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Scientific Note

Establishing a callogenesis protocol aimed at obtaining somatic embryos in leaf explants of African mahogany[1]

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ABSTRACT

African mahogany (*Khaya grandifoliola*) is a forest species of considerable economic interest, whose clonal propagation may benefit from the development of efficient tissue culture protocols. The present study aimed to evaluate callus induction in African mahogany leaves using concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BA). The optimal response occurred at a 2,4-D concentration of 67.86 μM, with predominantly friable characteristics, concentrated mainly in the midrib region, and to a lesser extent along the lateral margins of the explant. The BA concentration of 11.31 μM, in turn, promoted the formation of more compact calli, with white coloration, also predominating in the midrib region, with lower occurrence on the lateral margins of the explant.

KEYWORDS: *Khaya grandifoliola*, *in vitro* regeneration, plant growth regulators, plant biotechnology.

RESUMO

Estabelecimento de um protocolo de calogênese visando à obtenção de embriões somáticos em explantes foliares de mogno africano

O mogno africano (*Khaya grandifoliola*) é uma espécie florestal de grande interesse econômico, cuja propagação clonal pode ser beneficiada pelo desenvolvimento de protocolos eficazes de cultura de tecidos. Objetivou-se avaliar a indução de calos em folhas de mogno africano por meio de concentrações de ácido 2,4-diclorofenoxiacético (2,4-D) e benziladenina (BA). A melhor resposta ocorreu na concentração de 67,86 μM de 2,4-D, com características mais friáveis, concentrando-se, predominantemente, na região da nervura central, e em menor intensidade nas laterais do explante. Já a concentração de 11,31 μM de BA promoveu a formação de calos mais compactos, de coloração branca, predominando também na nervura central e com menor ocorrência nas laterais do explante.

PALAVRAS-CHAVE: *Khaya grandifoliola*, regeneração *in vitro*, fitorreguladores, biotecnologia vegetal.

INTRODUCTION

Plant biotechnology has proven to be a crucial tool for propagating timber species, particularly those of high commercial value that are threatened by predatory exploitation (Grattapaglia & Kirst 2008).

Techniques such as plant tissue culture enable the clonal multiplication of superior genotypes, thereby producing seedlings with high genetic and phytosanitary quality (Grattapaglia & Kirst 2008).

African mahogany (*Khaya grandifoliola*), widely recognized for its high-quality and durable wood, is one of the most valued species in the timber trade, whose cultivation represents an attractive strategy, as it produces wood with desirable characteristics while reducing pressure on natural forests (Santos et al. 2019).

In Africa, its region of origin, the species is subject to excessive exploitation, leading to degradation of natural habitats and a consequent significant decline in its populations (Santos et al. 2019), making it urgent to implement effective propagation and conservation strategies.

Seed-based propagation in forest species faces challenges, including low germination rates, genetic variability, and high costs, particularly under adverse climatic conditions that compromise seed viability (Tiebel et al. 2023). Cutting propagation also presents

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limitations, including low rooting rates in tropical woody species, which require specific techniques to achieve successful propagation (Santos et al. 2011). In this context, the development of callogenesis protocols focused on producing somatic embryos from leaf explants represents an innovative and promising approach (Ribeiro et al. 2017).

The use of adult plant material as a source of explants is important for clonal propagation, as it allows the multiplication of superior genotypes already evaluated under field conditions (George et al. 2008). However, tissues from adult plants show greater lignification, reduced regenerative capacity, and accumulation of phenolic compounds, which can hinder callus induction and somatic embryo formation, thereby compromising process efficiency (Corredoira et al. 2019).

