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Microplant production of *Cochlospermum regium* (Schrank) Pilg. by the indirect organogenesis: an important medicinal plant of the Cerrado Biome

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ABSTRACT: *Cochlospermum regium* is an endemic and endangered Brazilian Cerrado Biome species traditionally used for its therapeutic properties. However, a significant problem raised by growers is seed dormancy, leading to propagation difficulties. This research aimed to evaluate indirect organogenesis in *Cochlospermum regium* through callogenesis induction and bud regeneration. Cotyledon, hypocotyl, and radicle tissues from *in vitro* germinated seedlings were used as explants. TDZ, 2,4-D, and NAA were supplemented in a culture medium to induce morphogenic responses for 49 days. The explants were then transferred to a regeneration culture medium supplemented with BAP and NAA for 49 days. All tissues and PGR combinations prompted callogenesis, with hypocotyl as the most responsive tissue. The combination of hypocotyl tissue and TDZ-induced regeneration of adventitious buds, resulted in 5.3% of regeneration. Adventitious rooting was confirmed at 49 days of *in vitro* cultivation, making plant regeneration possible.
Keywords: *in vitro* culture; indirect regeneration; adventitious bud; plant regeneration.

Produção de microplantas de *Cochlospermum regium* (Schrank) Pilg. por organogênese indireta: uma importante planta medicinal do Bioma Cerrado

RESUMO: *Cochlospermum regium* é uma espécie endêmica e ameaçada do bioma do Cerrado brasileiro que tem sido tradicionalmente usada por suas propriedades terapêuticas. No entanto, um problema significativo enfrentado pelos cultivadores é a dormência das sementes, o que dificulta a propagação. Esta pesquisa teve como objetivo avaliar a organogênese indireta em *Cochlospermum regium* por meio da indução de calogênese e regeneração de gemas. Tecidos do cotilédone, hipocótilo e radícula de plântulas germinadas *in vitro* foram usados como explantes. TDZ, 2,4-D e ANA foram adicionados a um meio de cultura para induzir respostas morfogênicas durante 49 dias. Em seguida, os explantes foram transferidos para um meio de cultura de regeneração suplementado com BAP e ANA por 49 dias. Todos os tecidos e combinações de reguladores de crescimento induziram a calogênese, sendo o hipocótilo o tecido mais responsivo. A combinação de tecido de hipocótilo e TDZ induziu a regeneração de gemas adventícias, resultando em uma regeneração de 5,3%. A formação de raízes adventícias foi confirmada após 49 dias de cultivo *in vitro*, tornando possível a regeneração de plantas.
Palavras-chave: cultivo *in vitro*; regeneração indireta; gemas adventícias; regeneração de plantas.

1. INTRODUCTION

Cerrado Biome is a large savanna in South America, accounting for about 24% of the Brazilian territory. It is Brazil's second-largest biome, with numerous vegetation types (LIMA et al., 2017). *Cochlospermum regium* (Schrank) Pilger, a bush from the country's Midwest area, is among the endemic and endangered species of the Brazilian Cerrado (ROSSI et al., 2013). This species presents widespread medicinal indications, where the rhizome is used in teas for preventing infections and inflammations (CARVALHO et al., 2018; GAVILAN et al., 2018; GALVÃO et al., 2023). However, the importance of this species is not restricted only to folk medicine. There is evidence of analgesic and antidematogenic effects besides antibacterial and anti-inflammatory activities (CARVALHO et al., 2018; PEDROSO et al., 2019).

The low germination rates due to seed dormancy is a key factor that increases the extinction risk of endangered medicinal species from the Cerrado Biome (ROSSI et al., 2013). *Cochlospermum regium* seeds present integumentary dormancy as the hard integument prevents water permeability (JOHNSON-FULTON; WATSON, 2018). Vegetative propagation by *in vitro* culture is an alternative to various plant species, considering the difficulties of seminal propagation since it aims to meet both commercial purposes in seedling production and the conservation of genetic resources (SOUZA et al., 2019; SANTOS et al., 2022), contributing to increasing the seedling production of

Cochlospermum regium and its application for conservation and improvement programs.

