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Induction and maturation of somatic embryogenesis in mangaba tree a multipurpose Apocynaceae

Indução e maturação de embriogênese somática em mangabeira, uma Apocynaceae de usos múltiplos

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This study reported the first results of *in vitro* somatic embryogenesis induction in *Hancornia speciosa* Gomes (mangaba tree, Apocynaceae). To induce callus, the TC accession leaf and nodal segments were cultivated in culture medium I (Murashige and Skoog; MS salts, putrescine, 45 μM 2,4-dichlorophenoxyacetic acid; 2,4-D and 22.19 μM 6-benzylaminopurine; BA). For the next stage (multiplication), the callus masses were sub-cultured into a culture medium II (MS salts, 22.62 μM 2,4-D and 22.19 μM BA). After 90 days, embryogenic calluses were inoculated to somatic embryo maturation medium culture III (MS salts, polyethylene glycol; PEG (0 and 2%) and BA (44.39 and 66.58 μM). Pro-embryos and somatic embryos in the maturation stage were assessed after 60 days. The rstatix package from R software with non-parametric tests ($p < 0.05$) were used to analysis data of experiments I and II. The Tukey's test ($p < 0.05$) was performed on the data of experiment III. To induce and multiply embryogenic callus, 45 μM 2,4-D and 22.19 μM BA were efficient. More pro- and somatic embryo development were seen in the nodal explant cultured in the presence of 44.39 and 66.58 μM BA and 2% PEG. Putrescine does not promote the embryogenic masses of callus derived from leaf explants in the TC accession.
Keywords: *Hancornia speciosa* Gomes, micropropagation, somatic embryo.

Este estudo relatou os primeiros resultados de indução de embriogênese somática *in vitro* em *Hancornia speciosa* Gomes (mangabeira, Apocynaceae). Para induzir calogênse, segmentos foliares e nodais do acesso TC foram cultivados em meio de cultura I (Murashige e Skoog; sais MS, putrescina, 45 µM de ácido 2,4-diclorofenoxiacético – 2,4D; 22,19 µM de 6-benzilaminopurina - BAP). Para a próxima etapa (multiplicação), as massas de calos foram subcultivadas em meio de cultura II (sais MS, 22,62 µM de 2,4-D e 22,19 µM de BAP). Após 90 dias, os calos embriogênicos foram inoculados em meio de cultura III de maturação embrionária somática (sais MS, polietilenoglicol; PEG (0 e 2%) e BAP (44,39 e 66,58 µM). Foram avaliados pró-embriões e embriões somáticos em fase de maturação após 60 dias. Para análise dos dados dos experimentos I e II foi utilizado o pacote rstatix do software R com testes não paramétricos ($p < 0,05$). O teste de Tukey ($p < 0,05$) foi realizado nos dados do experimento III. Para indução e multiplicação de calos embriogênicos, 45 µM de 2,4-D e 22,19 µM de BAP foram eficientes. O maior desenvolvimento pré-embrionário e somático foi observado nos explantes nodais cultivados na presença de 44,39 e 66,58 µM de BAP e 2% de PEG. A putrescina não promoveu as massas embriogênicas de calos derivados de explantes foliares no acesso TC.
Palavras-chave: *Hancornia speciosa* Gomes, micropropagação, embriogênese somática.

1. INTRODUCTION

This Brazilian species, *Hancornia speciosa* Gomes (Apocynaceace), is one of native species due the ecological and medicinal proprieties. Several studies point to this species with medicinal potential for diseases such as cancer [1, 2]. The species presents high risk of genetic erosion as it occurs naturally in areas with high human pressure. Commercial plantations are still few in the country, and in addition, many mangaba gatherers depend on native populations to sell their fruits [3], reinforcing their socioeconomic role in traditional communities.

Mangaba trees are mainly propagated by seeds, which promotes a variation in the characteristics of interest, considering cross-fertilization carried out by insects [4]. Therefore, the development of asexual multiplication processes is important; however there are no promising results for traditional methods such as cuttings and grafting [5, 6]. According to Chilukamarri et al. (2021) [7] new approaches for efficient propagation are demand of ever-increasing population. Plant tissue culture techniques, as micropropagation, can be a strategy for commercial production and improved and restoring of mangaba tree natural populations. The morphogenetic route by which a somatic cell develops into a somatic embryo is known as somatic embryogenesis (SE) and can regenerate into a plant [8].