Callogenesis is characterized by the formation of callus from undifferentiated plant cells (Werner et al. 2009), which may subsequently differentiate into roots, shoots, or somatic embryos (Yusnita & Hapsoro 2011). Leaf explants are advantageous for callogenesis induction because they are readily available and exhibit a high regenerative capacity (Silva et al. 2020). The efficiency of callus induction depends on the composition of the culture medium, growth regulator concentrations, and environmental conditions (Golle et al. 2020). In this context, auxins and cytokinins are the plant growth regulators most widely employed to stimulate cell division and initiate the callogenic process (Huang et al. 2018), and determining their optimal concentrations is critical to maximizing the morphogenic response of leaf explants (Azevedo et al. 2021). Somatic embryogenesis constitutes a valuable alternative to conventional sexual propagation, allowing the rapid production of genetically uniform seedlings for commercial purposes (Maciel et al. 2003) and ensuring greater genetic homogeneity among the produced plants (Santos et al. 2017). Furthermore, the successful implementation of a callogenesis protocol for *Khaya grandifoliola* could contribute to the sustainable management of this species by enabling *in vitro* propagation under controlled conditions, thereby minimizing the risk of diseases and pests that affect conventional plantations (Gonzalez et al. 2012).

Despite advances in callogenesis and somatic embryogenesis induction techniques, several challenges remain to be addressed, including low induction efficiency, genotype-dependent responses, and difficulties in obtaining embryogenic tissues. Moreover, variation in explant responses to *in vitro* conditions requires careful attention due to differences in physiological status and morphogenic competence. For this reason, adapting methodologies to the specific physiological and genetic characteristics of African mahogany (*Khaya grandifoliola*) is essential to ensure consistent results (George et al. 2008). Accordingly, the present study aimed to establish an effective protocol for callogenesis induction to support somatic embryo production from leaf explants of *Khaya grandifoliola*. The research focused on optimizing culture conditions, including medium composition and hormone concentrations, to maximize callus formation rates (Moura et al. 2001).

The study was performed between March 2024 and February 2025 at the Universidade Federal de São João Del-Rei, in Sete Lagoas, Minas Gerais state, Brazil.

The experimental stages included preparation of culture medium, disinfestation, explant inoculation, *in vitro* culture, and morphological and anatomical analyses.

African mahogany leaf explants (*Khaya grandifoliola*) were obtained from seedlings originating from the Fazenda Origem (Figure 1). The plant material was washed under running water with detergent, disinfected in 70 % ethanol for 1 min, followed by 5 % sodium hypochlorite for 20 min, and rinsed three times in sterile distilled water. After being dried on autoclaved paper, the explants were immediately inoculated.

The inoculation was carried out in a laminar flow cabinet, with explants positioned so that the

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Figure 1. Appearance of the seedlings and leaves of *Khaya grandifoliola* used in the experiment. A) Seedlings maintained in a nursery under controlled irrigation; B) disinfested leaves used to obtain leaf explants.

abaxial surface was in contact with the culture medium. Three explants per flask were used and maintained in a growth room in darkness at 25 ± 2 ºC.

The basal culture medium consisted of Murashige & Skoog (MS) formulation, supplemented with 30 g L^{-1} of sucrose and 5.6 g L^{-1} of agar. Different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BA) were tested. The pH was adjusted to 5.7, and the medium was autoclaved at 120 ºC, for 20 min, with 30 mL distributed per flask.

The experimental design was completely randomized, comprising 10 treatments, three replications, and three explants per experimental unit. The explants (1 cm in diameter) were excised from the midrib region. The evaluated variables were callus formation, texture, fresh mass, and embryogenic characteristics, which were monitored over 30 days.

Treatments T1-T5 consisted of MS medium supplemented with concentrations of 2,4-D (11.31, 22.62, 45.24, 67.86, and 90.48 µM), whereas treatments T6-T10 consisted of MS medium supplemented with concentrations of BA (11.31, 22.62, 45.24, 67.86, and 90.48 µM).

Data were subjected to the Shapiro-Wilk and Bartlett tests, followed by analysis of variance (Anova) at 5 % of significance. The mean was adjusted by regression analysis using the R software (v. 3.0.1), with the ExpDes.pt package (Ferreira et al. 2013).

Callus formation was quantitatively assessed by the number of responsive explants in each treatment, by direct observation or with the aid of a stereoscopic magnifying glass. Callus texture was classified at 30 days as friable or compact, based on visual and tactile characteristics, with the coloration and structural appearance also recorded through images obtained under a stereomicroscope.

Furthermore, the calli obtained from the most efficient treatments were subcultured every 30 days to maintain growth and induce embryogenic characteristics, with weekly monitoring for morphological alterations.