This research aimed to evaluate callogenesis induction and adventitious bud regeneration in *Cochlospermum regium* for the determination of the best combination of tissues (cotyledon, hypocotyl, and radicle) with plant growth regulator (PGR) [thidiazuron (TDZ); 2,4-dichlorophenoxyacetic acid (2,4-D), and α-naphthaleneacetic acid (NAA)].

2. MATERIAL AND METHODS

2.1. Source of explants

Tissues collected from *in vitro* germinated seedlings were the source of explants. The seeds of *Cochlospermum regium* were collected from ripe fruits in September and October 2014 in a Cerrado area in 'Chapada dos Guimarães National Park, Highway MT - 251, in the town of Chapada dos Guimarães (authorization n°. 46295 from ICMBio - Chico Mendes Institute for Biodiversity Conservation, Brazil).

In vitro germination and callogenesis induction: Cotyledon (fully expanded), hypocotyl (collected from the median position with 1.0 cm of length), and radicle (collected from the median position with 1.0 cm of length) tissues were excised from *in vitro* germinated seedlings at 40 days (GAVILAN et al., 2018), and inoculated into test tubes (2.0×10.0 cm) containing 5.0 mL of the MS culture medium (MURASHIGE; SKOOG, 1962) supplemented with 1.0 mg L^{-1} of TDZ or 2,4-D or NAA. The experiment was conducted in random blocks in a factorial scheme (3×3) with three tissues and three PGRs, with ten replications. After 49 days of *in vitro* cultivation, the survival percentage, tissue oxidation, and percentage of callogenesis were calculated. A subculture was performed at 28 days.

2.2. Adventitious bud regeneration

Callus formed on the 49th day were used as explants and inoculated into test tubes (2.0×10.0 cm) containing 5.0 mL of the MS culture medium supplemented with 1.0 mg L^{-1} of benzylaminopurine (BAP) and 0.05 mg L^{-1} NAA (regeneration medium). The experiment was conducted in a random design in a factorial scheme (3×3) with three tissues and three PGRs (i.e., considering the treatments of induction to callogenesis), with ten replications. After 49 days of *in vitro* cultivation, the percentages of adventitious bud regeneration, tissue oxidation, and survival were evaluated. A subculture was performed after 28 days.

2.3. Shoot elongation and adventitious rooting

In vitro elongated shoots (>2.0 cm in length) were obtained in 91 days using the methodology developed by Gavilan et al. (2018). These shoots were inoculated into test tubes (2.0×10.0 cm) containing 10.0 mL of the MS culture medium added with 0.5 mg L^{-1} NAA and 0.05 mg L^{-1} BAP (rooting medium). *In vitro* adventitious rooting at 35 days of cultivation was evaluated.

2.4. Acclimatization and micro plant production

The microplates were acclimatized in a mini-incubator system (BRONDANI et al., 2018) for 36 days. The substrate used was decomposed pine bark and medium vermiculite.

2.5. Culture medium and growth conditions

MS culture medium was prepared with distilled water, 6 g L^{-1} of agar, and 30 g L^{-1} of sucrose. The pH value of the solution was adjusted to 5.8 with HCl (0.1 M) and NaOH (0.1 M) before adding the agar to the culture medium. After that, the culture medium was autoclaved at 121 ºC (~ 1 $kgf.cm^{-2}$) for 20 minutes. The procedure of explant manipulation was performed in a laminar flow chamber. The explants were incubated in a growth room at 25°C (± 2°C), 16 hours of photoperiod, and luminosity of 32 μmol m^{-2} s^{-1}.

2.5. Histological analysis

Tissue samples of *Cochlospermum regium* were prepared according to the methodology of Gavilan et al. (2018). The blocks were sectioned longitudinally to 5 μm thickness with an automatic rotary microtome Microm HM 355S (Thermo Scientific).

2.6. Statistical analysis

The data was submitted to the Shapiro-Wilk test ($P > 0.05$) for normality verification. Hartley's test ($P > 0.05$) was used for homogeneity of variance verification, and the Box-Cox test was used for data transformation. The data were submitted to analysis of variance (ANOVA, $P < 0.05$), and mean values were subjected to Duncan's test comparison analysis ($P < 0.05$). The analyses were processed using R software (R CORE TEAM, 2018).