In plant tissue cultures, micropropagation via SE can provide numerous benefits, including large-scale production, the improvement of commercially important plants, and the study of genetic and physiological changes related to the fate of a plant cell [9]. This approach is considered ideal for mass clonal propagation and breeding, and it is also a very useful tool for cryostorage of germplasm. One of the conditions for SE induction is the use of some type of stress (temperature, osmotic stress, injury, and others). A form of stress, whatever it may be, can promote an initial reaction that can leads to the dedifferentiation of embryogenically competent cells [10].

Several authors applied polyethylene glycol (PEG) for *in vitro* simulate of osmotic stress and stimulated the maturation of somatic embryos, how *Medicago truncatula*, *Abies alba* Mill, *Zea mays* L., *Passiflora edulis* Sims, *Vigna radiata* (L.) and *Abies nebrodensis* [11-16]. Endogenous levels of auxin and cytokinin are fundamental in regulation of somatic embryogenesis, and auxin is the main hormone able to control cell division and growth. Low levels can cause inhibition of the embryogenic capacity of the explant [17].

The results of SE protocols in mangaba tree are still lacking. The studies published in the literature refer only to initial stages of callus induction/growth and indirect organogenesis [18-20]. Thus, this work aimed to conduct studies of induction, multiplication and maturation of somatic embryogenesis in *Hancornia speciosa* Gomes in different *in vitro* conditions.

2. MATERIALS AND METHODS

2.1 Establishment of plantlets in vitro

Seeds were extracted from Terra Caída accession -TC ripe fruits collected at Active Germplasm Bank (BAG Mangaba) of Embrapa, Sergipe, Brazil. Following disinfestation, seeds were inoculated in a MS salts [21] medium, 30 g L^{-1} sucrose, and 3 g L^{-1} Phytagel™ (Sigma-Aldrich, USA). The culture medium's pH was controlled in 5.8. The growing room was used to store the cultures for 180 days at 25 ± 2°C in an indirect light and an average humidity of 70%, lighting time 12 h day^{-1}.

2.2 Induction and multiplication of embryogenic masses of callus

At 180 days of plantlet growth, leaf (0.5 cm^2) and nodal (1 cm length) explants were excised from aseptic plantlets of the TC accession. The explants were inoculated in Petri dishes of sterile polystyrene (50 × 10 mm) containing embryogenetic callus masses (EM) induction medium (culture medium I) composed by MS salts, putrescine hydrochloride (Sigma-Aldrich®, USA) at 0, 250, 500, 750, and 1000 μM; 45 μM 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich, USA); 22.19 μM 6-benzylaminopurine (BA; Sigma-Aldrich®, USA); 1 g L^{-1} activated charcoal (AC; Sigma-Aldrich®, USA), 30 g L^{-1} sucrose and 3 g L^{-1} Phytagel™. The abaxial surface of the leaf explants was placed in contact with the culture medium. The cultures were kept in the absence of light for 120 days at 25 ± 2°C and an average humidity of 70%. The ability to induce EM was recorded after this period. For multiplication stage, all the cultures of the previous step were sub-cultured into a multiplication medium (culture medium II) with MS salts, 45 μM 2,4-D, 22.19 μM BA, 1 g L^{-1} AC, 30 g L^{-1} sucrose, and 3 g L^{-1} Phytagel™. Using a Leica EZ4 magnifying glass, the EM were assessed after 90 days.

2.3 Maturation of somatic embryos

The EM from nodal explants that had previously been cultured in a medium II were transferred to a culture medium III for the purpose of maturation induction. This medium was composed by MS salts with 22.62 μM 2,4-D, 44.39 μM BA, 30 g L^{-1} sucrose, 1 g L^{-1} AC, and 3 g L^{-1} Phytagel^{TM}. The culture medium's pH was controlled in 5.8. The growing room was used to store the Petri dishes for 60 days at 25 ± 2°C in an indirect light and an average humidity of 70%.

