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Germplasm bank formation of *Cordia trichotoma* (Vell.) Arrab, ex Steud. by the micropropagation technique

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ABSTRACT: *Cordia trichotoma* is considered an economically important and priority species for forest plantations, but its vegetative propagation is challenging. This study aimed to define a micropropagation protocol for seedling production and forming a *C. trichotoma* germplasm bank. For *in vitro* establishment, three types of explants were tested, diaspores, seeds with coat, and seeds without coat. In the multiplication stage, different culture media (MS and WPM), activated charcoal concentrations (0 and 100 mg L^{-1}), and 6-benzylaminoperine concentrations (0.5, 1.0, and 2.0 mg L^{-1}) were tested. To evaluate elongation, the effects of culture medium (MS and WPM), activated charcoal concentration (0 or 100 mg L^{-1}), and naphthalene acetic acid supplementation (0.5, 1.0, or 2.0 mg L^{-1}) were evaluated. In the rooting stage, the activated charcoal concentration (0 and 100 mg L^{-1}) and the light conditions (presence and absence of light) were tested. The use of seeds without a coat, regardless of the time of exposure to active chlorine, resulted in 83% *in vitro* establishment. MS culture medium supplemented with 100 mg L^{-1} of activated charcoal yielded the most shoots per explant. Activated charcoal concentration of 100 mg L^{-1} resulted in the greatest shoot elongation. The absence of light and the use of activated charcoal in the culture medium did not influence adventitious rooting.
Keywords: plant tissue culture; *in vitro* culture; plant growth regulator; activated charcoal; luminosity.

Formação de banco de germoplasma de *Cordia trichotoma* (Vell.) Arrab, ex Steud. pela técnica de micropropagação

RESUMO: A *Cordia trichotoma* é considerada uma espécie economicamente importante e prioritária para plantios florestais, no entanto apresenta dificuldades de propagação vegetativa. O objetivo do trabalho foi definir um protocolo de micropropagação para a produção de mudas e formação de um banco de germoplasma de *C. trichotoma*. Para o estabelecimento *in vitro* foram testados três tipos de explantes: diásporo, semente com tegumento e semente sem tegumento. Aos 30 dias após a inoculação foram avaliadas a porcentagem de contaminação fúngica, bacteriana, oxidação dos tecidos, estabelecimento e germinação. Na fase de multiplicação foram testados o meio de cultura (MS e WPM), concentração de carvão ativado (0 e 100 mg L^{-1}) e suplementação com BAP (0,5; 1,0 e 2,0 mg L^{-1}). Após 60 dias da inoculação *in vitro* dos explantes foram avaliados a sobrevivência, o número de brotos e número de folhas. No alongamento foram avaliados o meio de cultura (MS e WPM), concentração de carvão ativado (0 e 100 mg L^{-1}) e suplementação com ANA (0,5; 1,0 e 2,0 mg L^{-1}). Após 60 dias da inoculação *in vitro* dos explantes foi avaliado o comprimento das brotações. Na fase de enraizamento foram testadas concentrações de carvão ativado (0 e 100 mg L^{-1}) e as condições de luminosidade (presença e ausência de luminosidade). Após 30 dias da inoculação *in vitro* dos explantes foi avaliada a indução de raízes adventícias. O uso de sementes sem o tegumento, independente do tempo de exposição ao cloro ativo, resultou em 83% de estabelecimento *in vitro*. A utilização do meio de cultura MS suplementado com 100 mg L^{-1} de carvão ativado apresentou os melhores resultados para o número de brotos por explantes. A concentração de 100 mg L^{-1} de carvão ativado resultou nos melhores valores para o alongamento de brotos. A ausência de luz e uso do carvão ativado no meio de cultura não influenciaram a formação de raízes adventícias.
Palavras-chave: cultura de tecidos de plantas; cultivo *in vitro*; regulador de crescimento vegetal; carvão ativado; luminosidade.

1. INTRODUCTION

The success of the world economy is directly related to the supply of resources and services from forests (ROCHA et al., 2020), given the increase in the world population and the demand for wood products. However, extracting natural resources in some situations is unsustainable, such as to meet the industrial need for large amounts of wood, especially in unmanaged areas (CARVALHO et al., 2020). In Brazil, native species used for this purpose are not normally cultivated on a large scale, but the growing global need for wood has accelerated the search for new species with silvicultural potential, mainly due to their easy adaptability

and occurrence in several Brazilian regions and the high added value of wood (ANDRADE et al., 2020).

Species such as *Cordia trichotoma* (Vell.) Arrab. ex Steud. have become increasingly relevant in this context. This species, popularly known as 'louro-pardo', belongs to the family Boraginaceae and is distributed in tropical and subtropical forests of Argentina, Bolivia, Paraguay, and Uruguay, as well as in Brazil, where it occurs from the northeast to the south regions (BERGHETTI et al., 2021). *C. trichotoma* is considered an economically important and priority species for forest plantations, mainly due to the high volumetric growth, quality, and multi-functionality of its wood, which is easily workable and so is used in the manufacture of luxury furniture, in addition to its ability to be used in environmental recovery strategies (FLECK et al., 2019). However, seed production of the species does not occur cyclically or continuously and depends on climatic conditions and the habit of pollinators, which is one of its main silvicultural problems (MACHADO et al., 2015). In addition, the seeds produced are recalcitrant and cannot be stored because they lose viability quickly (LOPES et al., 2019).

Vegetative propagation can be an important tool to overcome the problems of producing high-quality seedlings to form homogeneous plantations (LOPES et al., 2019). One of the techniques that can be applied for successful vegetative propagation is micropropagation, which enables, for example, the creation of an active germplasm bank *in vitro* that can be used for large-scale seedling production, thus reducing the pressure on the natural environment brought by the extraction of native species (SILVA et al., 2020).

This study aimed to define a micropropagation protocol for the seedling production and formation of a *C. trichotoma* germplasm bank.

2. MATERIAL AND METHODS

2.1. Study site and experimental material

The material for the experiments came from diaspores collected from four *C. trichotoma* trees with seven years old, selected according to trunk shape (rectilinear), diameter at breast height (21.4 to 23.9 cm), and health (absence of diseases and pest attacks), from a progeny and origin test in Trancoso, Porto Seguro District, state of Bahia, Brazil. Registration in SisGen under the number A2929EB.

