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Histological studies on induction of somatic embryogenesis in mangabeira

Estudos histológicos na indução de embriogênese somática em mangabeira

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ABSTRACT
The Hancornia speciosa Gomes (Apocynaceae), is a fruit tree known as mangabeira and officially listed as a genetic resource with food potential in Brazil. The aim of this investigation was to conduct histological studies on inducing maturation of somatic embryos from explants of Hancornia speciosa Gomes. For callus induction, leaf and nodal

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segments of the Terra Caída accession were cultured in media with different concentrations of putrescine (0, 250, 500, 750, and 1000 µM) in MS medium with 10 mg/L of 2,4D, 5 mg/L of BAP, 1 g/L of activated carbon, and 30 g/L of sucrose, and gelled with 3 g/L of Phytagel®. At 90 days of culture in multiplication medium, the embryogenic calluses were identified and selected for the maturation phase of somatic embryos in media composed of MS salts combined with two concentrations of BAP (10 and 15 mg/L) and two concentrations of polyethylene glycol – PEG 2000 (0% and 2%) with 30 g/L of sucrose, 1 g/L of activated carbon, and 4 g/L of Phytagel®. At 90 days of culture in multiplication medium (medium II) and at 60 days of culture in maturation medium (medium IV), the callus samples were fixed in FAA. After that, the samples were infiltrated in 100% ethanol + liquid resin (1:1) for 2 hours and then in activated resin = infiltration solution for 24 hours in the refrigerator. After drying, the slides were fixed with glass varnish and then evaluated in an optical microscope (Zeiss/Jenamed2), and photomicrographs (Axiocam software) were taken of the prepared slides. The results obtained in the different histological analyses of the Terra Caída accession of Hancornia speciosa Gomes are important to show the response of the nodal and leaf segments in inducing embryogenic calluses and possible maturation of somatic embryos, providing information that collaborates in validation of future protocols of somatic embryogenesis for this species.

Keywords: *Hancornia speciosa*, plant tissue culture, vegetal cell.

RESUMO

A Hancornia speciosa Gomes (Apocynaceae), árvore frutífera conhecida como mangabeira e oficialmente listada como recurso genético com potencial alimentar no Brasil. O objetivo desta investigação foi realizar estudos histológicos sobre a indução da maturação de embriões somáticos provenientes de explantes de Hancornia speciosa Gomes. Para indução de calos, segmentos foliares e nodais do acesso Terra Caída foram cultivados em meios com diferentes concentrações de putrescina (0, 250, 500, 750 e 1000 µM) em meio MS com 10 mg/L de 2,4D, 5 mg /L de BAP, 1 g/L de carvão ativado e 30 g/L de sacarose, e gelificado com 3 g/L de Phytagel®. Aos 90 dias de cultivo em meio de multiplicação, os calos embriogênicos foram identificados e selecionados para a fase de maturação dos embriões somáticos em meio composto por sais de MS combinados com duas concentrações de BAP (10 e 15 mg/L) e duas concentrações de polietilenoglicol – PEG 2000 (0% e 2%) com 30 g/L de sacarose, 1 g/L de carvão ativado e 4 g/L de Phytagel®. Aos 90 dias de cultivo em meio de multiplicação (meio II) e aos 60 dias de cultivo em meio de maturação (meio IV), as amostras de calos foram fixadas em FAA. Em seguida, as amostras foram infiltradas em etanol 100% + resina líquida (1:1) por 2 horas e depois em resina ativada = solução de infiltração por 24 horas na geladeira. Após a secagem, as lâminas foram fixadas com verniz vítreo e posteriormente avaliadas em microscópio óptico, sendo realizadas fotomicrografias das lâminas preparadas. Os resultados obtidos nas diferentes análises histológicas do acesso de mangabeira Terra Caída são importantes para mostrar a resposta dos segmentos nodais e foliares na indução de calos embriogênicos e possível maturação de embriões somáticos, fornecendo

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informações que colaboram na validação de futuros protocolos de embriogênese somática para esta espécie.

Palavras-chave: *Hancornia speciosa*, cultura de tecidos de plantas, célula vegetal.