After that period, the EM were sub-cultured into a medium for inducing maturation by hydric stress (culture medium IV): MS salts, BA (44.39 and 66.58 μM), PEG 4000 (Vetec, 1254): 0 and 2%, 30 g L^{-1} sucrose, 1 g L^{-1} AC, and 4 g L^{-1} Phytagel^{TM}. The same conditions for storage described before was used.

Pro-embryos and somatic embryos in the maturation stage were assessed after 30 and 60 days, using a Leica EZ4 magnifying glass. The tests were set up in a completely randomized design in a factorial 2×2 (BA x PEG) with five replications, and each experimental unit was composed by a Petri dish with five individual masses of callus.

2.4 Statistical analyses

The rstatix package [22] from R software [23] with non-parametric Kruskal Wallis and Mann-Whitney-Wilcoxon tests ($p < 0.05$) were used to analysis data of experiments I and II. The Tukey's test ($p < 0.05$) was performed on the data of experiment III.

3. RESULTS AND DISCUSSION

3.1. Induction and multiplication of embryogenic masses of callus (EM)

Masses of callus induction began at 40 days of culture. The putrescine associated with the type of explant was not significant for callus induction. Nevertheless, it is noteworthy that the greatest formation of calluses (100%) for both explants occurred in the absence of putrescine (Figure 1).

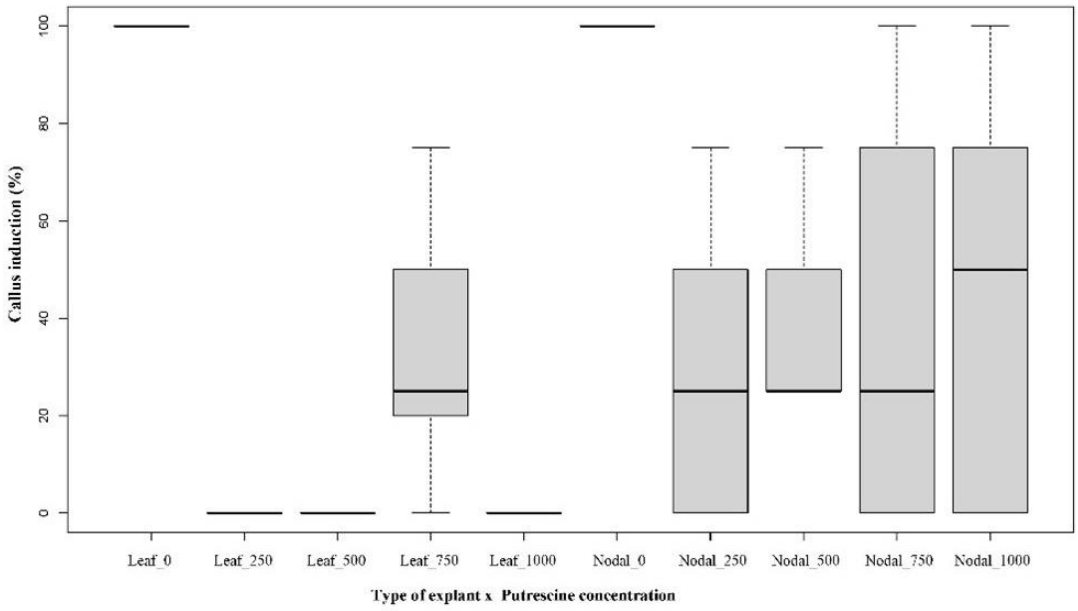

Figure 1: Effect of TC accession explants (leaf and nodal segments) and putrescine (0, 250, 500, 750, and 1000 µM) on callus induction (%) at 120 days of in vitro culture.

Putrescine is a type of polyamine whose beneficial effects are related to *in vitro* regeneration and SE. In addition, its promotional role in conversion of somatic embryos or regeneration has been confirmed in various species [24]. Based on that, it can be considered that the use of this polyamine might not have performed its role through being associated with the initial stage of SE, where only the induction of callogenesis occurred. Thus, the putrescine for *Hancornia speciosa* Gomes was not considered a determining factor for *in vitro* callus development. The induction media with the presence of 2,4-D and BA were more responsive in that step.

