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In vitro culture of *Stryphnodendron adstringens* Mart. Coville

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ABSTRACT

Stryphnodendron adstringens (Mart.) Coville is a species native to Cerrado with high medicinal potential. It is an important tannin source, is used mainly due to its healing, anti-inflammatory, and anti-septic activities. Due to the difficulties in sexual propagation, the objective of this work was to study some aspects of its the *in vitro* culture. The concentration of salts influenced the establishment of the initial explant *in vitro*, with a higher percentage of germination in the culture medium without salts. The combination of concentrations of 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA promoted greater shoot formation, and the concentration of 2.0 mg L^{-1} IBA promoted greater histogenesis. The composition of the culture medium influenced the establishment and development of *S. adstringens.*

Index terms: Native Brazilian tree; tissue culture; micropropagation; growth regulator.

INTRODUCTION

The Cerrado is the second largest Brazilian biome in terms of territorial extension and diversity of plants, occupying about 23% of the national territory (PIMENTEL et al., 2021). It presents the richest flora among the savannas throughout the world, with a high level of endemism, especially when tree species are considered. It is one of the 'hotspots' for worldwide biodiversity conservation with a large part of its extension occupied by farmers (BORGES et al., 2015).

Many plant species from Cerrado are important due to their medicinal properties like *Inga laurina* (STEIN et al., 2010; BELÉM et al., 2021), *Cariocar cariaceum* (SANTOS et al., 2006; BELÉM et al., 2021), *Hancornia speciosa* (PRUDENTE et al., 2017; VARGEM et al., 2022) and *Stryphnodendron adstringens* (Mart.) Coville (PORTO et al., 2014; VARGEM et al., 2022). *S. adstringens* is a specie belonging to the Fabaceae family, subfamily Mimosoideae, popularly known in Brazil as barbatimão (FERNANDES et al., 2022; LIMA et al., 2022; TROPICOS, 2022). It is widely used as an antiseptic (SOARES et al., 2008; SOUZA et al., 2007), anti-inflammatory (FALCÃO et al., 2005) a wound healing (RIBEIRO et al., 2022).

The species has a big attention problem, since its seeds have tegument dormancy and show low germination when intact, which makes seedlings development a difficult process (MARTINS; NAKAGAWA, 2008; SOUZA et al., 2021). Furthermore, the development of the seedlings and the plant in the field occurs slowly and in a heterogeneous form (LORENZI, 1992), therefore, studies are important to determine a better propagation method for *S. adstringens.*

Among the methods of plant propagation, micropropagation is a biotechnological alternative for the rapid propagation occupying little space throughout the year. Few reports on the micropropagation of *S. adstringens* have been carried out in the past (NICIOLI et al., 2008; CASTRO et al., 2009; MORAES; CERDEIRA; LOURENÇO, 2021).

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In this context, this work had the objective of evaluating aspects of the *in vitro* propagation of *S. adstringens*.

MATERIAL AND METHODS

In vitro germination

Ripe fruits were harvested from natural populations, in an area of campestral formation with an aspect of Cerrado *stricto sensu*, from a natural population in the south of Minas Gerais State, Brazil, located at 918.0 m altitude, latitude 21°14'S and longitude 44.9°00'W GRW. After harvesting, the seeds were removed manually from the fruits, transferred to a transparent glass flask, and stored in the refrigerator at 4 °C for 90 days.

The seeds were washed under running water with detergent for 20 minutes and transferred to a laminar flow chamber, where they were immersed in alcohol 70% (v/v) for 60 seconds and in sodium hypochlorite solution (NaOCl) with 2% active chlorine for 10 minutes. Afterward, seeds were washed three times in autoclaved distilled water and inoculated on culture media MS (MURASHIGE; SKOOG, 1962) and WPM (LLOYD; MCCOWN, 1980) with different salt concentration [MS 50% (MS/2) and WPM 50% (WPM/2] and the control (absence of salts), supplemented with 3% sucrose, solidified with 0.7% agar and pH adjusted to 5.8 before autoclaving at 120 °C for 20 minutes.

After the inoculation, seeds were maintained in a growth room under irradiance of 36 mmol m^{-2} s^{-1} photons, 16-hour photoperiod, and temperature of 25±2 °C. An evaluation was performed on the 15th and the 30th day of incubation, and the germinating length radicle and shoot were evaluated. It was considered germinated, seed that presented a protruded radicle.