1 INTRODUCTION

Brazilian flora is an excellent source of forest genetic resources, with potential for extraction activities practiced by local communities and applied in agroindustries, especially for food production. One of these important genetic resources is *Hancornia speciosa* Gomes (Apocynaceae), a fruit tree known as mangabeira and officially listed as a genetic resource with food potential in Brazil (NUNES et al., 2022). Popularly known as mangaba, the species presents a wide distribution in the Neotropics, being reported in Brazil, Paraguay, Bolivia and Peru (COLLEVATTI et al., 2018; FAJARDO et al., 2018). In Brazil, it can be seen from Amapá, in the North, to Paraná, in the South, in different phytophysiognomies associated with the Cerrado and the Atlantic Forest (SILVA et al., 2021).

The species is used in folk medicine and different parts of the plant have been used to treat various diseases, such as gastric ulcers and hypertension (DÓREA et al. 2021). The fruits are consumed in natura and used in the preparation of jellies, juices, cookies, ice cream, playing an important role for the local economy (COLLEVATTI et al., 2016; CHAVES et al., 2020).

The species does not have a large-scale propagation method; seed propagation is the most common method. Considering this aspect, plant tissue culture techniques have been successfully applied to numerous fruit and forest species. Somatic embryogenesis (SE) is the process through which the somatic embryo develops from a somatic cell, which may regenerate into a plant under appropriate conditions (GUAN et al., 2016).

This approach is considered ideal for mass clonal propagation and breeding, and it is also a very useful tool for cryostorage of germplasm. These advantages have led to intensive studies attempting to establish reproductive protocols for efficient production and maturation of somatic embryos (PAIS, 2019).

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Embryogenic callus cultures contain differentiated embryogenically competent cells that regenerate complete plants (EFFERTH, 2019). The embryogenic calluses are the friable calluses, of intense yellow color, reduced growth, and anatomical forms similar to those present in meristematic cells (SILVA et al., 2020). The somatic embryos, in turn, are comparable to the zygotic embryos and exhibit similar developmental stages – globular, heart-shaped, torpedo, and cotyledonary (GARCIA et al., 2019).

Studies have not yet been reported in the literature that show histological aspects about the steps for obtaining efficient protocols of SE for mangabeira. Thus, the aim of this investigation was to conduct histological studies on inducing maturation of somatic embryos from explants of *Hancornia speciosa* Gomes.

2 MATERIAL AND METHODS

For callus induction, leaf and nodal segments of the Terra Caída accession were cultured in media with different concentrations of putrescine (0, 250, 500, 750, and 1000 µM) in MS medium with 10 mg/L of 2,4D, 5 mg/L of BAP, 1 g/L of activated carbon, and 30 g/L of sucrose, and gelled with 3 g/L of Phytagel®. After 120 days, the explants were transferred to a multiplication medium composed of MS supplemented with 10 mg/L of 2,4D, 5 mg/L of BAP, 1 g/L of activated carbon, 30 g/L of sucrose, and 3 g/L of Phytagel®. At 90 days of culture in multiplication medium, the embryogenic calluses were identified and selected for the maturation phase of somatic embryos in media composed of MS salts combined with two concentrations of BAP (10 and 15 mg/L) and two concentrations of polyethylene glycol – PEG 2000 (0% and 2%) with 30 g/L of sucrose, 1 g/L of activated carbon, and 4 g/L of Phytagel®.

At 90 days of culture in multiplication medium (medium II) and at 60 days of culture in maturation medium (medium IV), the callus samples were fixed in FAA formaldehyde (CASTRO et al., 2009). The samples were transferred to 70%, 80%, 90%, and 100% alcohol at 1-hour intervals for ethanol dehydration. After that, the samples were infiltrated in 100% ethanol + liquid resin (1:1) for 2 hours and then in activated resin = infiltration solution (TECHNOVIT 7100) for 24 hours in the refrigerator. Soon

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afterwards, the inclusion solution was prepared, adding the hardener to the infiltration resin or activated resin (following the proportions recommended by the manufacturer).

The infiltrated plant fragments were placed in 6 × 8 mm histological molds of Leica®. The polymerization process occurred at ambient temperature. The blocks were kept in low humidity and stored in well-sealed containers containing silica gel. Having included the material, microtomy was performed using the semiautomatic rotary microtome Slee Mainz®. After placing the block, sections were made of 6-µm thickness; the sections on the slides were stained with toluidine blue (pH 4.8) and washed with distilled water. After drying, the slides were fixed with glass varnish and then evaluated in an optical microscope (Zeiss/Jenamed2), and photomicrographs (Axiocam software) were taken of the prepared slides.