2.2. *In vitro* establishment

Diaspores and seeds (explants) were washed five times in autoclaved deionized water and immersed in a 70% hydroalcoholic solution (v/v) for 20 seconds with constant agitation inside a horizontal laminar flow chamber. Explants were washed again with autoclaved deionized water. They were immersed in a NaClO solution at 2.0-2.5% of active chlorine (v/v) (Clarix®) according to treatments (Table 1). Finally, the diaspores and seeds were washed in autoclaved deionized water five times.

The explants were inoculated vertically, under aseptic conditions, in 2.0 × 10.0 cm test tubes containing 10 mL of MS culture medium (MURASHIGE; SKOOG, 1962). The tubes were sealed with polyvinyl chloride (PVC) plastic film and placed in a growth room under a temperature of 24 °C (± 1 °C), a photoperiod of 16 hours, and an irradiance of 40 μmol m^{-2} s^{-1}. The equipment was disinfected with 70% of hydroalcoholic solution throughout the process. The culture medium was supplemented with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar, prepared with deionized water, and the pH was adjusted to 5.8 (± 0.05) with NaOH (0.1 M) and HCl (0.1 M) before autoclaving and agar addition. The culture medium was autoclaved at 121 °C (approximately 1.0 kgf cm^{-2}) for 20 minutes. After 30 days of *in vitro* inoculation of the explants, the percentages of *in vitro* establishment (absence of fungal and bacterial contamination), fungal and bacterial contamination, tissue oxidation, and germination were evaluated.

The experiment was conducted in a completely randomized factorial design (3×3) considering the type of explant (diaspores, seeds with coat, or seeds without coat) and the exposure time to NaClO (5, 10, or 20 minutes). Each treatment had 40 replicates, with one explant per replicate.

Table 1. Treatments used to evaluate the *in vitro* establishment of diaspores and seeds of *Cordia trichotoma*.
Tabela 1. Tratamentos utilizados para avaliar o estabelecimento *in vitro* de diásporos e sementes de *Cordia trichotoma*.

Treatment	Explant[1]	Time[2]
T1	DP	5
T2	DP	10
T3	DP	20
T4	WC	5
T5	WC	10
T6	WC	20
T7	NC	5
T8	NC	10
T9	NC	20

[1] DP: diaspore; WC: seed with seed coat; NC: seed without seed coat. [2] Immersion in NaClO (minutes).

2.3. *In vitro* multiplication

After seed germination (30 days), the seedlings were sectioned, and their apical meristem was inoculated vertically in a 2.0 × 10.0 cm test tube containing 10 mL of multiplication medium, according to Table 2.

Table 2. Treatments used to evaluate the *in vitro* multiplication of *Cordia trichotoma*.
Tabela 2. Tratamentos utilizados para avaliar a multiplicação *in vitro* de *Cordia trichotoma*.

Treatment	Culture medium	Activated charcoal (mg L^{-1})	BAP (mg L^{-1})
T1	MS	0.0	0.5
T2	MS	0.0	1.0
T3	MS	0.0	2.0
T4	WPM	0.0	0.5
T5	WPM	0.0	1.0
T6	WPM	0.0	2.0
T7	MS	100.0	0.5
T8	MS	100.0	1.0
T9	MS	100.0	2.0
T10	WPM	100.0	0.5
T11	WPM	100.0	1.0
T12	WPM	100.0	2.0

MS (MURASHIGE; SKOOG, 1962): 6 mg L^{-1} of agar and 30 mg L^{-1} of sucrose. WPM (LLOYD; MCCOWN, 1980): 6 mg L^{-1} of agar and 20 mg L^{-1} of sucrose. BAP: 6-benzylaminopurine.

The cultivation conditions, asepsis and preparation of the culture medium were the same in 2.2. item.

In all treatments evaluated, supplementation with α-naphthaleneacetic acid (NAA) was performed at a

concentration of 0.1 mg L^{-1}. After 60 days of *in vitro* inoculation of the explants, survival, number of shoots, and number of leaves were evaluated.

The experiment was conducted in a completely randomized factorial design (2 × 2 × 3) considering the culture medium (MS or WPM), activated charcoal concentration (0.0 or 100.0 mg L^{-1}), and BAP supplementation (0.5, 1.0, or 2.0 mg L^{-1}). Each treatment had 15 replicates, with one explant per replicate.

2.4. *In vitro* elongation

The shoot (explant) produced in the *in vitro* multiplication stage was standardized to 1.0 cm in length and two leaves. Table 3 shows shoots were inoculated under aseptic conditions in a 2.0 × 10.0 cm test tube containing 10 mL of elongation medium.

Table 3. Treatments used to evaluate *in vitro* elongation of *Cordia trichotoma*.
Tabela 3. Tratamentos utilizados para avaliar o alongamento *in vitro* de *Cordia trichotoma*.

Treatment	Culture medium	Activated charcoal (mg L^{-1})	NAA (mg L^{-1})
T1	MS	0.0	0.5
T2	MS	0.0	1.0
T3	MS	0.0	2.0
T4	WPM	0.0	0.5
T5	WPM	0.0	1.0
T6	WPM	0.0	2.0
T7	MS	100.0	0.5
T8	MS	100.0	1.0
T9	MS	100.0	2.0
T10	WPM	100.0	0.5
T11	WPM	100.0	1.0
T12	WPM	100.0	2.0

MS (MURASHIGE; SKOOG, 1962): 6 mg L^{-1} of agar and 30 mg L^{-1} of sucrose. WPM (LLOYD; MCCOWN, 1980): 6 mg L^{-1} of agar and 20 mg L^{-1} of sucrose. NAA: α-naphthaleneacetic acid.

The cultivation conditions, asepsis and preparation of the culture medium were the same in 2.2. item.

In all treatments evaluated, supplementation with BAP was performed at a concentration of 0.1 mg L^{-1}. The shoot length was evaluated after 60 days of *in vitro* inoculation of the explants.