The statistical design was completely randomized, with 15 replicates per treatment, each one comprised of a test tube containing one seed. Data were submitted to the analysis of variance (ANOVA) and performed using the SISVAR® statistical software (FERREIRA, 2014) with means compared by Tukey test at 5% significance.

Effect of BAP and NAA

Nodal segments obtained from *in vitro* germinated plants with approximately 1.0 cm long and two stem buds were inoculated on MS medium containing 3% sucrose, supplemented with different concentrations of 6-benzylaminopurine (BAP) (0.0, 1.0, 2.0, and 4.0 mg L^{-1}) and naphthalene acetic acid (NAA) (0.0, 0.01, 0.1, and 1.0 mg L^{-1}). The medium was solidified with 0.7% agar. The pH was adjusted to 5.8 before autoclaving at 120 °C for 20 minutes.

After the inoculation, the segments were maintained in a growth room at the temperature of 25 ± 2 °C, irradiance of photons 36 mol $m^{-2}s^{-1}$, and a 16-hour photoperiod. After 60 days, the number of shoots per explant, length (cm) and number of buds of the longest shoot, and the presence of calluses (%) were evaluated.

The statistical design used was completely randomized in a factorial scheme (4 BAP concentrations x 4 NAA concentrations) constituted of 10 replications per treatment, each one comprised of a test tube containing one stem segment. Data were analyzed via polynomial regression with SISVAR® statistical software (FERREIRA, 2014).

The obtained calluses were analyzed by scanning electron microscopy. It was fixed in Karnovsky (2.5% glutaraldehyde and 2.5% paraformaldehyde) in cacodylate buffer, pH 7.0, for a minimum of one hour at room temperature, then, cut with a scalpel in liquid nitrogen and the fragments were washed three times (10 minutes) in 0.05M cacodylate buffer and post-fixed in 1% osmium tetroxide and 0.05M cacodylate buffer for 1-2 hours. Dehydration was performed by increasing concentrations of acetone (30%, 50%, 70%, and 90%) for 10 minutes each after the dehydration, samples were critical-point dried in liquid CO_2 and metalized with a layer of gold. The observations were performed in LEO Evo 40 electron microscopy, operating between 10 and 20 kV.

Effect of IBA on *in vitro* rooting

Shoots with approximately 5 cm in length oriented from the best result obtained previously were inoculated on 50% MS, and 3% sucrose, supplemented with different concentrations of indole butyric acid - IBA (0.0, 0.5, 1.0, 2.0 mg L^{-1}). The medium was solidified with 0.7% agar and its pH was adjusted to 5.9 before autoclaving at 120 °C for 20 minutes.

After the inoculation, the shoots were kept in a growth room in the absence of light, at a

temperature of 25 ± 2 °C. After 15 days, the shoots were transferred to an 50% MS and 0.1% active charcoal, without the addition of the growth regulator and were maintained in a growth room at the temperature of 25 ± 2 °C, irradiance of 36 mol $m^{-2}s^{-1}$ photons and 16-hour photoperiod. The evaluation was performed on the 15th, 30th, and 45th day after the second inoculation when the number of roots and the presence or absence of calluses on the base of the explants were observed.

A completely randomized experimental design was used, with 10 replications per treatment, each one composed of a test tube containing one shoot originating from the *in vitro* culture. Data were submitted to the analysis of variance with (ANOVA) using SISVAR® statistical software, with means compared by the Scott-Knott test at 5% significance.

RESULTS AND DISCUSSION

In vitro germination

Seeds began to germinate with the protrusion of the radicle after the 5th day of inoculation, demonstrating that for germination of this species, an incision on the seed is not necessary.

The culture media MS, WPM with half the concentration of its salts and the control (absence of salts) did not present significant differences on the 15th day of evaluation (Figure 1). However, on the 30th day, salt concentration did influence the initial *in vitro* explant establishment. The medium with absence of salts presented higher germination percentage (93.3%), which did not statistically differ from the WPM/2 medium.

It is possible that with the absence of macro and micronutrients (control) the culture medium of *S. adstringens* had released higher quantity of water to the explants compared to the other media, which unleashed the process of radicle protrusion, due to the awakening of the embryo by imbibition.

The seedlings germinated on the WPM medium with half the concentration of its salts showed a higher average length of the radicle (4.66 cm) as well as the aerial part (2.9 cm), being important sources of secondary explants, but they did not significantly differ from the control treatment (Figure 2).