Calluses were classified as embryogenic if they had anatomical forms similar to meristematic cells, with dense cytoplasm, thinner walls, a more evident large central nucleus and nucleoli, isodiametric cell shape, and small and fragmented vacuoles. The other calluses, with the presence of elongated parenchyma cells with wide intercellular spaces, not very evident nuclei, and well-developed vacuoles, were classified as non-embryogenic (BARTOS et al., 2018).

3 RESULTS AND DISCUSSION

The study of somatic embryogenesis through histological observations includes the use of various techniques that allow detailed analysis of morphogenetic events that occur when plants are cultured in vitro. It is possible to know and monitor the development of cells and tissues of the plant material during the different steps of the somatic embryogenesis process. It is also possible to identify the most responsive tissues and/or cells, as well as the embryogenic markers at the beginning of the process.

For histological analyses, calluses were selected with friable characteristics of the nodal and leaf segments of the TC accession at 60 days of culture in the multiplication medium. In the calluses originating from the nodal segments, different morphological structures were observed (Figure 1). In the histological studies, the round agglomerated cell (rac) type, characterized by scattered rounded cells or cells associated with cell

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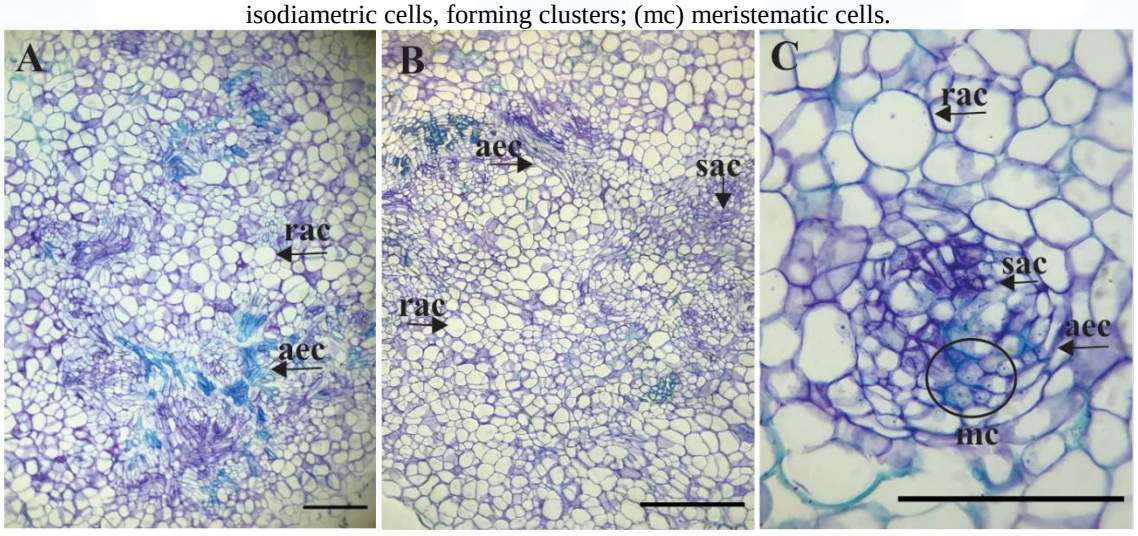
clusters; agglomerated elongated cells (aec), scattered elongated cells or cells associated with cell clusters; and small agglomerated cells (sac), formed by small isodiametric cells, forming clusters were detected. Cells in dedifferentiation were observed in all the histological sections analyzed. In some photomicrographs, the presence of meristematic cells (mc), cells with meristematic characteristics, can also be seen, characterized by an isodiametric shape, the presence of voluminous nuclei, visible nucleoli, and a dense cytoplasm with a high nucleus:cytoplasm ratio (Figure 1C). The presence of meristematic cells characterizes the callus as embryogenic, with the ability of dedifferentiating in an embryo.

Figure 1. Anatomical structures in callus cells originating from nodal segments of the mangabeira TC accession at 60 days of in vitro culture in MS medium supplemented with 10 mg/L of 2,4-D + 5 mg/L of BAP + 1 g/L of activated charcoal + 30 g/L of sucrose + 3 g/L of Phytagel®. Structure types (A-B: 10X objective, 50 µm scale; C: 40X objective, 100 µm scale): round agglomerated cells (rac), scattered rounded cells or cells associated with cell clusters; agglomerated elongated cells (aec), scattered elongated cells or cells associated with cell clusters; and small agglomerated cells (sac), small isodiametric cells, forming clusters; (mc) meristematic cells.