In addition, the callus induction and morphology depend on biotic and abiotic variables. The composition of the culture media, concentrations of plant growth regulators, sucrose, pH and the incubation conditions are reported according to Osman et al. (2016) [25]. In relation to the type of explant used, significant differences were detected in induction of callus of the TC accession. However, in the absence of putrescine, the nodal and leaf segments were not statistically different, and they led to greater development of masses of callus.

There was no difference in the percentage of callus development in leaf explants at the concentrations of 0 and 750 μM putrescine. However, both treatments differed from the results shown at the concentrations of 250, 500, and 1000 μM putrescine. Those concentrations showed no ability of callogenesis from the leaf explants, which show a highly sensitivity to polyamine in the culture medium compared to the nodal segments. In regard to this point, at all concentrations of putrescine, the nodal explant was responsive and induced masses of callus. The same response was observed for all the concentrations of putrescine studied, which did not differ from each other. In spite of the results obtained, the best responses of induction occurred in culture medium without putrescine, both for the nodal and leaf explants.

Thus, it can be inferred that auxins stimulate callus formation in nodal and leaf explants of this species. Its presence associated with cytokinin according to Slesak et al. (2017) [26] is linked to response of an *in vitro* tissue. The masses obtained during the 120 days of *in vitro* culture had translucent white or yellowish color, compact size, and sensitivity to touch. These characteristics are common in friable calluses and corroborate the results obtained in studies of indirect organogenesis of *H. speciosa*. Prudente et al. (2016) [18] reported that mangaba masses of callus cultured with BA and 2,4-D are friable and have white color initially. Silva et al. (2018) [27], in studies with *Caesalpinia ferrea* (Brazilian ironwood), verified that masses of callus coming from leaf and nodal explants had heterogeneous color, with areas of translucent, dark brown, and light or dark green colors.

With the results obtained in experiment I, the masses of callus were sub-cultured in a MS medium containing 2,4-D and BA. Over 60 days of culture, high proliferation and development was observed. In studies with indirect organogenesis in leaf explants of *H. speciosa*, Costa et al. (2022) [20] demonstrated that BA (4.44 μM) and NAA (10.74 μM), promoted greater induction on leaf explants as of the 60th day of *in vitro* culture. This result shows that the species may have late callus development. The scoring scale for evaluation of the development of these masses was established by the frequency observed with the aid of a Leica EZ4 magnifying glass. Thus, it was inferred that the concentrations of regulators used in the culture medium II were efficient for the masses growth. At 90 days of *in vitro* culture, the ability of the callus masses in producing EM was also evaluated. With the aid of a magnifying glass and a linked camera, visual observations were made of the characteristics of the clusters of embryogenic masses that were characterized by white, yellowish and/or greenish color, sometimes with reddish dots (similar to the color of the fruit), grainy texture, glassy appearance, and ease of dissociation in both types of explants. The callus translucent white or yellowish appearance is considered friable and with potential for forming somatic embryos [28].

The observations made led to the finding that the different growth regulators, as well as their concentrations, influenced the nodal and leaf callus dedifferentiation in an EM at 90 days of culture in multiplication medium II. However, there was no significant effect of the type of explant, which shows that both the leaf and the nodal explants of the TC accession are able to develop EM (Figure 2).