The experiment was conducted in a completely randomized factorial design (2 × 2 × 3) considering the culture medium (MS or WPM), activated charcoal concentration (0.0 or 100.0 mg L^{-1}) and NAA supplementation (0.5, 1.0, or 2.0 mg L^{-1}). Each treatment had 15 replicates, with one explant per replicate.

2.5. *In vitro* rooting

Elongated shoots were selected and standardized to 2 cm in length. They were inoculated under aseptic conditions in a 2.0 × 10.0 cm test tube containing 10 mL of rooting medium supplemented with 1.0 mg L^{-1} NAA, 0.1 mg L^{-1} BAP, and 0.1 mg L^{-1} of indole-3-butyric acid (IBA). The treatments tested consisted of two activated charcoal concentrations (0.0 or 100.0 mg L^{-1}) and two light conditions (dark or 40 μmol m^{-2} s^{-1}) (Table 4). A cardboard box in a black polyethylene plastic bag created a dark environment.

The temperature, asepsis and preparation of the culture medium were the same in 2.2. item.

After 30 days of *in vitro* inoculation of the explants, rooting was evaluated.

Table 4. Treatments used to evaluate the *in vitro* rooting of *Cordia trichotoma*.
Tabela 4. Tratamentos utilizados para avaliar o enraizamento *in vitro* de *Cordia trichotoma*.

Treatment	Activated charcoal (mg L^{-1})	Luminosity
T1	0.0	Presence
T2	0.0	Absence
T3	100.0	Presence
T4	100.0	Absence

The experiment was conducted in a completely randomized factorial design (2 × 2) considering the activated charcoal concentrations (0.0 or 100.0 mg L^{-1}) and the light conditions (light or dark). Each treatment had 15 replicates, with one explant per replicate.

2.6. Data analysis

The analyses were done in R Core Team Software (2018) using the ExpDes package, version 1.1.2 (FERREIRA et al., 2013). The data collected from the experiments were analyzed for homoscedasticity and normality of residuals using the Hartley ($P > 0.05$) and Shapiro-Wilk tests ($P > 0.05$), respectively. When nonparametric, the data were transformed by the Box-Cox test and subjected to analysis of variance (ANOVA, $P < 0.05$). The mean values were compared by Tukey's test ($P < 0.05$).

3. RESULTS

3.1. *In vitro* establishment

The factors evaluated as influencers of the *in vitro* establishment of *C. trichotoma* (type of explant and immersion time in sodium hypochlorite, Table 1) showed interaction for germination percentage.

Seeds without coats had a significantly higher mean *in vitro* establishment percentage (83.0%) than the other treatments (< 33.0%) (Figure 1A). In comparison, immersion time did not result in significantly different responses at 5% significance (29.0-42.6%) (Figure 1B). There was no difference in the percentage of tissue oxidation for the type of explant, considering that the frequency was low (< 3.5%) (Figure 1C).

Regarding *in vitro* germination, seeds without coat and asepsis for 5 minutes in NaClO had the highest percentage of germination (90.0%), differing statistically from the other types of explants (0.0-50.0%) (Figure 1D). After asepsis for 10 minutes, seeds with coat showed 30.0% of germination, and seeds without coat showed 20.0% of germination, both significantly more than the diaspore. After immersion in NaClO for 20 minutes, seeds with and without coat had the mean germination percentages, 50.0% and 45.0%, respectively, which were similar (Figure 1D).

Seeds without coats had significantly lower mean fungal contamination (8.0%) than the other treatments (65.0-80.0%) (Figure 2A). Immersion in sodium hypochlorite for 20 minutes resulted in the lowest fungal contamination (46.0%), showing a significant difference relative to asepsis for 5 (60.0%) and 10 minutes (58.0%) (Figure 2B).

Regarding bacterial contamination, seeds with seed coat showed no contamination by bacterial, with a significant difference to other treatments (5.0-12.0%) (Figure 2C). The

asepsis time did not statistically affect the bacterial contamination (4.0-8.0%) (Figure 2D).

The use of diaspores was not suitable for most of the evaluated traits. It resulted in the highest means of fungal and bacterial contamination, with 80.0% (Figure 2A) and 12.0% (Figure 2C), respectively, and the lowest *in vitro* establishment (Figure 1A) and germination (Figure 1D) percentages (4.0 and 0.0%, respectively). The immersion time in NaClO did not differ statistically at 5% significance for tissue oxidation (0.0-5.0%) (Figure 2E).