Histological studies were characterized by descriptive interpretation.

3. RESULTS

Cotyledon tissue and hypocotyl had better responses concerning survival percentages (90.1% and 75.3%, respectively), which did not differ significantly (Figure 1A). The lowest value for survival percentages was observed in radicle tissues (66.4%) (Figure 1A). Tissues cultivated in a culture medium supplemented with NAA (61.7%) had the lowest survival percentages compared to those grown with 2,4-D (82.9%) and TDZ (86.6%) (Figure 1B).

The supplementation of TDZ and NAA in the culture medium increased the percentage of tissue oxidation (78.2% at 100.0%), regardless of the evaluated tissues. When associated with hypocotyl and 2,4-D, it results in lower tissue oxidation (58.0%) (Figure 1C).

Callogenesis analysis showed that the explants from the cotyledon and hypocotyl presented the highest predisposition to callus formation (78.7% at 92.0%), independent of the PGR (Figure 1B) - all treatments induced callogenesis. The combination of 2,4-D, TDZ, or NAA, with the cotyledon and hypocotyl, presented the highest averages (Figure 1D). Radicle tissue revealed inferior results concerning callogenesis induction (31.2% at 78.5%) (Figure 1D).

TDZ presented the best survival percentage, especially when the explant applied was the hypocotyl (77.7%) and radicle (75.6%). Only for cotyledonary explants, the 2,4-D was superior in survival percentage (74.4%) (Figure 2A).

A considerable percentage of tissue oxidation was observed in all treatments, with higher occurrences in hypocotyl and TDZ combination (77.7%), radicle and TDZ

(72.9%), and radicle and NAA (75.0%) combinations (Figure 2B). Although the highest oxidation percentages were observed when TDZ was combined with hypocotyl and radicle, it did not make the experiment unfeasible since there was adventitious bud regeneration only from PGR. The 2,4-D and NAA had lower tissue oxidation percentages than TDZ, considering hypocotyl tissues, but this response was reversed when cotyledon was cultivated (Figure 2B).

Only the callus from TDZ and hypocotyl tissue combination resulted in adventitious bud regeneration (5.3%) (Figure 3A), which differed significantly from the other PGRs (i.e., 2,4-D and NAA). Callus originated from the supplementation with 2,4-D, and NAA did not produce adventitious buds when submitted to the regeneration culture medium for 49 days. At 35 days of *in vitro* culture, Adventitious rooting was observed, with 80% rooting. The rooted shoots were transferred to acclimatization for 36 days, and no mortality was observed. (i.e., there was 100% of survival of rooted plants).