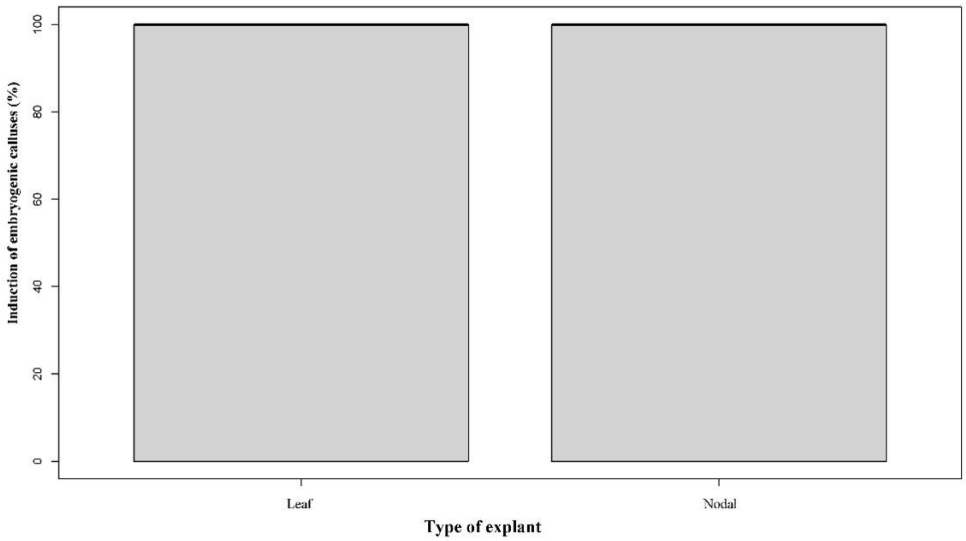

Figure 2: Effect of TC accession explants (nodal and leaf segments) on the induction of embryogenic calluses (%) at 120 days of in vitro culture.

Somatic embryos were observed in the globular, codiform and cotyledonary stages at the edges of the EM present on the nodal and leaf explants (Figure 3).

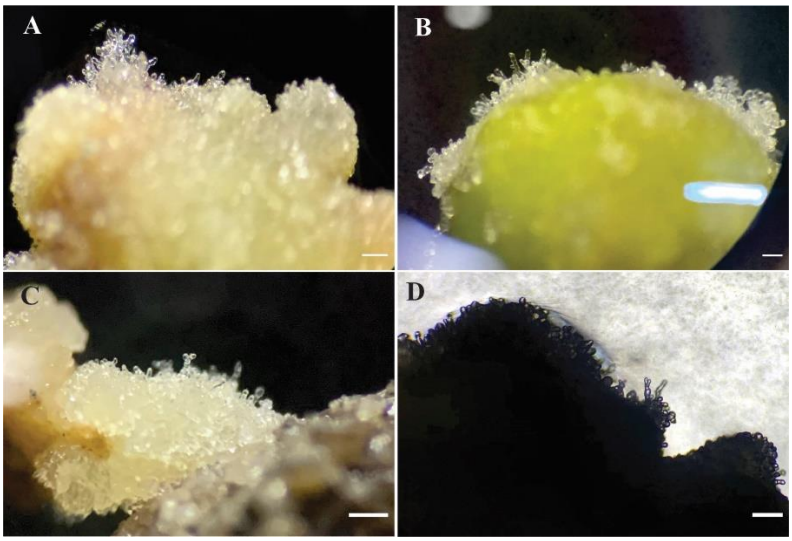

Figure 3: Masses of embryogenic calluses with globular, codiform and cotyledonary shape in explants of the TC accession at 120 days of in vitro culture. A- C: Calluses on nodal segment; B-D: Calluses on leaf segment. Bar= 10 μm scale.

These results may be related to the auxins in the multiplication medium, which play a critical role in morphogenesis process [29]. Mahendran and Bai (2016) [30] 2,4-D report that the high number of embryos, and its ability in activation of the embryogenic morphogenetic route may be related to its efficiency in inducing stress genes, contributing to cell reprogramming of the somatic cells in the direction of embryogenesis.

Nevertheless, the response to these growth regulators varies between species, as well as according to the classes, types, and concentrations of growth regulators used. Prudente et al. (2016) [18] observed that the combination between the endogenous concentration of phytohormones in an internodal segment of *H. speciosa* in the presence of 7.38 μM of 2,4-D stimulated the cell division of competent regions of the parenchyma, directing dedifferentiating

cells to form sprouts in callus formations through indirect organogenesis. In the studies of Bastos et al. (2007) [31], with the *in vitro* culture of cotyledons of mangaba tree plantlets without the presence of auxins and cytokinins, they did not obtain responses for the regeneration of microplants.

Just as for 2,4-D, the effectiveness of the presence of BA in culture media for initiating embryogenic calluses has also been found in diverse studies. Recent reports of Jouini et al. (2023) [16] indicate that mature zygotic embryos of *Abies nebrodensis* (Sicilian fir) cultured with cytokinin (BA) presented EC. The authors concluded that 10 weeks after the beginning of the culture, the highest rate of EC (5%) was observed for 4.43 μM of BA, followed by the combination of 4.43 μM of BA and 4.52 μM of 2,4-D (3.5%). That characterized the beginning of EC as dependent on BA, a cytokinin.