Source: Authors

In the calluses originating from leaf segments, rac, aec, and sac cells were also detected (Figure 2). According to Chiavegatto et al. (2015), the cells with rac and aec shape have greater probability of forming embryogenic calluses, as was shown in this study. The beginning of dedifferentiation of the cells in a globular structure could also be seen, as well as the formation of a structure similar to the cotyledonary somatic embryo

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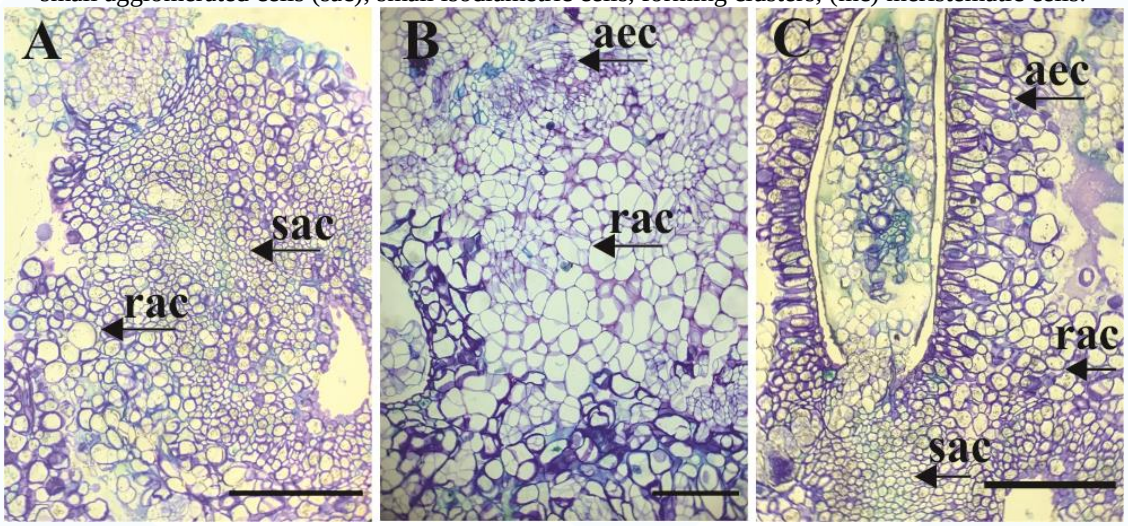
in development (Figure 2A-C). According to Meira et al. (2019), histological studies show characteristics of the existing cells, if they are elongated or rounded, if they have an expanded vacuole, and they show the presence of the cell matrix and intercellular spaces.

Figure 2. Anatomical structures in callus cells originating from leaf segments of the mangabeira accession Terra Caída - TC at 60 days of in vitro culture in MS medium with 10 mg/L of 2,4-D + 5 mg/L of BAP + 1 g/L of activated charcoal + 30 g/L of sucrose + 3 g/L of Phytagel®. Structure types (20X objective, 50 µm scale): round agglomerated cells (rac), scattered rounded cells or cells associated with cell clusters; agglomerated elongated cells (aec), scattered elongated cells or cells associated with cell clusters; and small agglomerated cells (sac), small isodiametric cells, forming clusters; (mc) meristematic cells.



Source: Authors

After 60 days of culture in maturation media, calluses of nodal segments with proembryos and embryos in maturation (Figure 3A) of the mangabeira TC accession were also selected for histological analyses. A greater presence of somatic embryos in the maturation stage could be seen in the culture media with the presence of 2% PEG. Thus, a compact callus with the presence of somatic embryos in maturation of white and beige color was selected to show the presence of the structures necessary for conversion into a somatic embryo. According to Zhao et al. (2022), in studies of morphohistological analysis of embryogenic calluses in *Pulsatilla tongk*angensis Y. N. Lee & T. C., in the globular stages, in the heart-shaped and torpedo form, the development of somatic embryos occurred from milky-white structures, characterized by deep coloring, with small cells with a rich cytoplasm and with very little vascular tissue in the embryos in development, as detected in the present study.