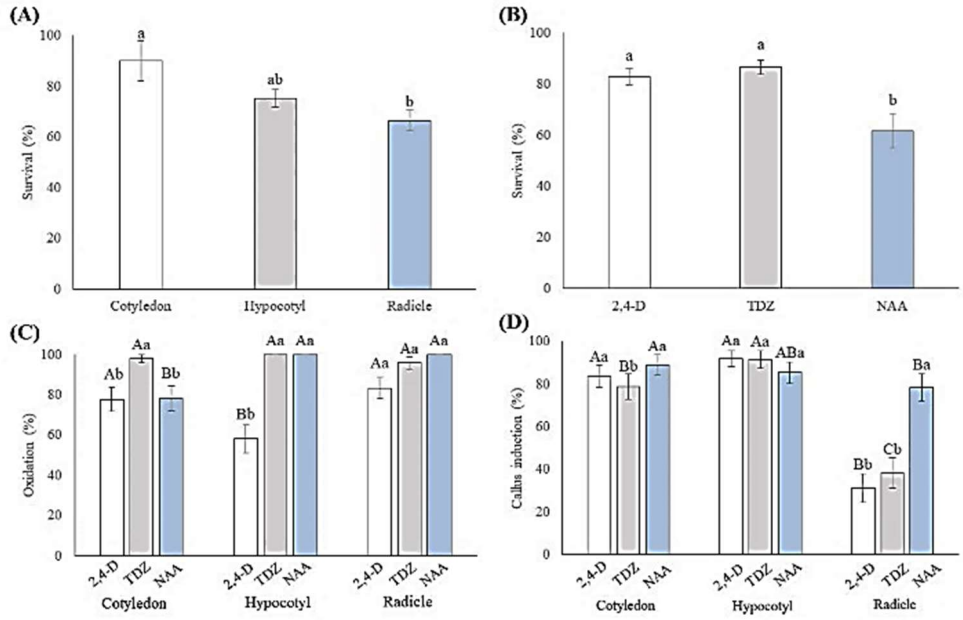

Figure 1. Callogenesis induction in *Cochlospermum regium* at 49 days of *in vitro* culture, considering tissues and PGRs. A-B – Mean value of survival percentage according to explant tissue and PGR. C – Mean value of tissue oxidation percentage; D – Mean value of callus induction percentage; C-D – Mean values followed by equal capital letters considering the same PGR and equal lowercase letters considering the same tissue do not differ statistically by Duncan's test. A-B – Mean values followed by equal lowercase letters do not differ statistically by Duncan's test. Data is presented as Mean value (± standard error).

Figura 2. Indução de calogênese em *Cochlospermum regium* após 49 dias de cultivo *in vitro*, levando em consideração tecidos e reguladores de crescimento. A-B - Valor médio da porcentagem de sobrevivência de acordo com o tipo de tecido de explante e reguladores de crescimento. C - Valor médio da porcentagem de oxidação tecidual; D - Valor médio da porcentagem de indução de calos; C-D - Valores médios seguidos por letras maiúsculas iguais, considerando o mesmo regulador de crescimento, e letras minúsculas iguais, considerando o mesmo tipo de tecido, não apresentam diferenças estatisticamente significativas de acordo com o teste de Duncan. A-B: os valores médios seguidos por letras minúsculas iguais não diferem estatisticamente de acordo com o teste de Duncan. Os dados são apresentados como Valor médio (± erro padrão).

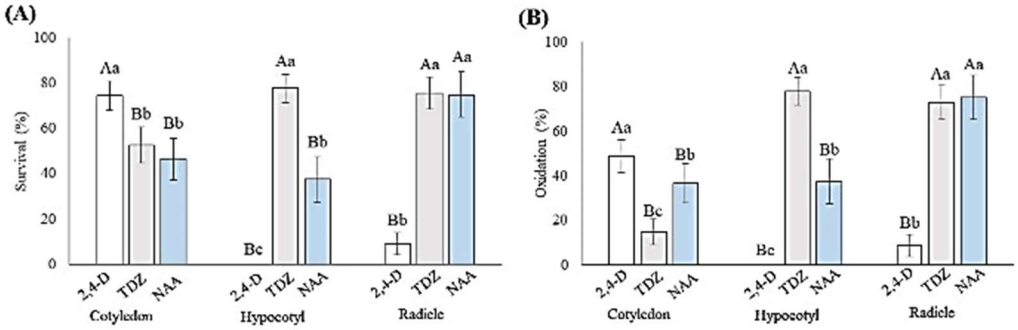

Figure 2. Adventitious bud regeneration in *Cochlospermum regium* at 49 days of *in vitro* culture, considering tissues and PGRs. A – Mean value of survival percentage; B – Mean value of tissue oxidation percentage; Mean values followed by equal capital letters considering the same PGR and equal lowercase letters contemplating the same tissue do not differ statistically by Duncan's test. Data are presented as Mean value (± standard error).

Figura 2. Regeneração de gemas adventícias em *Cochlospermum regium* após 49 dias de cultivo *in vitro*, considerando tecidos e reguladores de crescimento. A - Valor médio da porcentagem de sobrevivência; B - Valor médio da porcentagem de oxidação tecidual; Valores médios seguidos por letras maiúsculas iguais, considerando o mesmo regulador de crescimento, e letras minúsculas iguais, considerando o mesmo tipo de tecido, não apresentam diferenças estatisticamente significativas de acordo com o teste de Duncan. Os dados são apresentados como Valor médio (± erro padrão).