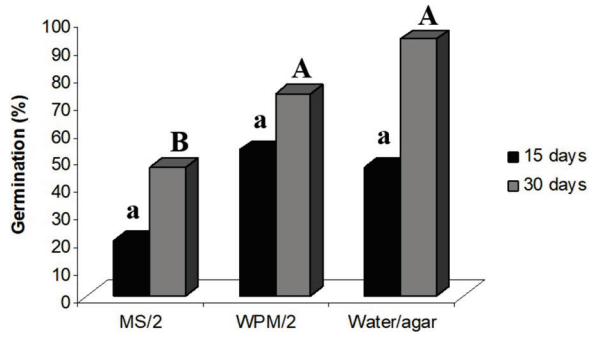

Figure 1: Germination percentage of *S. adstringens* seeds at the 15th and 30th days of *in vitro* culture submitted to culture media with different salt concentration.

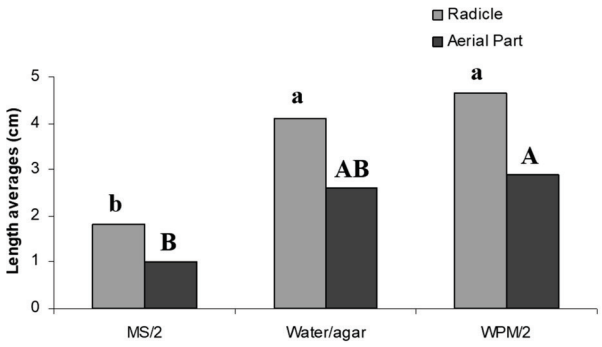

Figure 2: Root (cm) and aerial part (cm) lengths of *S. adstringens* as a function of the culture media with different salt concentration.

The MS/2 medium has a higher salt concentration in relation to the WPM/2 medium. According to our results, the use of culture media with an absence or lower salt concentration (WPM/2) in the *in vitro* seed initial establishment of *S. adstringens* is responsible for the higher percentage of germination. It is also less expensive since it uses fewer salts in relation to the MS/2 medium (Figure 3).

Souza et al. (2003), studying *Lychnophora pinaster* Mart., verified that the culture media with fewer concentration of salts were more efficient in the germination of embryos and in the development of plantlets. Similar results were also observed in *Byrsonima intermedia* A. Juss. seeds by Nogueira et al. (2004), where the presence of salts and carbohydrates possibly interfered in the osmotic regulation of the culture medium and, as a consequence, in the availability of water for the imbibition process during germination.

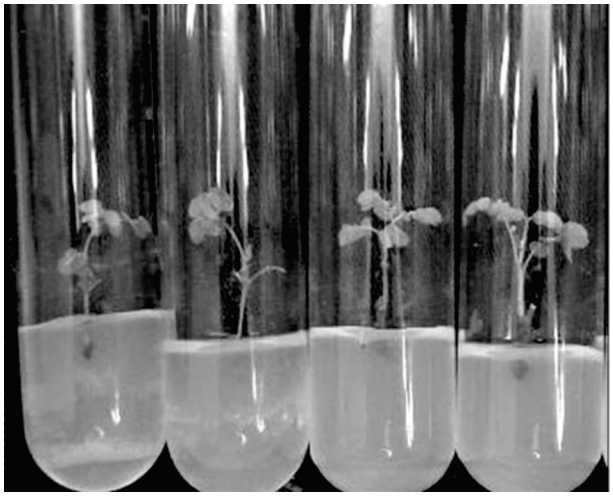

Figure 3: Visual aspects of *S*. *adstringens* shoots at the 45th day of *in vitro* culture.

Pessanha et al. (2022) did not find any statistical difference in the germination of *Dalbergia nigra* (Vell.) Allemão ex Benth., where both culture media tested showed no significant difference, however, the seedlings of the WPM culture medium presented well-developed leaves, while those obtained from MS culture medium showed more leaf senescence. The WPM medium was also more suitable for the development of *Hancornia speciosa* Gomes explants (PIRES et al., 2020). According to Hazubska-Przybył (2019) and Phillips and Garda (2019) the concentration of nitrate and ammonium ions in WPM culture medium (25% of the concentration present in MS culture medium), in addition to more potassium and a high level of sulfate ions, are factors that contribute to the development of woody species.