Considering the results obtained in the induction and multiplication tests, it can be inferred that this step provided important information on the components of the culture media and the response of the nodal and leaf explants of the TC accession for studies on induction of embryogenic callus (Figure 4).

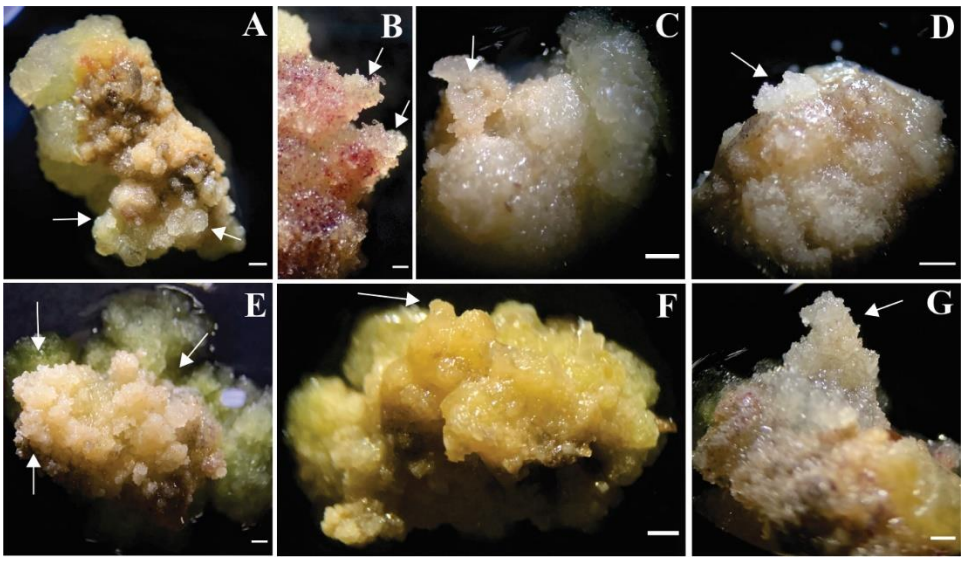

Figure 4: Embryogenic calluses, pro-embryos and somatic embryos induced in the TC accession. A-E: Embryogenic calluses and pro-embryos of beige color at 30 days of culture in MS medium supplemented with 66.58 μM BA and 2% PEG. B-G: Calluses with the presence of globular embryos with glassy appearance. C-D: Embryogenic calluses of beige color with the presence of a cotyledonary embryo in differentiation. F: Embryogenic calluses of beige color with the presence of a codiform embryo in differentiation. Bar= 10 μm scale.

3.2. Maturation of somatic embryos

For maturation of the TC accession somatic embryos, the cultures were first maintained in culture medium III (MS salts, 22.62 μM 2,4 D, 44.39 μM BA, 30 g L^{-1} sucrose, 1 g L^{-1} AC, and 3 g L^{-1} Phytagel™ The composition of this medium considered the adaptation of the cultures to a lower concentration of auxin (2,4-D) and greater concentration of cytokinin (BA) for beginning the maturation of the somatic embryos for 60 days. However, Arnold (2008) [32] reported that the 2,4-D is important in the initial stage of SE, while the development and maturation of the somatic embryo this auxin can be absence or in low levels.

Asghar et al. (2022) [17] reported that it is important for there to be a proportion between auxin and cytokinin considering their role on the cell differentiation or dedifferentiation. There was no significant effect on the BA (44.39 and 66.58 μM) × PEG (0 and 2%) interaction. The two concentrations of BA used also did not significantly affect the formation of pro-embryos and embryos in maturation stage (Table 1, Figure 5).

Table 1. Presence of proembryos and embryos in maturation in nodal explants of the mangabeira accession Terra Caída - TC in MS culture medium with different concentrations of BAP and PEG at 60 days of in vitro culture.