For anatomical analysis, calli demonstrating embryogenic characteristics were fixed in 70 % FAA for 3 days, transferred to 70 % ethanol, and dehydrated through a series of graded ethanol solutions. Subsequently, they were infiltrated and embedded in historesin (Leica®) and sectioned (3-5 µm) using a rotary microtome (Zeiss®, HYRAX M 55). Slides were stained with toluidine blue (0.1 %) and analyzed under light microscopy to assess cellular characteristics associated with somatic embryogenesis.

Callus induction was observed in both 2,4-D and BA treatments, with evident morphological differences between calli formed under each condition. Calli induced with 2,4-D displayed a whitish to yellowish coloration and friable texture, characteristics frequently associated with calli bearing embryogenic potential (Silva et al. 2014). In contrast, the BA-derived calli had a more compact texture and a brownish coloration, which does not correspond to the typical characteristics of embryogenic calli (Garcia et al. 2019).

These differences reflect the specific mode of action of each growth regulator. The synthetic auxin 2,4-D is widely recognized as promoting intense and disorganized cell divisions, thereby favoring the formation of friable calli with high potential for somatic embryogenesis (Garcia et al. 2019). On the contrary, BA, a cytokinin, tends to induce calli with greater predisposition to organogenesis (Garcia et al. 2019).

Similar results have been reported in *Kalanchoe pinnata*, where the application of 4.52 µM of 2,4-D combined with 8.88 µM of BA resulted in 91 % of callus induction and surface coverage of 50-100 % of the leaf area, representing the most efficient protocol (Santos et al. 2014). The combination of 9.06 µM of 2,4-D and 8.88 µM of BA produced 100 % of induction, but with leaf coverage limited to 25 % (Santos et al. 2014). In *Eugenia involucrata*, leaf discs subjected to 5 µM of 2,4-D and 5 µM of BA under dark conditions exhibited a high callogenesis frequency (Golle et al. 2020).

The concentration of 67.86 µM of 2,4-D resulted in 100 % of leaf explants forming callus, with a friable texture consistent with embryogenic potential (Figure 2). Callus formation was concentrated predominantly in the midrib region of the explant, with more discrete occurrence along the lateral margins during the first 30 days. At 60 days after inoculation, callus formation was also observed in other regions of the explant (Figure 3). According to Silva et al. (2003), 2,4-D is widely recognized as one of the most effective auxins for inducing friable callus in *in vitro* cultures, owing to its ability to stimulate dedifferentiation and cell proliferation in plant tissues.

The predominance of friable calli in the midrib region suggests that the concentration of 67.86 µM of 2,4-D favored the induction of tissues with characteristics that may be associated with somatic embryogenesis, as indicated by Magalhães et al. (2006), who noted that embryogenic calli generally present a looser texture and lighter coloration, in contrast to nonembryogenic calli, which tend to be more compact and opaquer.

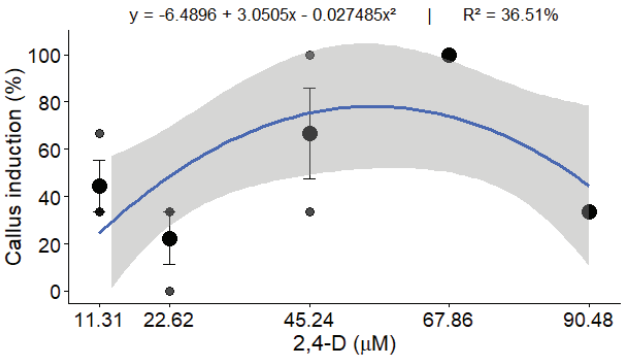

Figure 2. Polynomial quadratic regression model for the percentage of callogenic response in *Khaya grandifoliola* leaf explants as a function of treatments with concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) after 30 days of *in vitro* culture on Murashige & Skoog (MS) medium. Each point represents the mean percentage of explants showing callus formation obtained from three replicates with three explants per experimental unit. The highest callogenic response (100 %) was obtained at 67.86 µM of 2,4-D.