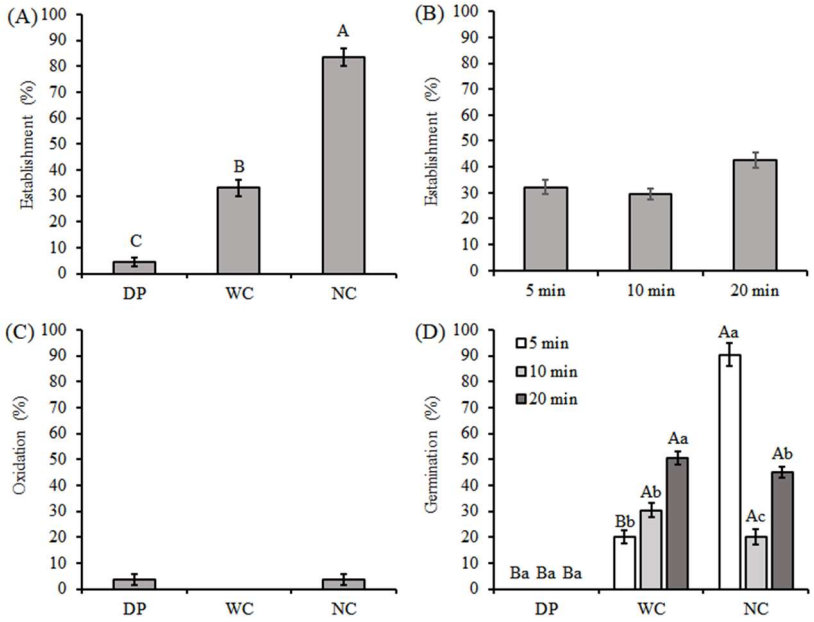

Figure 1. Features of the *in vitro* establishment of *Cordia trichotoma* at 30 days. (A) *In vitro* establishment according to type of explant (DP: diaspore; WC: seed with seed coat; NC: seed without coat); (B) *In vitro* establishment according to immersion time in NaClO (5, 10, or 20 minutes); (C) Tissue oxidation according to type of explant; (D) *In vitro* germination according to type of explant (Table 1). In (A): Means followed by the same letters do not differ by Tukey's test ($P < 0.05$). Error bars represent the standard error of the mean. In (D): Means followed by the different uppercase letters represent significant differences when comparing different types of explant given the same immersion time in NaClO, and different lowercase letters represent significant differences when comparing immersion time in NaClO within the same type of explant by Tukey's test ($P < 0.05$). Error bars represent the standard error of the mean.
Figura 1. Características do estabelecimento *in vitro* de *Cordia trichotoma* aos 30 dias. (A) Estabelecimento *in vitro* de acordo com o tipo de explante (DP: diásporo; WC: semente com tegumento; NC: semente sem tegumento); (B) Estabelecimento *in vitro* de acordo com o tempo de imersão em NaClO (5, 10 ou 20 minutos); (C) Oxidação tecidual de acordo com o tipo de explante; (D) Germinação *in vitro* de acordo com o tipo de explante (Tabela 1). Em (A): Médias seguidas das mesmas letras não diferem entre si pelo teste de Tukey ($P < 0,05$). As barras de erro representam o erro padrão da média. Em (D): Médias seguidas de diferentes letras maiúsculas representam diferenças significativas quando se comparam diferentes tipos de explantes para o mesmo tempo de imersão em NaClO; e diferentes letras minúsculas representam diferenças significativas na comparação do tempo de imersão em NaClO dentro do mesmo tipo de explante pelo teste de Tukey ($P < 0,05$). As barras de erro representam o erro padrão da média.

3.2. *In vitro* multiplication

The culture media and activated charcoal used in the *in vitro* multiplication stage at 60 days interacted (Figure 3A). The concentration of BAP did not significantly influence the number of shoots (1.4-1.6 shoots per explant) (Figure 3B).

Better results were obtained without activated charcoal and MS culture medium, with an average of 2.7 shoots per explant, which differed statistically from the other treatments (0.8-1.2 shoots per explant) (Figure 3A).

Culture medium (Figures 4A and 5A), activated charcoal (Figures 4B and 5B), and BAP concentrations (Figures 4C and 5C) did not influence the survival in the *in vitro* culture and number of leaves of *C. trichotoma*. There was no significant difference between the treatments, with an average value of 77.0% of survival and 2.3 leaves per explant, regardless of the factor.

3.3. *In vitro* elongation

There was no interaction for the factors analyzed for their effects on *in vitro* elongation (culture medium, NAA concentration, and activated charcoal) (Figures 6A-C). There was a significant effect of activated carbon concentrations on shoot length.

The culture medium (1.8-2.1 cm) (Figure 6A) and NAA concentrations (1.9-2.0 cm) (Figure 6C) did not induce different shoot lengths. However, the presence of activated charcoal in the culture medium induced greater shoot length (3.0 cm) at 60 days of culture than the absence (0.9 cm) (Figure 6B). The explants of *C. trichotoma* showed that the use of activated charcoal was required for their development.

3.4. *In vitro* rooting

The spontaneous rooting of the explants did not differ regarding the luminosity (Figure 7A) or the use of activated charcoal in the culture medium (Figure 7B) 30 days after inoculation. The presence of adventitious roots was observed both in the absence and presence of luminosity (54.5 and 45.5% of rooting, respectively, Figure 7A) and in the absence and presence of activated charcoal as a supplement to the culture medium (58.6 and 41.4% of rooting, respectively).

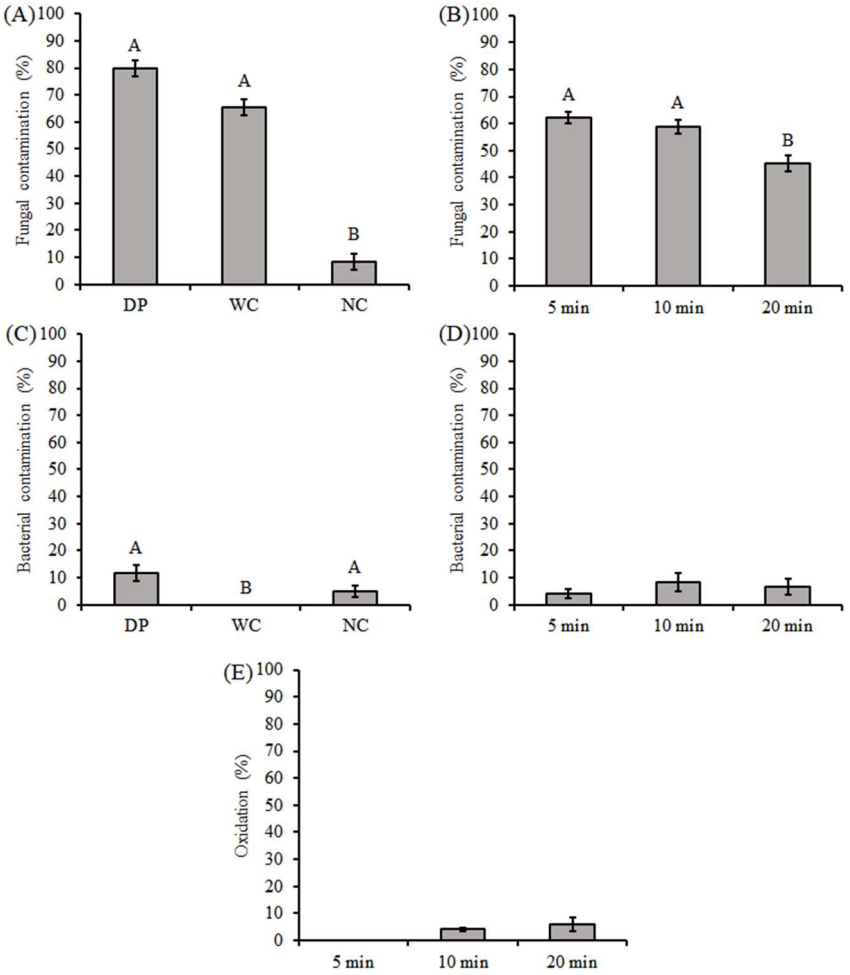

Figure 2. Features evaluated in the *in vitro* establishment of *Cordia trichotoma* at 30 days. (A) Fungal contamination according to type of explant (DP: diaspore; WC: seed with seed coat; NC: seed without coat); (B) Fungal contamination according to immersion time in NaClO (5, 10, or 20 minutes); (C) Bacterial contamination according to type of explant; (D) Bacterial contamination according to immersion time in NaClO; (E) Tissue oxidation according to immersion time in NaClO (Table 1). In (A-C): Means followed by the same letters do not differ by Tukey's test ($P < 0.05$). Error bars represent the standard error of the mean.