The studies on the culture media that favor *in vitro* germination are important, not only to maximize the germination rate but also to obtain uniform plantlets with genetic quality and adequate phytosanitation for *S*. *adstringens*.

Influence of BAP and NAA on shoot induction

The interaction between BAP and NAA presented significant effect on the number of shoots, number of buds and the length of the biggest shoot.

The use of 2.0 mg L^{-1} BAP in the absence of NAA was efficient in the multiplication of *S*. *adstringens* presenting, simultaneously, higher averages of number of shoots (3.0 per explant), number of buds (2.6 per explants), and length of the biggest shoot (1.18 cm) (Figures 4 and 5). These results demonstrate that the presence of cytokinin in the culture medium is fundamental for the multiplication of the aerial part and the formation of shoots in *S*. *adstringens*.

The treatments 0.01 mg L^{-1} NAA + 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA + 0.0 mg L^{-1} BAP induced the formation of an average number of 2.5 shoots and buds per explant. However, there was an increase in the length of most of the shoots, with 1.5 cm on average, which can favor the rooting phase.

Brum, Silva and Pasqual (2002) found the best results in the *in vitro* propagation of fig tree (*Ficus carica* L.) when only BAP was added to the culture medium, the higher number of shoots being formed between the concentrations of 2 and 4 mg L^{-1}, without the addition of NAA. Similar results were obtained by Erfa et al. (2022), in which the best combination of cytokinin and auxin concentrations to regenerate callus to form adventitious shoots in *F. carica* was 2 mg L^{-1} BAP + 0.25 mg L^{-1} NAA. As for the induction of shoots in the stem segments of pequi, Santos et al. (2006) obtained an increase in the number of shoots with the combined use of BAP and NAA, with a predominance of the first, thus promoting higher rate of multiplication.

It is observed that as the BAP concentration is increased in the culture medium, there is a considerable increase in the formation of shoots and lower formation of calluses observed in the relation between BAP and NAA. Conversely, there is a reduction in the number of shoots with the increase of NAA concentration added to the culture medium. In the concentration of 1.0 mg L^{-1}, the reduction of shoots becomes very pronounced, with an average of only 0.3 shoots per explant (Tables 1, 2, and 3).

Santos-Serejo et al. (2006) affirm that auxins, in the majority of cases, are not needed or used in very low concentrations, because they tend to stimulate the formation of calluses. Therefore, elevated concentrations of NAA resulted in a pronounced decrease in the induction of shoots, buds, and the length in *S. adstringens* and triggered the formation of calluses on the stem explants, being prejudicial or making the development of the aerial parts impracticable.

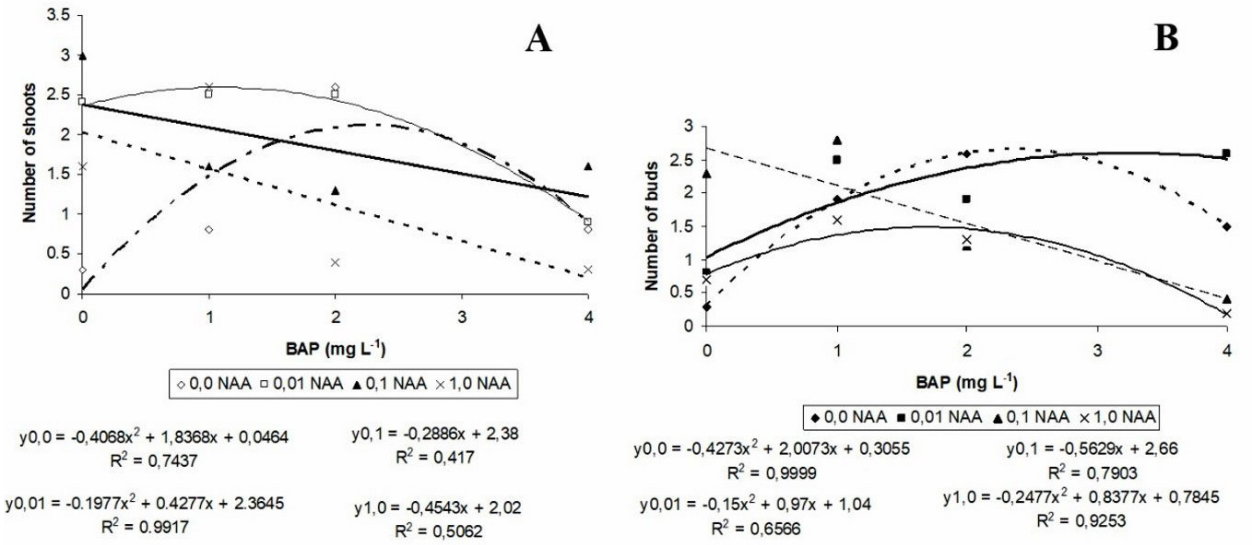

Figure 4: Number of shoots in stem segments (A) and number of buds (B) of *S. adstringens* in different concentrations of BAP and NAA.