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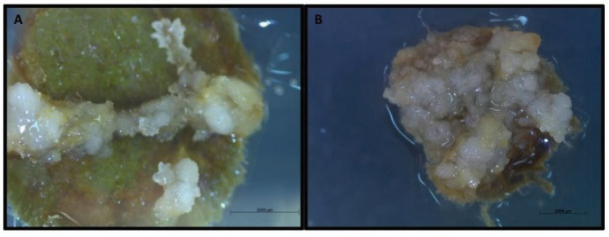

Figure 3. Appearance of callogenic masses formed during the *in vitro* culture of *Khaya grandifoliola* leaf explants on Murashige & Skoog (MS) culture medium containing 67.86 µM of 2,4-dichlorophenoxyacetic acid (2,4-D) and maintained in the dark at 25 ± 2 ºC. A) Friable callogenic mass with embryogenic characteristics formed predominantly in the midrib region after 30 days of culture; B) expansion of the friable callogenic mass throughout the leaf explant after 60 days of culture, indicating progressive callus development.

Additionally, callus formation in the midrib region may indicate a hormonal redistribution that favors cell division in different areas of the explant, as reported by Werner et al. (2009), who found comparable patterns in callus induction from leaflet discs of Brazilian rosewood (*Caesalpinia echinata*) treated with concentrations of 2,4-D.

Gomes-Copeland et al. (2017) also reported consistent results on callus induction in *Poincianella pyramidalis*, demonstrating that 2,4-D application can promote callus formation from different types of explants. The concentration of 4.91 mg L^{-1} of 2,4-D was effective in inducing leaf calli after 30 days of *in vitro* culture, indicating the potential of this regulator for micropropagation protocols.

Regarding the results obtained with BA at different concentrations, the best callogenic response was observed at 11.31 µM, with 100 % of leaf explants forming compact white callus (Figure 4). These calli were predominantly concentrated in the midrib region, with a lower occurrence at the lateral margins of the explant during the first 30 days. At 60 days after inoculation, callus formation was also observed in other regions of the explant (Figure 5). As noted by Botin & Carvalho (2015), cytokinins such as BA are widely used in *in vitro* studies for their ability to stimulate cell division, thus facilitating callus formation. At the 11.31 µM concentration, the formation of compact white calli suggests that

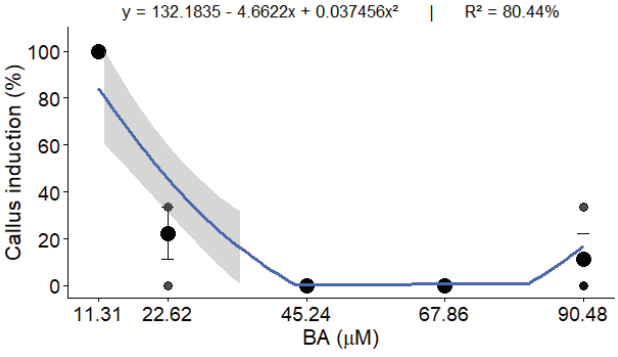

Figure 4. Polynomial quadratic regression model for the percentage of callogenic response in *Khaya grandifoliola* leaf explants as a function of treatments with concentrations of 6-benzylaminopurine (BA) after 30 days of *in vitro* culture on Murashige & Skoog (MS) medium. Each point represents the mean percentage of explants showing callus formation obtained from three replicates with three explants per experimental unit. The highest callogenic response (100 %) was obtained at 11.31 µM of BA.

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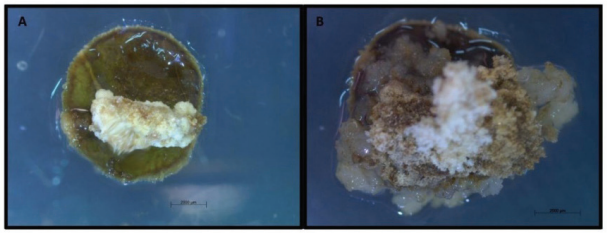

Figure 5. Appearance of callogenic masses formed during the *in vitro* culture of *Khaya grandifoliola* leaf explants on Murashige & Skoog (MS) culture medium containing 11.31 µM of 6-benzylaminopurine (BA) and maintained in the dark at 25 ± 2 ºC. A) Compact white callogenic mass without embryogenic characteristics formed predominantly in the midrib region after 30 days of culture; B) expansion of the compact callogenic mass throughout the leaf explant after 60 days of culture, indicating continued callus development without embryogenic characteristics.

the hormone induced a cellular response resulting in dense tissues, characteristic of calli that, although less friable, are nonetheless indicative of good developmental potential.