Figura 2. Características avaliadas no estabelecimento *in vitro* de *Cordia trichotoma* aos 30 dias. (A) Contaminação fúngica de acordo com o tipo de explante (DP: diásporo; WC: semente com tegumento; NC: semente sem tegumento); (B) Contaminação fúngica de acordo com o tempo de imersão em NaClO (5, 10 ou 20 minutos); (C) Contaminação bacteriana de acordo com o tipo de explante; (D) Contaminação bacteriana de acordo com o tempo de imersão em NaClO; (E) Oxidação tecidual de acordo com o tempo de imersão em NaClO (Tabela 1). Em (A-C): Médias seguidas das mesmas letras não diferem entre si pelo teste de Tukey ($P < 0,05$). As barras de erro representam o erro padrão da média.

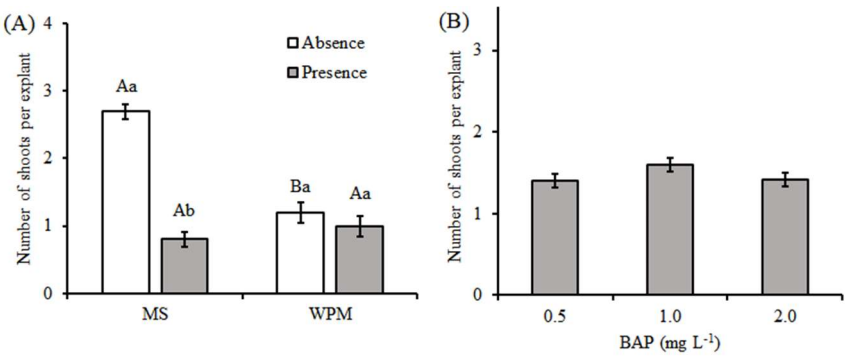

Figure 3. Number of shoots per explant (NS) evaluated in the *in vitro* multiplication of *Cordia trichotoma* at 60 days. (A) NS according to the culture medium and activated charcoal concentration (Absence: 0 or Presence: 100 mg L^{-1}). (B) NS according to BAP concentration (0.5, 1.0, or 2.0 mg L^{-1}) (Table 2). In (A): Means followed by the different uppercase letters represent significant differences when comparing the same activated charcoal concentrations given the different culture media, and different lowercase letters represent significant differences when comparing different activated charcoal concentrations within the same culture media by Tukey's test ($P < 0.05$). Error bars represent the standard error of the mean. In (B): Error bars represent the standard error of the mean.

Figura 3. Número de brotações por explante (NB) avaliadas durante a multiplicação *in vitro* de *Cordia trichotoma* aos 60 dias. (A) NB de acordo com o meio de cultura e concentração de carvão ativado (Ausência: 0 ou Presença: 100 mg L^{-1}); (B) NB de acordo com a concentração de

BAP (benzilaminopurina) (0,5; 1,0 ou 2,0 mg L^{-1}) (Tabela 2). Em (A): Médias seguidas de diferentes letras maiúsculas representam diferenças significativas quando se comparam a mesma concentração de carvão ativado para diferente meio de cultura; e diferentes letras minúsculas representam diferenças significativas na comparação de diferentes concentrações de carvão ativado dentro do mesmo meio de cultura pelo teste de Tukey ($P < 0,05$). As barras de erro representam o erro padrão da média. Em (B): As barras de erro representam o erro padrão da média.

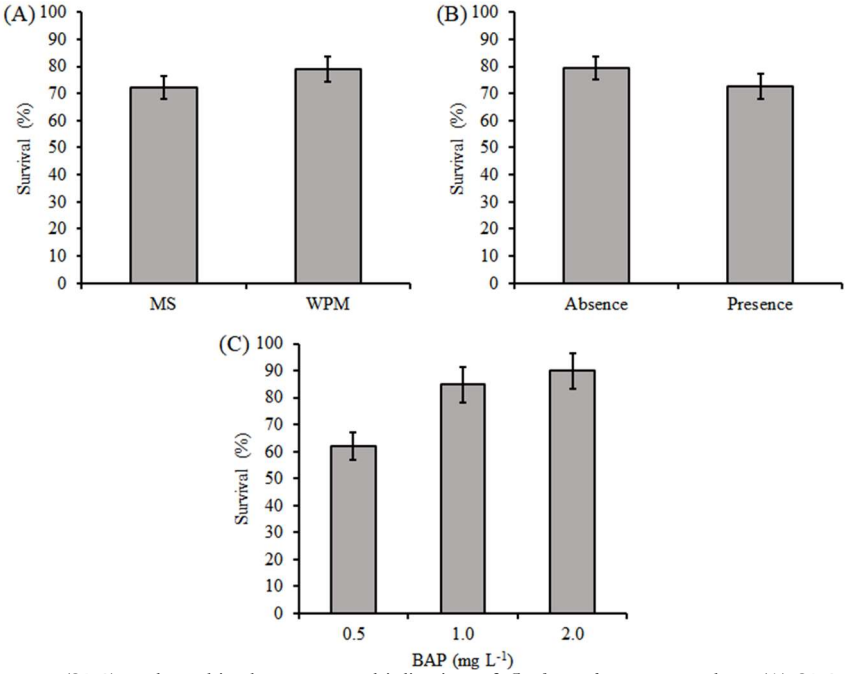

Figure. 4. Survival percentage (SUP) evaluated in the *in vitro* multiplication of *Cordia trichotoma* at 60 days. (A) SUP according to the culture medium (MS or WPM); (B) SUP according to activated charcoal concentrations (Absence: 0 or Presence: 100 mg L^{-1}); (C) SUP according to BAP concentrations (0.5, 1.0, or 2.0 mg L^{-1}) (Table 2). In (A), (B) and (C): Error bars represent the standard error of the mean.