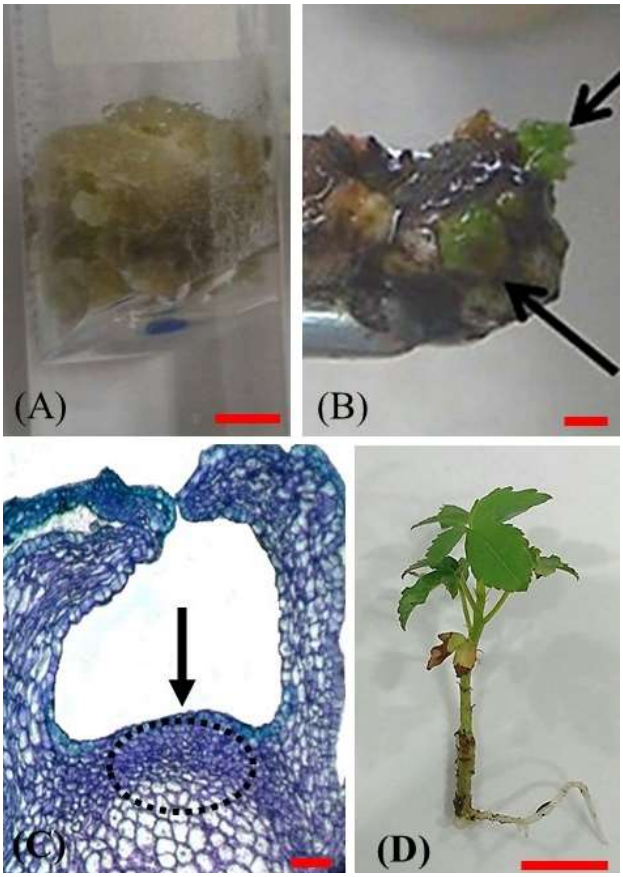

Figure 3. A detail of indirect organogenesis in *Cochlospermum regium*. A – Callogenesis induction from hypocotyl in MS medium supplemented with 1.0 mg L^{-1} TDZ at 49 days (scale: 0.5 cm). B – Adventitious bud (callogenic tissue from hypocotyl and 1.0 mg L^{-1} TDZ combination) in regeneration medium at 49 days (arrows, scale: 0.5 cm). C – Longitudinal section of adventitious bud (bud induced from hypocotyl and 1.0 mg L^{-1} TDZ combination) showing the formation of meristematic cells – shoot tip at 49 days (arrow, scale: 50 μm). D – Detail of acclimatized microplant at 35 days (scale: 1.0 cm).

Figura 3. Detalhes da organogênese indireta em *Cochlospermum regium*. A - Indução de calogênese a partir do hipocótilo em meio MS suplementado com 1,0 mg L^{-1} de TDZ aos 49 dias (escala: 0,5 cm). B - Brotos adventícios (tecido calogênico do hipocótilo e combinação de 1,0 mg L^{-1} de TDZ) em meio de regeneração aos 49 dias (setas, escala: 0,5 cm). C - Corte longitudinal da gema adventícia (gema induzida pelo hipocótilo e combinação de 1,0 mg L^{-1} de TDZ) mostrando a formação de células meristemáticas aos 49 dias (seta, escala: 50 μm). D - Detalhe de uma microplanta aclimatizada aos 35 dias (escala: 1,0 cm).

4. DISCUSSION

Hypocotyl tissues were the most responsive to callus induction, which is a positive result since there was high cellular competence (HARTMANN et al., 2011; TAMBARUSSI et al., 2015). Studies have reported the efficiency of the callus hypocotyl and cotyledon induction, such as those developed for *Eucalyptus saligna* (SILVA et al., 2015), *Eucalyptus globulus* (SALLA et al., 2018), *Eucalyptus microcorys* (FARIA et al., 2021) and *Eucalyptus cloeziana* (OLIVEIRA et al., 2022). The hypocotyl tissues presented higher callus induction for the organogenesis of *Ficus religiosa*, but bud proliferation was lower when compared with cotyledonary explants. The callogenesis induction and bud proliferation were high (HESAMI; DANESHVAR, 2018).