The white coloration of the formed calli, combined with their density, may indicate that these tissues are not fully differentiated, or that they lack embryogenic characteristics, as described by Carvalho Filho et al. (2023), who emphasized that calli of this nature generally present a firm and opaque texture, devoid of the typical signs of embryogenic calli, which are lighter and less compact. The predominant formation of these calli in the midrib region suggests that BA-induced callogenesis may have been more effective in areas with the highest hormone concentration, hence stimulating cell division more intensely there.

On the other hand, reduced callus formation along the lateral margins of the explant may indicate uneven hormone distribution, resulting in a higher concentration in the central region. This pattern may be relevant for optimizing the use of BA in induction protocols, maximizing cellular development in the central area of the explant. A similar study conducted by Ibrahim & Al-Nema (2023) also found significant callus induction in *Jatropha curcas* leaf explants, highlighting that the application of 0.5 mg L^{-1} of BA was imperative for effective callus induction, achieving a response of up to 80 %.

Huang et al. (2018) also reported consistent results for callus induction in *Rosa hybrida* leaf explants, demonstrating that the application of cytokinins such as BA can elicit varied responses in callus and adventitious bud formation, depending on the concentration used and its interactions with other regulators. Consequently, the use of 1.0 mg L^{-1} of BA combined with 3.0 mg L^{-1} of 2,4-D proved promising for callus induction at 100 %, and may be explored in future *in vitro* applications.

The anatomical characterization of calli formed from *Khaya grandifoliola* leaf explants was performed exclusively for calli induced in medium containing 2,4-D, since they demonstrated a macroscopic morphology compatible with embryogenic characteristics. Calli formed in the presence of BA were not subjected to anatomical analysis, as they showed no visual evidence of the typical cellular organization of embryogenic tissues.

In calli induced with 2,4-D, histological evaluation revealed friable cellular masses composed of small and isodiametric cells, with prominent nuclei and relatively uniform cellular organization (Figure 6). These attributes are considered indicative of intense metabolic activity and embryogenic potential, according to criteria described in the literature (Garcia et al. 2019). The presence of prominent nuclei and the absence of extensive vacuolization confirm cell viability and the possibility of progression to somatic embryogenesis (Silva et al. 2014).

The observed friable texture and cell organization reinforce previous descriptions of embryogenic calli in forest species, in which induction by the synthetic auxin 2,4-D plays a central role in the cellular reprogramming required to acquire embryogenic competence (Bartos et al. 2018).

Similar results were observed in *Elaeis guineensis* leaf explants, where a concentration of 15 µM of 2,4-D resulted in the formation of friable calli, followed by the induction of embryogenic structures, demonstrating the efficacy of 2,4-D in inducing calli with embryogenic potential (Yusnita & Hapsoro 2011). In *Coffea arabica* L. cv. Obat, the combination of 4 mg L^{-1} of 2,4-D and 2 mg L^{-1} of kinetin favored the induction of mixed primary calli, with a higher frequency of friable embryogenic calli in the presence of BA (8 mg L^{-1}), associated or not with 2,4-D (Maciel et al. 2003).

