be required where all the stages of formation of the embryogenic tissue, as well as of the somatic embryo, are involved.

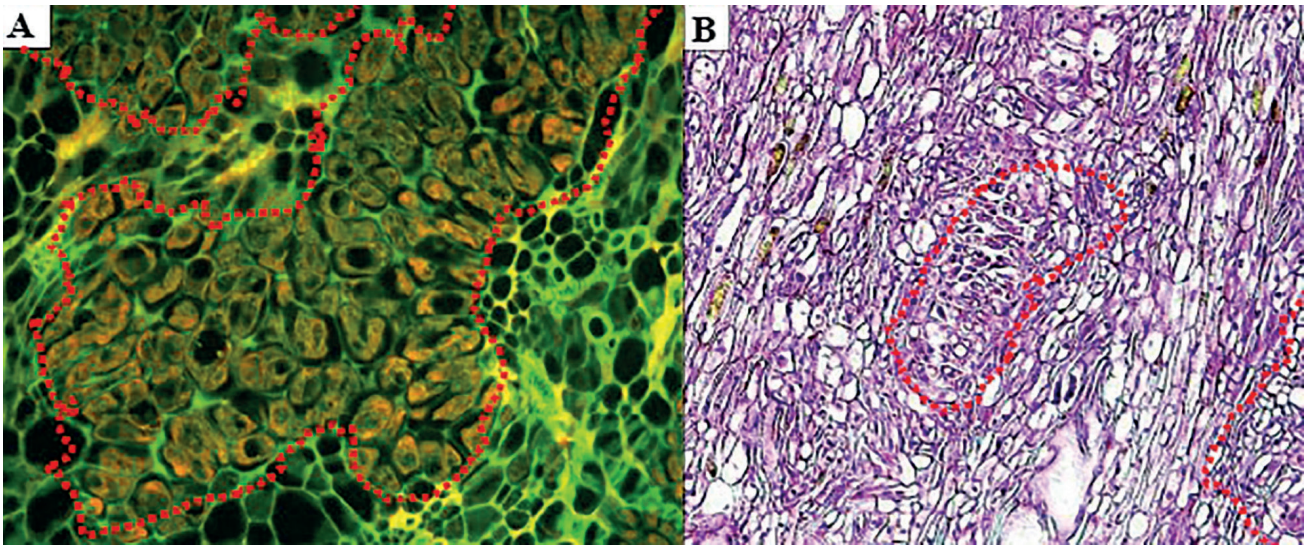
Previously, results have been obtained by other researchers, which provide information about the factors that affect the embryogenic process, from the process beginning, to the appearance of somatic embryos, either of unicellular or multicellular origin, that were taken into account in this study (12, 14).



CM = meristematic cells CC = conductive cells

(A) Light Micrograph, (B) Fluorescence Micrograph 10x

Figure 7. Micrograph of conductive cells close to meristematic cells of embryogenic calli of Coco Cumbé (*Parajubaea cocoides* Burret)



(A) areas of cells in fluorescence in constant cell division 40x, (B) zones of cells in cell division10x

Figure 8. Microphotograph of a histological section of embryogenic callus of Coco Cumbé (*Parajubaea cocoides* Burret)

In some cases the two types of origins can be given in the same callus, as indicated in the study carried out with the species *Euterpe oleracea* (15), where results were reported that, in addition to embryos of multicellular origin, were found embryos of unicellular origin, a situation that has been revalidated by means of the results in histological studies, using more sophisticated equipment and indicating the sequence of embryogenic structures in other palm species (12).

In addition, cells containing starch granules, which were located near the meristematic cells (16), were observed; these starch-containing cells had no embryogenic characteristics. The granules were more visible when using fluorescence techniques (Figure 9).

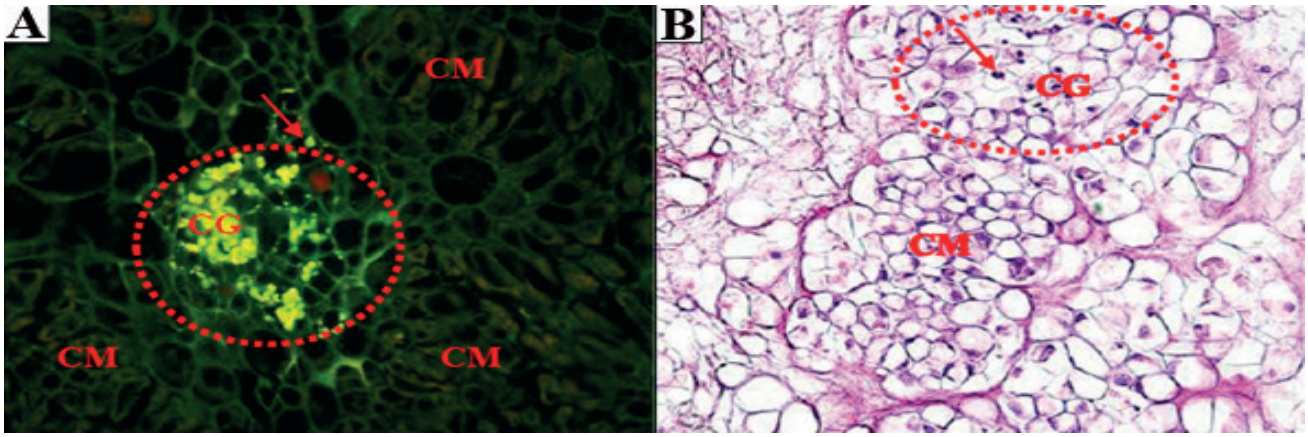
This fact was also observed in the palm *Acrocomia aculeata*, where it was indicated that the starch reserves in the callus occurred mainly in cells that did not exhibit embryogenic characteristics (12).