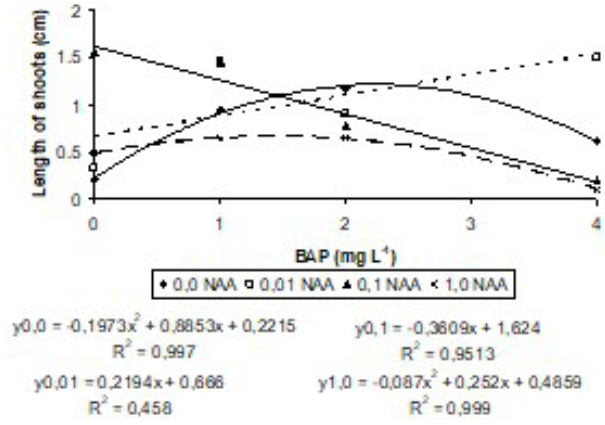

Figure 5: Length of the biggest shoot of *S. adstringens* in different concentrations of BAP and NAA

Table 1: Number of shoots of *S. adstringens* as a function of the interaction BAP x NAA.

NAA (mg L^{-1})	BAP (mg L^{-1})			
	0	1	2	4
0	0.3 bB	2.4 aA	3.0 aA	1.6 aA
0.01	0.8 aB	2.5 aA	1.6 aA	2.6 aA
0.1	2.6 aA	2.5 aA	1.3 bA	0.4 bB
1	0.8 aA	0.9 aA	0.6 bA	0.3 bB

*Means followed by the same lower-case letters in the column and upper-case letters in the row did not differ significantly according to the Scott-Knott test at 5% probability.

Table 2: Number of buds of *S. adstringens* as a function of the interaction BAP x NAA.

NAA (mg L^{-1})	BAP (mg L^{-1})			
	0	1	2	4
0	0.3 bB	1.9 aA	2.6 aA	1.5 aA
0.01	0.8 aB	2.5 aA	1.9 aA	2.6 aA
0.1	2.3 aA	2.8 aA	1.2 bA	0.4 bB
1	0.7 aB	1.6 aA	1.3 aA	0.2 bB

*Means followed by the same lower-case letters in the column and upper-case letters in the row did not differ significantly according to the Scott-Knott test at 5% probability.

The addition of higher NAA concentration probably provoked an imbalance in the endogenous relation of auxins and cytokinins of the explants, decreasing the number of shoots produced. The addition of the cytokinin BAP showed a benefic effect on the multiplication of *S. adstringens*, being related to the breaking of the apical dormancy and induction of the proliferation of axillary buds.

The electron microscopy demonstrated that elongate-shaped cells were predominant, characteristic of cells without embryogenic potential. However, it was possible to distinguish small regions with spherical organization, similar to somatic embryos in the globular stage. It is possible to visualize the formation of leaf primordia from the organogenic callus of *S. adstringens*.

Table 3: Length of shoots of *S. adstringens* as a function of the interaction BAP x NAA.

NAA (mg L^{-1})	BAP (mg L^{-1})			
	0	1	2	4
0	0.21 bB	0.94 aA	1.18 aA	0.94 aA
0.01	0.33 bB	1.47 aA	0.9 bA	1.5 aA
0.1	1.55 aA	1.45 aA	0.77 bA	0.2 bB
1	0.49 aB	0.64 aA	0.65 bA	0.05 bB

* Means followed by the same lower-case letters in the column and upper-case letters in the row did not differ significantly according to the Scott-Knott test at 5% probability.

In some situations, after a period of culture, the shoots present a decrease in vigor. This process is associated with the production of phenolic compounds or other factors such as the maturity of the explants (PAIVA; PAIVA, 2001). The ethylene accumulation produced by the tissues can interfere with the morphogenesis of the cultures, promoting the formation of calluses or accelerating the senescence of the cultures (GRATTAPAGLIA; MACHADO, 1998).