In *Brassica oleracea* L. var. *leucocephala*, calli with embryogenic characteristics were obtained in media supplemented with 5.0 mg L^{-1} of 2,4-D, and histological characterization revealed the superiority

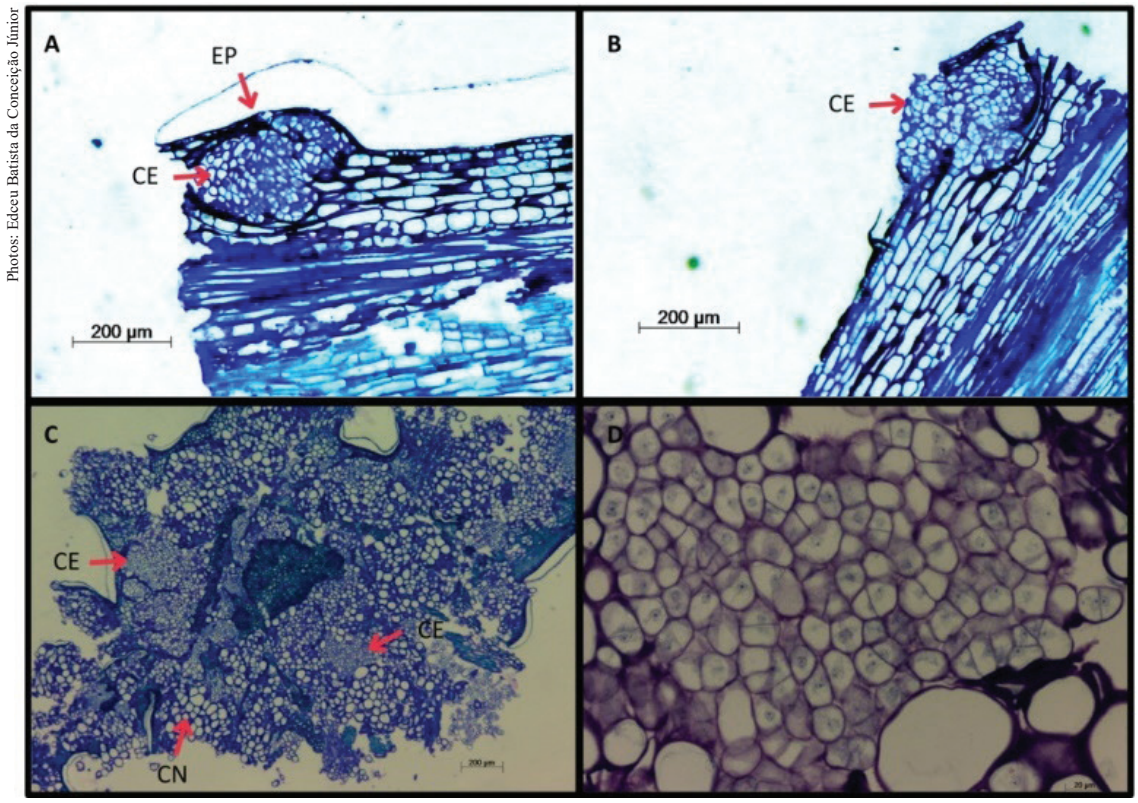

Figure 6. Anatomical appearance of callus formation in *Khaya grandifoliola* leaf explants cultivated *in vitro* on Murashige & Skoog (MS) medium containing 67.86 µM of 2,4-dichlorophenoxyacetic acid (2,4-D) and maintained in the dark at 25 ± 2 ºC. A) General view of callus formation after 30 days of culture; B) rupture of the leaf epidermis caused by proliferating embryogenic cells; C) anatomical organization of the callus after 60 days of culture showing embryogenic and non-embryogenic regions; D) detail of embryogenic cells characterized by isodiametric shape, prominent nuclei, small vacuoles, cell clusters, and active cell division. CE: embryogenic cells; CN: non-embryogenic cells; EP: epidermis.

of the interaction between media containing 2,4-D and BAP for the regeneration of calli and somatic embryos (Donato et al. 2000).

These results indicate that 2,4-D is effective in promoting the formation of calli with anatomical characteristics compatible with somatic embryogenesis in *Khaya grandifoliola*, representing a potential agent for regeneration protocols through embryogenesis in this species.

The use of leaf fragments as explant material for callus induction in *Khaya grandifoliola* proved technically viable and relevant to the development of *in vitro* propagation protocols for the species. Among the evaluated treatments, the concentration of 67.86 µM of 2,4-D yielded the best results, stimulating the formation of friable, light-colored calli predominantly located along the midrib, with a lower incidence at the explant margins. This morphogenic response suggests the presence of characteristics associated with embryogenic potential.

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