Figura. 4. Porcentagem de sobrevivência (POS) avaliada durante a multiplicação *in vitro* de *Cordia trichotoma* at 60 days. (A) POS de acordo com o meio de cultura (MS ou WPM); (B) POS de acordo com as concentrações de carvão ativado (Ausência: 0 ou Presença: 100 mg L^{-1}); (C) POS de acordo com as concentrações de BAP (benzilaminopurina) (0,5; 1,0 ou 2,0 mg L^{-1}) (Tabela 2). Em (A), (B) e (C): As barras de erro representam o erro padrão da média.

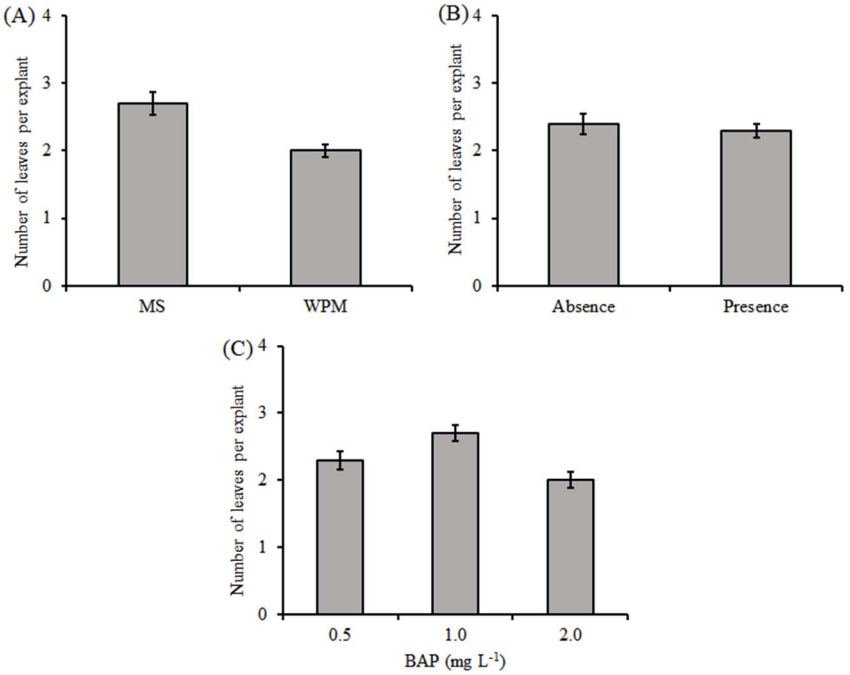

Figure 5. Number of leaves per explant (NL) evaluated in the *in vitro* multiplication of *Cordia trichotoma* at 60 days. (A) NL according to the culture medium (MS or WPM); (B) NL according to activated charcoal concentrations (Absence: 0 or Presence: 100 mg L^{-1}); (C) NL according to BAP concentrations (0.5, 1.0, or 2.0 mg L^{-1}) (Table 2). In (A), (B) and (C): Error bars represent the standard error of the mean.

Figura 5. Número de folhas por explante (NF) avaliada durante a multiplicação *in vitro* de *Cordia trichotoma* aos 60 dias. (A) NF de acordo com o meio de cultura (MS ou WPM); (B) NF de acordo com as concentrações de carvão ativado (Ausência: 0 ou Presença: 100 mg L^{-1}); (C) NF de acordo com as concentrações de BAP (0,5; 1,0 ou 2,0 mg L^{-1}) (Tabela 2). Em (A), (B) e (C): As barras de erro representam o erro padrão da média.

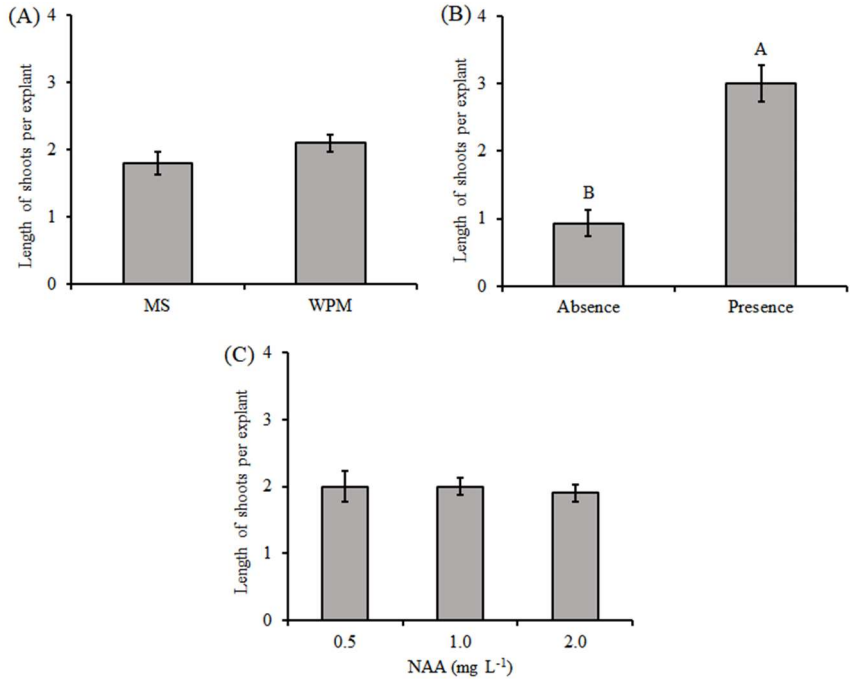

Figure 6. Length shoots per explant (SL) evaluated in the *in vitro* elongation of *Cordia trichotoma* at 60 days. (A) SL according to the culture medium (MS or WPM); (B) NL according to activated charcoal concentrations (Absence: 0 or Presence: 100 mg L^{-1}); (C) NL according to NAA concentrations (0.5, 1.0, or 2.0 mg L^{-1}) (Table 3). In (B): Means followed by the same letters do not differ by Tukey's test ($P < 0.05$). Error bars represent the standard error of the mean.