Effect of IBA on *in vitro* rooting

The results regarding the formation of roots, which were significantly influenced by the different IBA concentration are shown in Figure 7. With the increase of the concentration of this auxin in the culture medium, this species has a higher development of roots. IBA in the concentration of 2.0 mg L^{-1} promotes rooting in 80% of the explants.

The lowest percentage of rooting, observed in the absence of IBA, should be associated with the exogenous necessity of the auxin. The aerial parts originated in the multiplication provide IBA, which is used to stimulate the formation and development of adventitious roots on the stems of the vegetative segments of the plants. Therefore, IBA is essential for rooting (Figure 8).

Similar behavior was observed for the number of roots, where the usage of a higher concentration of IBA (2.0 mg L^{-1}) induced the formation of a higher number of roots, with an average of 3.4 roots per explant (Figure 9). However, significant differences were not observed when 1.0 mg L^{-1} IBA was used, with an average of 3.2 roots per shoot.

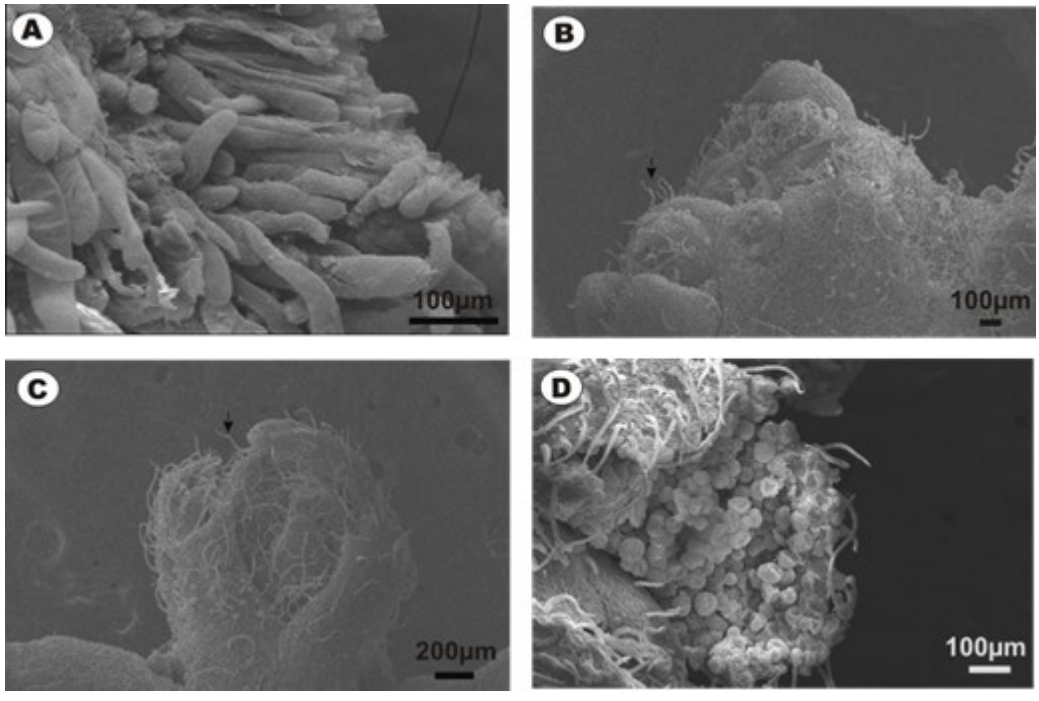

Figure 6: Scanning electron micrographs of calluses obtained from stem segments of *S. adstringens*. Aspects of the callus surface with elongated cells (A), callus with an early process of shoot formation (B), leaf primordium, detail of trichomes on the surface (C), and isodiametric cells on the callus surface (D).

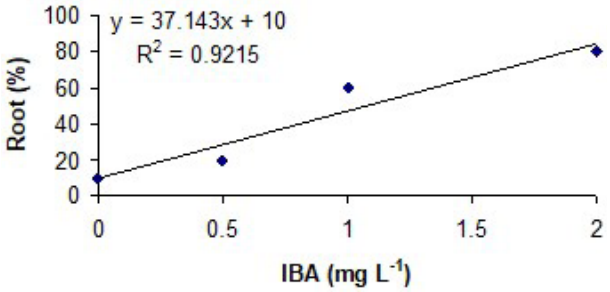

Figure 7: Formation of roots in shoots originated from *in vitro* culture of *S. adstringens* under different concentrations of IBA.