It was also pointed out that the presence of these reserve elements in cells adjacent to the embryogenic ones is due to a phenomenon that commonly indicates the acquisition of an embryogenic competence (17). However, the likely role of starch in the process of somatic embryogenesis is not yet clear, but it is believed that the starch storage may be related to the low mitotic activity of the cells containing it, since the embryogenic cells have higher mitotic activity when less is the presence of starch. However, a study in *Euterpe oleracea* (11) mentioned that both starch and other reserve substances increase in the cells with the consequent formation of somatic embryos.

As for the characterization of the somatic embryos, it was observed that these were in the globular state and had their own characteristics as their whitish coloration, defined and smooth rounded form, similar to a globe; constituted by meristematic cells, organized and distributed throughout their environment, similar to that found by other authors (17, 18). It was determined that the somatic embryo was in its first stage (globular stage), where tissue was observed as the protoderma of the embryo, which belongs to the outermost layer of the cells that are in constant division and that its presence Is more noticeable in the early stages of development of the embryo and will subsequently become the definitive epidermis of the plant (Figure 10). Through histological studies carried out on several species of palms such as *Bactris gasipaes* Kunth, *Acrocomia aculeata* and *Euterpe oleracea*, it was observed that the globular structures are formed by numerous spherical and organized cells, in addition to the presence of a well-defined protoderm as a basic characteristic of the first stage of embryonic development (11, 12, 14).

CONCLUSIONS

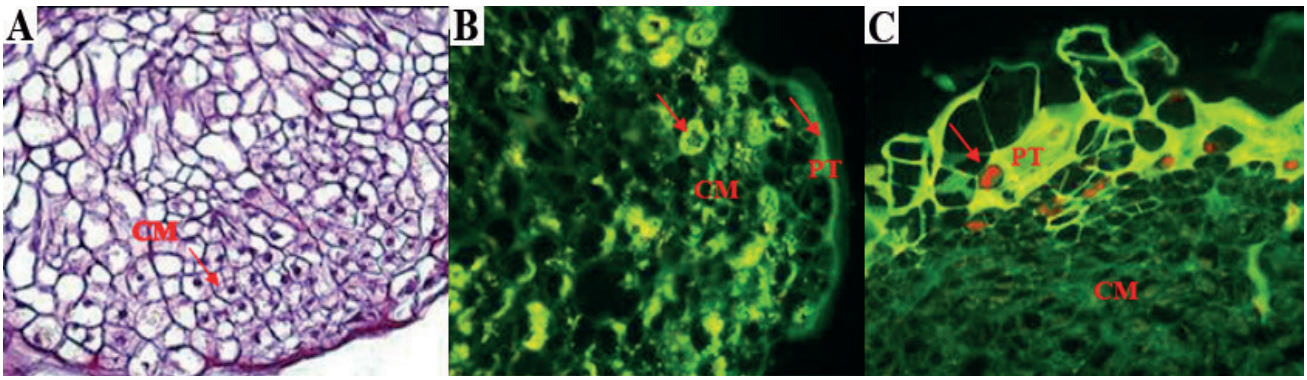
- The histological analysis of corns from the Coco Cumbé palm (*Parajubaea cocoides* Burret), confirmed that they were embryogenic, due to the presence of meristematic cells.
- The presence of starch granules in cells that were close to the embryogenic ones, implies the formation of somatic embryos.
- Embryos that were in the globular stage are characterized by having a well-defined layer of cells and in constant cell division, located in its periphery, denominated protoderm.



CM= meristematic cells CG= cells with starch granules

(A) (*Parajubaea cocoides* Burret) 40x, (B) non-embryogenic cells with starch granules, close to meristematic zones 40x

Figure 9. Cells with starch granules in fluorescence, close to meristematic zones of embryogenic calli of Palma Coco Cumbé



CM= meristematic cells PT= protoderm

(A) part of an embryo with organized meristematic cells 40x; (B) embryo in florescence with meristematic cells and protoderma 40x; (C) cell division in the protoderm and adjacent meristematic cells 100x

Figure 10. Microphotograph of a histological section of a somatic embryo in a globular state from Palma Coco Cumbé (*Parajubaea cocoides* Burret)

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