Figura 6. Comprimento de broto por explante (CB) avaliado durante o alongamento *in vitro* de *Cordia trichotoma* aos 60 dias. (A) CB de acordo com o meio de cultura (MS ou WPM); (B) CB de acordo com as concentrações de carvão ativado (Presença: 0 ou Ausência: 100 mg L^{-1}); (C) CB de acordo com as concentrações de ANA (ácido α-naftalenoacético) (0,5; 1,0 ou 2,0 mg L^{-1}) (Tabela 3). Em (B): Médias seguidas das mesmas letras não diferem entre si pelo teste de Tukey ($P < 0,05$). As barras de erro representam o erro padrão da média.

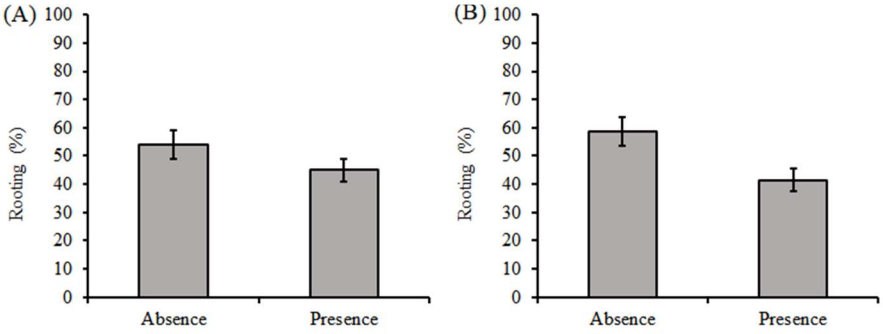

Figure 7. Rooting percentage (RP) evaluated in the *in vitro* rooting of *Cordia trichotoma* at 30 days. (A) RP according to luminosity (Absence: 0 or Presence: 40 µmol m^{-2} s^{-1}); (B) RP according to activated charcoal concentrations (Absence: 0 or Presence: 100 mg L^{-1}) (Table 4). In (A) and (B): Error bars represent the standard error of the mean.

Figura 7. Porcentagem de enraizamento (PE) avaliado durante o enraizamento *in vitro* de *Cordia trichotoma* aos 30 dias. (A) PE de acordo com a luminosidade (Ausência: 0 ou Presença: 40 µmol m^{-2} s^{-1}); (B) PE de acordo com as concentrações de carvão ativado (Ausência: 0 ou Presença: 100 mg L^{-1}) (Tabela 4). Em (A) e (B): As barras de erro representam o erro padrão da média.

4. DISCUSSION

4.1. *In vitro* establishment

In vitro germination is a way to propagate species that have difficulty germinating under natural conditions, as they are killed by animals and pathogens or by unfavorable environmental conditions (MASCOT-GÓMEZ et al., 2020). This approach enables the production of seedlings on a large scale in a shorter time and space, in addition to allowing the production of plants with high phytosanitary quality (FRANCESCHI et al., 2019).

One of the main barriers to *in vitro* propagation is contamination by microorganisms, especially in woody species, when the mother plants used for the collection are adult plants growing in the field (Oliveira et al., 2013), as is the case for *C. trichotoma*. These microorganisms, which include fungi and bacteria, may be of exogenous origin, occurring on the surface of the tissue, or endogenous, growing within the organism (PASQUALINI et al., 2019). Thus, disinfection of explants by aseptic methods is essential to establish contaminant-free seedlings and allow the continuity of the other micropropagation stages (LEITE et al., 2024). The asepsis protocols vary according to the species and type of explant used - there are different disinfectants, including ethanol and chlorine compounds such as sodium hypochlorite and calcium hypochlorite (Costa et al., 2007), along with different immersion times (MOLINARI et al., 2021).

Regarding fungal contamination, Bevilacqua et al. (2011) observed that using 2.5% sodium hypochlorite solution and a greater immersion time reduced contamination of *Calendula*

officinalis, the lowest contamination coming with immersion for 30 minutes. This occurs because sodium hypochlorite is adsorbed on the seed surface and, even after several washes with deionized water, can still react with organic compounds (MOLINARI et al., 2021). In *Paliavana sericiflora*, immersion in 1.25% sodium hypochlorite for 10 minutes, followed by washing in distilled water, resulted in 100.0% of seedlings being free of contaminants (LEITE et al., 2024). However, due to the difference between species and types of explants, the concentrations and immersion times in sodium hypochlorite may not influence seed germination, as observed by Lencina et al. (2014) in *Apuleia leiocarpa*, or other variables evaluated, such as oxidation, bacterial contamination, and *in vitro* establishment, as reported in *C. trichotoma*.

In another study, the seed coat removal resulted in the highest percentages of *in vitro* germination of *Calendula officinalis* (74.4%) (BEVILACQUA et al., 2011). Here, the reduction in fungal contamination in *C. trichotoma* seeds without coats was linked to higher percentages of *in vitro* establishment (83.0%) and one of the highest percentages of germination with an immersion time of 5 minutes in NaClO (90.0%). The scarification of the seed coat by the corrosive action of sodium hypochlorite increases the seed's permeability to water, oxygen, and solutes, which may facilitate the removal or oxidation of germination inhibitors (HISIÃO et al., 1981). In the present study, sodium hypochlorite may have removed the phenolic compounds present in the seeds, which could be germination inhibitors, as the oxidation percentages were low (< 3.5%).

The fact that the diaspores of *C. trichotoma* did not present satisfactory results may be linked to the structure and composition of these dispersal units, since they may contain tissues other than seed tissues or may have fungal on them (Machado; Silva, 2013), leading to the greater contamination and lower establishment observed.

4.2. *In vitro* multiplication

This study aimed to improve vegetative propagation protocols and, consequently, plant development, seeking to establish production systems that maximize the efficient production of seedlings on a commercial scale. Our results on the morphological characteristics of *C. trichotoma* provided information to optimize the production of seedlings through micropropagation. The species showed different responses to the factors evaluated (culture medium, activated charcoal and BAP concentrations) in the *in vitro* multiplication stage. The use of MS culture medium provided better results for the number of shoots, and there was no significant difference in the number of leaves and survival. This was contrary to the results found by Fick (2007), who observed greater growth of propagules in the WPM medium in terms of several shoots and number of leaves.