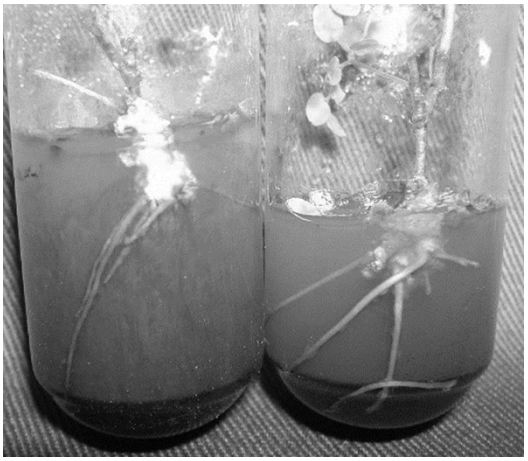

Figure 8: Visual aspect of adventitious roots of *in vitro* shoots in the presence of IBA in the MS medium of *S. adstringens*.

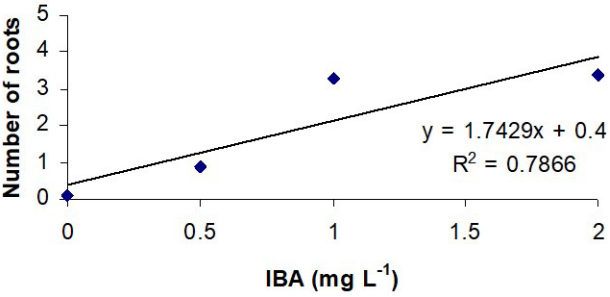

Figure 9: Average number of roots formed in *S. adstringens* explants inoculated on MS medium in the presence of IBA.

Besides auxin, the presence of active charcoal can facilitate root development, by stimulating the dark condition, in which the roots usually present a better development. Charcoal also has the capacity to remove rhizogenesis inhibitors and to fixate auxins, being, in general, beneficial to root elongation (GRATTAPAGLIA; MACHADO, 1998).

Sota et al. (2021) observed that for *in vitro* cultivation of *Malus sylvestris* (L.) Mill. there is a positive correlation between the formation of roots and new shoots and a concentration of activated carbon in the nutrient medium. Santos et al. (2006), studying the micropropagation of *Caryocar brasiliense*, verified that the use of active charcoal in the culture medium favors the induction and development of the roots. They also report that the presence of IBA is essential in the rooting process in shoots of this fructiferous species as well as those concentrations higher than 3 mg L^{-1} provoked a sudden decrease in root induction. A favorable effect of IBA in the rhizogenesis process was also observed by Soares et al. (2007) in the concentration of 3 mg L^{-1}, in their studies on *Hancornia speciosa* Gomes.

With the addition of IBA there was a callus formation on the explant base, a result similar to the one found by Radmann, Gonçalves and Fortes (2003), where a higher intensity in the callus formation was observed during the rooting of blackberry when IBA was added to the culture medium. According to Radmann, Gonçalves and Fortes (2003), the moment that auxin is applied, there is an increase in its concentration on the explant base and, if all of the other physiological requirements are satisfied, callus formation occurs, resulting from the activation of the cambial cells and of the adventitious root.

Even though IBA can induce root formation, even better than NAA in some species, it can also provoke undesirable effects due to its toxicity to plant tissues. Nicioli (2008), while evaluating the influence of the auxin NAA on the *in vitro* rooting of S. adstringens, did not observe callus formation on the explant bases and obtained a good percentage of rooted explants. In the study by Bonfá, Nascimento and Werner (2021), IBA favored the emergence of adventitious roots in most explants of *Cedrela fissilis* Vell., although no statistical differences were observed between treatments with auxin. According to Pasqual (1998) cited by Soares et al. (2007) genotypic differences in the responses to auxin can be caused by the differentiated ability of the tissue to absorb or metabolize synthetic auxin.

CONCLUSIONS

Culture medium with absence of salts promoted higher *in vitro* germination of *S. adstringens*. The combination of 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA promoted higher formation of shoots. There are ultra-structural differences among the callus cells originating from stem segments, where it is possible to visualize leaf primordial initiating on organogenic calluses. The concentration of 2.0 mg L^{-1} IBA promotes the highest formation of roots.

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