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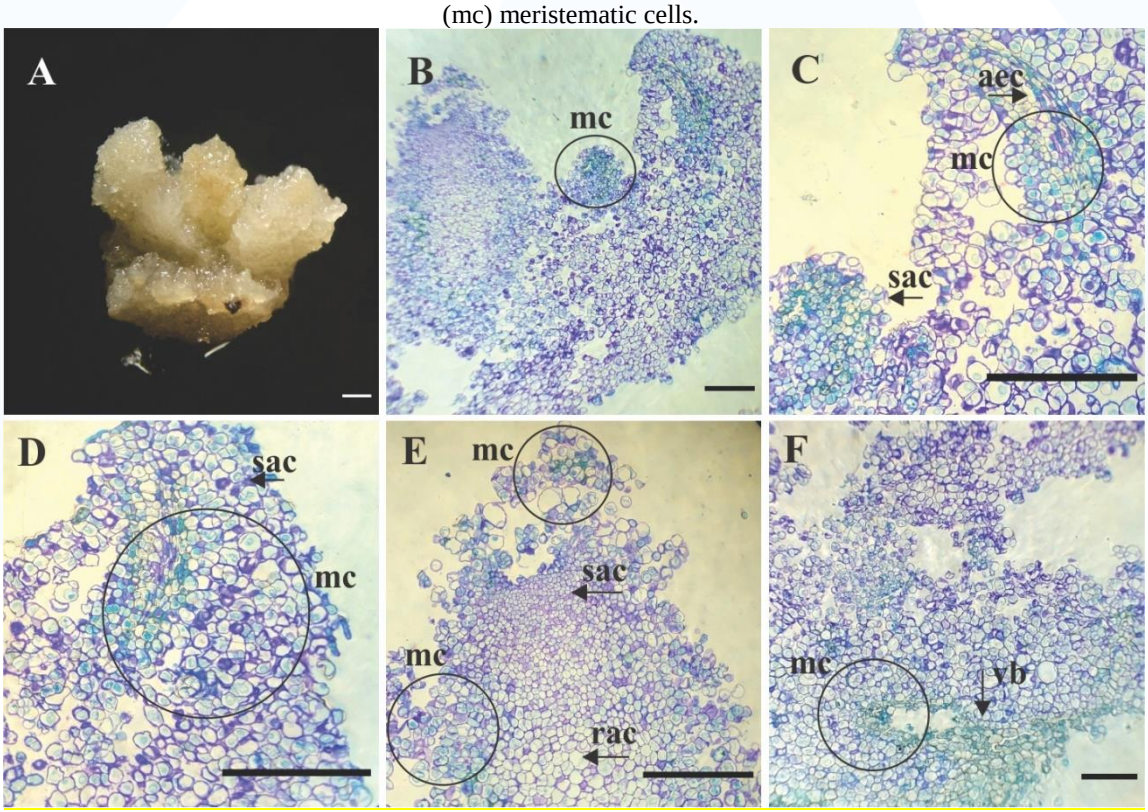
Cells considered meristematic, of small size and circular shape, with a well-defined nucleus and forming clusters, were observed in all parts of the embryo in maturation in the embryogenic callus (Figure 3). sibly in an additional more prolonged subculture.

Figure 3. Anatomical structures of calluses in nodal segments of the mangabeira accession Terra Caída - TC at 60 days of in vitro culture in MS medium supplemented with 15 mg/L of BAP + 2% PEG + 30 g/L of sucrose + 1 g/L of activated charcoal + 4 g/L of Phytagel®. Structure types (A: 4X objective, 10 µm scale; B-F: 10X objective, 50 µm scale; C-D-E: 20X objective, 50 µm scale): round agglomerated cells (rac), scattered rounded cells or cells associated with cell clusters; agglomerated elongated cells (aec), scattered elongated cells or cells associated with cell clusters; and small agglomerated cells (sac), small isodiametric cells, forming clusters; (vb) vascular bundle, structure of the vascular bundle of the stem; (mc) meristematic cells.



Source: Authors

This result corroborates the results obtained by Du et al. (2020), where embryogenic cells of *Paeonia* Sect. Moutan (tree peony) were small, suborbicular, well arranged, and with a prominent nucleus and dense cytoplasm, whereas the non-embryogenic cells were large, with irregular shape and with large vacuoles. According to Meira et al. (2019), when there is an advanced stage of embryonic development, it is still

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possible to observe characteristic elements, such as the ground meristem, procambium, protoderm, and vessel elements; which was not observed in the histological sections of this study. Nevertheless, it can be inferred that the embryo was in a stage of maturation and able to be converted into a somatic embryo pos

4 CONCLUSIONS

The results obtained in the different histological analyses of the Terra Caída accession of *Hancornia speciosa* Gomes are important to show the response of the nodal and leaf segments in inducing embryogenic calluses and possible maturation of somatic embryos, providing information that collaborates in validation of future protocols of somatic embryogenesis for this species.

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