With the choice of culture medium, we aim to provide the nutrients and other compounds necessary for the development of explants. MS medium (Murashige; Skoog, 1962) proved to be the culture medium that provided the greatest development of the shoots and inducing buds with a rosy appearance, so its use is advised for *in vitro* multiplication of the species. BAP concentration (0.5 to 2.0 mg L^{-1}) did not influence the number of shots.

The addition of plant growth regulators to the culture medium and the appropriate balance between them show that organogenetic processes depend on these phytohormones (COSTA et al., 2010). The results of this study corroborate those observed in several woody species, such as *Myracrodruon urundeuva* (Andrade et al., 2000) and *Cabralea canjerana* (Rocha et al., 2007), in which low numbers of shoots were also observed. In those studies, this characteristic was related to the genotype of the selected plant.

There was no difference between the MS and WPM culture media regarding the number of leaves. The increase in the number of leaves during the *in vitro* multiplication stage has important implications since the bud is formed at the insertion site between the stem and the leaf, which may originate a new shoot and thus increase the number of plants generated.

4.3. *In vitro* elongation

We sought to establish a production system that maximized the efficient production of seedlings on a commercial scale. Our results on the morphological characteristics of *C. trichotoma* will allow the optimization of seedling production through micropropagation.

Of the factors we evaluated, only activated charcoal showed a significant difference in shoot length. In these *C. trichotoma* explants, the supplementation of activated charcoal in the culture medium (100 mg L^{-1}) provided the highest means for shoot length. The MS and WPM culture media used in micropropagation provided substances essential for plant tissue growth and controlled the seedling development pattern. When the medium becomes inadequate, it can cause symptoms of nutritional deficiency, physiological disorders, and even death of the explants (OLIVEIRA et al., 2016). NAA concentration did not influence the length of shoots of *C. trichotoma*, considering the variation from 0.5 to 2.0 mg L^{-1}.

Different basic culture media can be used in the *in vitro* elongation stage, depending on the needs of each plant species. Studies that define the concentration of mineral salts, plant growth regulators, and antioxidants for *in vitro* culture are essential for adjusting the culture medium (MASCARENHAS et al., 2019). Similar results have been found for the *in vitro* elongation of *Eucalyptus globulus* under different combinations of NAA, in which concentrations close to 0.5 mg L^{-1} were more efficient for shoot length (CORDEIRO et al., 2014). In contrast, Silva et al. (2013), studying *Caesalpinia pyramidalis*, found an increase in the length of shoots without plant growth regulators, which showed that higher concentrations of this auxin were inhibitory.

Another important factor in the composition of the culture medium in the present study was the activated charcoal, providing better results when compared to its absence. These results are similar to those found by Kim et al. (2019) using 1.0 g L^{-1} activated charcoal in the *in vitro* regeneration and development of *Pecteilis radiata*. In the explants of *Abelmoschus esculentus* (Irshad et al., 2017) and *Lupinus mutabilis* (Mamani et al., 2014), the use of antioxidants (PVP and activated charcoal) reduced the effects of phenolic oxidation, promoting greater development in the micropropagation of the cultures. The addition of activated charcoal to the culture medium increases the adsorption of substances that inhibit plant development, such as phenols and ethylene, and minimizes the toxicity of plant growth regulators or exogenous substances with harmful effects, contributing to greater proliferation of shoots and the growth of explants *in vitro* (KIM et al., 2019). These effects are due to a pore network in the structure of the activated charcoal,

in which many inhibitory substances in the medium or released by the explants can remain adsorbed. However, the addition of an antioxidant (activated charcoal, PVP, citric acid, or ascorbic acid) to the culture medium can have positive or negative effects, depending on the species, type of explant or culture stage being propagated (PANKAJ et al., 2014).

4.4. *In vitro* rooting

The rooting stage is one of the most important in the micropropagation process and is recommended to obtain a functional root system with normal structure, which may favour the survival and *ex vitro* growth of plants, avoiding possible losses during acclimatization (Leite et al., 2024); thus, it is essential to improve the protocols for this stage. In the present study, the rooting percentages were considered satisfactory at 45.5-58.6%, and the fact that the absence and presence of light and the supplementation of activated charcoal did not influence the rooting process may be related to the plant growth regulators at the same concentrations in all treatments evaluated.

The difference in light can indirectly affect adventitious rooting, influencing the hormonal balance between auxins and cytokinins and the availability and distribution of carbohydrates throughout the plant (DE ALMEIDA et al., 2017). Activated charcoal can simulate darkness, where the roots usually develop better, and chemically, it has an adsorbent effect, retaining part of all the elements that make up the culture medium. However, the use of this component does not always prove advantageous, given that activated charcoal can act as both a growth promoter and inhibitor, because in addition to adsorbing toxic compounds, it can adsorb key nutrients for growth, causing them to be gradually released (VILLA et al., 2014).

Furthermore, it is important to note that the seedlings that serve as explant sources for *in vitro* inoculation are of seminal origin, which indicates the low ontogenetic age of the plants, a fact that can influence the ability of cells to respond to stimuli and redifferentiate, thus inducing adventitious rooting more easily due to the juvenility of the tissues (DE ALMEIDA et al., 2017). Overall, the protocol developed efficiently produced C. trichotoma seedlings and formed a germplasm bank in 180 days of *in vitro* cultivation.

5. CONCLUSIONS

Seeds without coat result in 83.0% of *in vitro* establishment at 30 days, regardless of the time of exposure to active chlorine. High germination (90.0%) occurred in Seeds without a coat and 5 minutes of exposure to active chlorine.

MS culture medium without activated charcoal is the most appropriate medium for the *in vitro* multiplication stage.

The activated charcoal concentration of 100.0 mg L^{-1} obtained the greatest shoot length per explant in the elongation stage. The luminosity and activated charcoal supplemented in the culture medium did not influence the adventitious rooting stage.

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