Some of the most popular PGRs for callus induction in plant tissues are TDZ (ZORZ et al., 2020) and 2,4-D (OLIVEIRA et al., 2017). However, in this study, NAA was the most responsive one independently of the evaluated tissue, as observed in *Asparagus densiflorus*, where adding NAA to the culture medium increased the callus induction efficiency (PINDEL, 2017). This result with the *Cochlospermum regium* correlates to the fact that NAA is more stable in its ability to withstand physical and enzymatic degradation in the *in vitro* culture (SOUZA et al., 2010), having a residual effect during the callogenesis induction phase. NAA and 2,4-D present auxin action and were able to start cell division and control cell growth and elongation processes (SETH; PANIGRAHI, 2018), which may favor the induction of allogeneic masses *in vitro* conditions (ZANG et al., 2019).

The presence of adventitious buds confirmed the TDZ action in the hypocotyl tissue of *Cochlospermum regium*. Adventitious buds originating from the callus tissues display the development of an aerial organ (e.g., adventitious shoot, shoot tip, and leaf) - an important path for the acquisition of plants through indirect organogenesis (SILVA et al., 2015).

The fact that the explants submitted to callogenesis with TDZ were the only ones responsive for adventitious bud regeneration is due to cytokine-like substances (GUO et al., 2011), unlike NAA and 2,4-D, which have auxin activity. Therefore, adventitious bud regeneration associated only with treatments with TDZ is probably related to its cytokine action. Thus, PGR contributed to maintaining hormonal balance for directing the organogenesis events and differentiation of undifferentiated callus cells in adventitious buds. TDZ induces the formation of adventitious buds and somatic embryos more frequently than auxin-cytokine-based treatments (SANDHU et al., 2018). This PGR is known for the induction of adventitious buds and the proliferation of axillary shoots in woody species (HARTMANN et al., 2011).

TDZ efficiency to regenerate adventitious buds (Figure 3B) was confirmed by the presence of meristematic cells (Figure 3C), and similar results were observed by Gao et al. (2021) in the callus of *Pinus koraiensis*. Aggarwal et al. (2010) found intense meristematic activity in cells of the superficial layer in tissues of *Eucalyptus tereticornis*, and these cells were later organized into buds. Also, the higher oxidation percentages of explants in TDZ treatments did not interfere with morphogenesis. The phenolic compounds may enhance adventitious organogenesis (HARTMANN et al., 2011), and probably the higher production of phenols, which handles the oxidation of explants in treatments with TDZ, implies the morphogenic activity of the hypocotyl's cells.

The highest frequency of adventitious bud regeneration was observed in the treatments associated with BAP and TDZ in tissues of *Eucalyptus cloeziana* (ZORZ et al., 2020), corroborating the *Cochlospermum regium* results. In contrast, with *F. religiosa* explants, a more significant regeneration of buds occurred with the combination of indol-3-acetic acid (IAA) and TDZ (HESAMI; DANESHVAR, 2018). The species needs a long *in vitro* cultivation (e.g., numerous subcultures) to present an organogenic response to a chemical stimulus, which could increase bud proliferation and favor general development (HARTMANN et al., 2011).

Rooting and acclimatization showed satisfactory results for producing micropropagated cotton plants, considering the high values of *ex vitro* established plants. According to data from Inácio et al. (2014), about 80% of the plants obtained from *in vitro* cultivation had at least twice the root area when compared with the aerial part, favoring acclimatization and obtaining new plants, allowing the production of seedlings as observed in our study.

5. CONCLUSIONS

All tissues and PGRs induced callogenesis, especially the hypocotyl tissue. TDZ combined with the hypocotyl tissue was the only combination that resulted in adventitious bud regeneration.

Adventitious rooting was confirmed in 35 days of *in vitro* culture under a rooting medium (i.e., 80%) and acclimatization with 100% survival at 36 days; therefore, obtaining a complete plant in 300 days is possible. These results can contribute to the propagation of the species for medicinal applications and assistance in programs to protect endangered species.

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