TREATMENT	CONCENTRATION	PROEMBRYOS AND EMBRYOS IN MATURATION
BAP	10 mg/L	56.25 A
	15 mg/L	57.50 A
PEG	0%	47.5 A
	2%	66.25 B

Mean values followed by the same uppercase letter in the column do not differ from each other by Tukey's test at 5% significance.

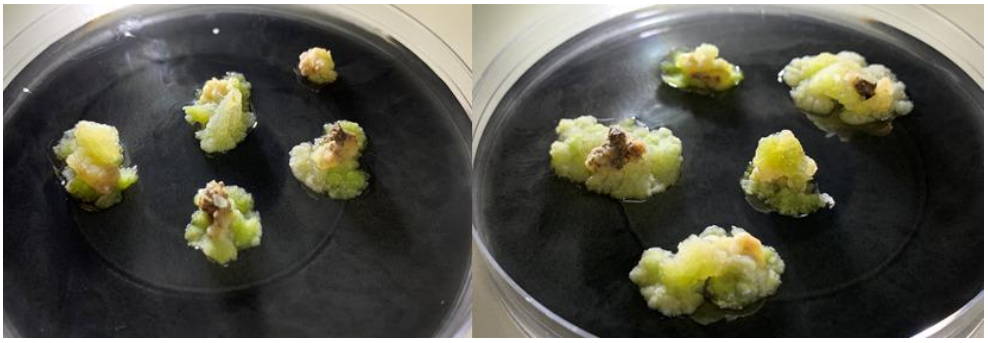

Figure 5: Masses of calluses with pro-embryo and somatic embryos induced on nodal segments of TC accession in the presence of BA. A- 44.39 μM and B- 66.58 μM at 30 days of in vitro culture

The presence of 2% PEG led to greater ability in induction of pro-embryos and embryos in maturation with potential for conversion into plantlets compared to the culture media without PEG. According to Abdel-Rahman et al. (2022) [13], the changes observed in *Zea mays* L. (maize) callus in the presence of 10 and 20% PEG suggest that PEG as a water regulator may be related to increase the protein metabolism and its related genes with the morphogenetic responses.

PEG has been applied to induce somatic embryo development. PEG at 6% promoted the maturation of a globular and cotyledonary somatic embryo stages of *Passiflora edulis Sims* (passion fruit) [14]. Its combination with growth regulators is also reported for maturation. Embryos of *Abies nebrodensis* (Sicilian fir) cultivated with 37.83 μM abscisic acid (ABA), 8% PEG, and 4% maltose have reached advanced stages of development [16]. Salaj et al. (2020) [12] established SE from immature zygotic embryos of *Abies alba* Mill. (European silver fir) upon observing the maturation of somatic embryos with ABA (37.84 μM), 7.5% PEG, and 3% maltose.

Somatic embryos in an advanced stage of maturation were not observed in the masses up to 60 days of culture in maturation medium. On other hand, globular, torpedo, and cotyledonary somatic embryos was detected. Oliveira and Aloufa (2022) [33] during the initial maturation stage of *Plinia cauliflora* (Brazilian grape tree) also obtained improved the torpedo and cotyledonary somatic embryos at PEG 3%.

The maturation and regeneration of somatic embryos are also depending by the concentration of nutrients of the MS medium and phenolic acids from oxidation process [34]. The osmotic stress and the presence of cytokinins are crucial to simulate the processes that naturally occur in embryonic development in seeds [35].

There is a scarcity of publications on the micropropagation of *H. speciosa* by somatic embryogenesis and there is a need to establish protocols for clonal propagation of the species. Considering this, the results obtained in the present work demonstrate the competence of leaf and nodal explants in following the morphogenetic route of somatic embryo production. It is

necessary to establish adjustments in the somatic embryo maturation stage for the regeneration of seedlings of this species.

4. CONCLUSIONS

The current study shows that somatic embryogenesis is promising for the micropropagation of *Hancornia speciosa*. However, putrescine does not induce embryogenic callus in leaf explants in the TC accession. The formation of embryogenic callus is promoted in MS medium with 45.25 μM 2,4-D and 22.19 μM BA. Nodal and leaf explants can develop somatic embryos. Embryo maturation is enhanced by 2% PEG and nodal callus exposed to 44.39 and 66.58 μM BA.

5. ACKNOWLEDGEMENTS

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